ผลกึ่งเฉียบพลันของสารสกัดบัวบกด้วยเอทานอลต่อเอนไซม์ไซโตโครมพี 450 และค่าเคมีคลินิกในเลือดหนูขาว

ว่าที่เรืออากาศโทหญิงชัชนี ผ่องจิตต์

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SUBACUTE EFFECTS OF *CENTELLA ASIATICA* ETHANOLIC EXTRACT ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ชัชนี ผ่องจิตต์: ผลกึ่งเฉียบพลันของสารสกัดบัวบกด้วยเอทานอลต่อเอนไซม์ไซโตโครม พี 450 ในตับ และค่าเคมีคลินิกในเลือดหนูขาว. (SUBACUTE EFFECTS OF *CENTELLA ASIATICA* ETHANOLIC EXTRACT ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS) อ. ที่ปรึกษา: [ผศ. พ.ต.ท.หญิง ดร. สมทรง ลาวัณย์ประเสริฐ], อ.ที่ปรึกษาร่วม: [รศ. นวลศรี นิวัติศัยวงศ์] หน้า. ISBN 974-17- 4447-1

บัวบกเป็นพืชในวงศ์ Umbelliferae มีชื่อทางวิทยาศาสตร์ว่า *Centella asiatica* (Linn.) Urban การศึกษานี้ใช้ต้น และใบของบัวบกมาสกัดด้วย 80%เอทานอล และนำมาศึกษาผลกึ่งเฉียบพลันต่อเอนไซโตโครม พี 450 (CYP) ที่เกี่ยวข้องกับการกระตุ้นฤทธิ์ของสารก่อมะเร็ง ได้แก่ CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 และ CYP3A ในตับหนูขาว นอกจากนี้ได้ตรวจค่าเคมีคลินิกและโลหิตวิทยาด้วย การศึกษานี้ใช้หนูขาวเพศผู้พันธุ์วิสตาร์จำนวน 30 ตัว แบ่งโดยการสุ่มเป็น 3 กลุ่ม กลุ่มละ 10 ตัว กลุ่มแรกได้รับน้ำกลั่นโดยป้อนทางปากวันละครั้ง เป็นเวลา 30 วัน เป็นกลุ่มควบคุม กลุ่มที่ 2 และ 3 ได้รับสารสกัดบัวบกด้วยเอทานอลในขนาด 250 มก./ กก./ วัน และ1,000 มก./ กก./ วัน ตามลำดับ โดยการป้อนทางปากวันละครั้ง เป็นเวลา 30 วัน เมื่อครบระยะเวลา ทำให้หนูหมดความรู้สึก เก็บตัวอย่างเลือดจากหัวใจเพื่อตรวจค่าโลหิตวิทยา และแยกซีรัมเพื่อตรวจค่าเคมีคลินิก นำตับมาเตรียมไมโครโซมเพื่อวัดสมรรถนะของเอนไซม์ ผลการทดลองพบว่าสารสกัดบัวบกด้วยเอทานอลไม่มีผลเปลี่ยนแปลง total CYP contents และสมรรถนะของ CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 และ CYP3A เมื่อเปรียบเทียบกับกลุ่มควบคุม สารสกัดบัวบกด้วยเอทานอลไม่มีผลต่อค่าเคมีคลินิก และค่าโลหิตวิทยาดังต่อไปนี้ : glucose, blood urea nitrogen (BUN), creatinine (Cr), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, triglyceride (TG), total cholesterol, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), uric acid, electrolytes (Na, K, Cl), complete blood count (CBC), white blood cell (WBC) count, % differential WBC, platelet count, red blood cell (RBC) morphology, และRBC indices (mean corpuscular volume; MCV, mean corpuscular hemoglobin; MCH, mean corpuscular hemoglobin concentration; MCHC) ผลจากการทดลองนี้แสดงให้เห็นว่า การได้รับสารสกัดบัวบกด้วยเอทานอลแบบกึ่งเฉียบพลันไม่มีผลเพิ่มและ/หรือลดความเสี่ยงของการเกิดพิษ การก่อกลายพันธุ์ และ/หรือ การก่อมะเร็งจากสารแปลกปลอมที่ถูกกระตุ้นโดย CYPs เหล่านี้ รวมทั้งแสดงให้เห็นว่าสารสกัดบัวบกด้วยเอทานอลไม่เกิดอันตรกิริยาระหว่างยาเมื่อได้รับร่วมกับยา/สารใดๆ ที่ถูกเปลี่ยนแปลงโดย CYPs เหล่านี้ จากการที่สารสกัดบัวบกด้วยเอทานอลในขนาดที่มีฤทธิ์ทางเภสัชวิทยาไม่ทำให้เกิดผลพิษใดๆ ต่ออวัยวะ/ระบบที่สำคัญของร่างกาย น่าจะบ่งชี้ว่าบัวบกน่าจะเป็นพืชพื้นบ้านที่มีศักยภาพสูงที่จะนำมาพัฒนาเพื่อใช้เป็นยารักษาโรคได้อย่างปลอดภัยในอนาคต

ภาควิชาเภสัชวิทยา ลายมือชื่อนิสิต.....

สาขาวิชาเภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2546 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEYWORDS: *CENTELLA ASIATICA* / BUA BOK / HEPATIC CYTOCHROME P450 / CLINICAL BLOOD CHEMISTRY

CHATCHANEE PHONGJIT: (SUBACUTE EFFECTS OF *CENTELLA ASIATICA* ETHANOLIC EXTRACT ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS). THESIS ADVISOR: [ASST. PROF. POL. LT. COL. DR. SOMSONG LAWANPRASERT], THESIS CO-ADVISORS : [ASSO. PROF. NAUNSRI NIWATTISAIWONG], [121] pp. ISBN 974-17-4447-1

Bua bok is a plant in family Umbelliferae. It's scientific name is *Centella asiatica* (Linn.) Urban. In this study, stems and leaves of *C. asiatica* were extracted with 80% ethanol. Subacute effect of the extract was investigated on rat hepatic cytochrome P450 (CYP) involving in carcinogenic bioactivation such as CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A. Effects of this extract on clinical blood chemistry and hematology were also determined. Thirty male Wistar rats were randomly divided into 3 treatment groups. Each group comprised 10 rats. Rats in the first group were given water orally once daily for 30 days, serving as a control group. The second and the third groups were given *C. asiatica* ethanolic extract orally at dosages of 250 mg / kg/ day and 1,000 mg / kg/ day for 30 days, respectively. At the end of the treatment period, rats were anesthesized. Blood samples were collected by heart puncture and serum samples were prepared for determining hematology and clinical blood chemistry, respectively. Microsomes were prepared from livers for enzyme assays. The results showed that *C. asiatica* ethanolic extract did not affect total CYP contents and the activities of CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A as compared to the control group. *C. asiatica* ethanolic extract did not produce any changes on these following clinical blood chemistry and hematology : glucose, blood urea nitrogen (BUN), creatinine (Cr), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin, direct bilirubin, triglyceride (TG), total cholesterol, low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), uric acid, electrolytes (Na, K, Cl), complete blood count (CBC), white blood cell (WBC) count, % differential WBC, platelet count, red blood cell (RBC) morphology, and RBC indices (mean corpuscular volume; MCV, mean corpuscular hemoglobin; MCH, mean corpuscular hemoglobin concentration; MCHC). Results from this study implied that *C. asiatica* should not be able to produce an increase and/or decrease risks of toxicities, mutagenicity and/or carcinogenicity from xenobiotics that are bioactivated by these CYPs. Furthermore, drug-drug interactions between *C. asiatica* and other drugs that are metabolized by these CYPs would not be expected. Since *C. asiatica* caused no harmful effects on various important organs/systems at the doses of pharmacologically active, this plant is valuable to be developed for using therapeutically in the future.

Department of Pharmacology	Student's signature……..
Field of study Pharmacology	Advisor's signature……...
Academic year 2003	Co-advisor's signature…….

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List of Abbreviations

β	= beta
°C	= degree celcius
μg	= microgram
μL	= microlitre
μmol	= micromole
μM	= micromolar
α	= alpha
ALP	= alkaline phosphatase
ALT	= alanine aminotransferase
AST	= aspartate aminotransferase
BROD	= benzyloxyresorufin O-dealkylation
BSA	= bovine serum albumin
BUN	= blood urea nitrogen
BW	= body weight
C. asiatica	= *Centella asiatica*
cm	= centimetre
cont'd	= continued
CYP	= cytochrome P450
dL	= decilitre
DMSO	= dimethylsulfoxide
etc.	= et cetera (and the rest)
EDTA	= ethylenediaminetetraacetic acid
EROD	= ethoxyresorufin O-dealkylation
et al.	= et alii (and others)
g	= gram
g	= gravity

List of Abbreviations (cont'd)

G6P	= glucose 6-phosphate
G6PD	= glucose 6-phosphate dehydrogenase
GST	= glutathione S-transferase
h	= hour
Hb	= Hemoglobin
Hct	= Hematocrit
HDL-C	= high density lipoprotein-cholesterol
hRf	= high retention value factor
i.e.	= id est (that is)
i.p.	= intraperitonial
kg	= kilogram
L	= litre
LDL-C	= low density lipoprotein-cholesterol
M	= molar
mg	= milligram
mg/kg	= milligram per kilogram body weight
min	= minute
mm	= millimetre
mL	= millilitre
mM	= millimolar
MROD	= methoxyresorufin O-dealkylation
MW	= molecular weight
NADP	= nicotinamide adenine dinucleotide phosphate
NADPH	= nicotinamide adenine dinucleotide phosphate (reduced form)
nm	= nanometer

List of Abbreviations (cont'd)

nmol	= nanomole
pmol	= picromole
PROD	= pentoxyresorufin O-dealkylation
qs	= quantum sufficit (as much as suffices)
RBC	= red blood cell
rpm	= revolution per minute
SCr	= serum creatinine
SEM	= standard error of mean
SGOT	= serum glutamic oxaloacetic transaminase
SGPT	= serum glutamic pyruvic transaminase
TCA	= trichloroacetic acid
TLC	= thin layer chromatography
Tris	= tris (hydroxymethyl) aminomethane
U	= unit
UDPGT	= uridine 5'-diphospho-glucuronyltransferase
v/v	= volume by volume
vols	= volumes
w/v	= weight by volume
WBC	= white blood cell

CHAPTER I

INTRODUCTION

Centella asiatica (Linn.) Urban is a plant in family Umbelliferae. It has many common names in Thai such as Bua bok, Phak nok, Pa-na-e-khaa-doh, etc. This plant has another synonymous names as Indian Pennywort, Indian Water Navelwort, Asiatic pennywort, Gotu Kola (อรนุช โชคชัยเจริญพร, 2540; คณะกรรมการจัดทำตำรายา, 2545). The plant is a common weed, grown widespread throughout tropics and subtropics, in sunny or slightly shade, along streambanks and also between stones of pathways. Whole plants have been used as food ingredient, beverage and traditional medicine such as treatment of poor memory, Alzheimer's disease, gastrointestinal ulcers, eczema, psoriasis, hypertension, venous insufficiency, cancer, etc. (Caldecott T, 2002). In Thai traditional pharmacopoeia, this plant has been widely used for treatment of skin diseases, wounds, and used as diuretic, antidiarrhoeal, blood purifier, and antisnake venom compound (อรนุช โชคชัยเจริญพร, 2540).

C. asiatica contains many chemical constituents such as flavonoid glycosides (e.g. quercetin-3-glycoside, kaempferol-3-glycoside), free amino acids (e.g. alanine, serine, aminobutyrate, aspartate, glutamate, histidine, lysine, threonine), terpenoids (e.g. mono and sesquiterpene e.g. α-pinene, β-pinene, myrcene, bornyl acetate), triterpenes (e.g. asiaticoside, asiatic acid, brahmic acid, brahmoside, braminoside, centoic acid, centellic acid, madecassic acid, madecassoside) (Carol, et al., 1996).

Physiological and pharmacological properties of *C. asiatica* have been reported. Numerous *in vitro* and *in vivo* studies were performed regarding wound healing effects of either the active ingredients of *C. asiatica* or an extract of *C. asiatica* (Tenni, *et al.,* 1988; Maquart, et al., 1990; Bonte, et al., 1994, Sunilkumar, et al., 1998, Shukla, *et al*., 1999). Effects of the major triterpenic acid (asiatic acid, madecassic acid and asiaticoside) of *C. asiatica* were investigated *in vitro* using human skin fibroblasts. The results showed that

these three compounds increased collagen type I synthesis (Tenni, *et al*., 1988; Maquart, *et al*., 1990; Bonte, *et al*., 1994) as well as increased fibronectin synthesis, thereby facilitating wound healing effects (Tenni, *et a*l., 1988). Various formulations (ointment, cream and gel) of aqueous extract of *C. asiatica* were studied *in vivo* by applying topically on the open wounds in rats. The results demonstrated an increase of cellular proliferation and collagen synthesis at the wound site, thus accelerated wound healing effects (Sunilkumar, *et al*., 1998). Administration of topical solution of asiaticoside (0.2%) in guinea pig punch wounds was shown to produce an increase of hydroxyproline, tensile strength, and collagen content. Futhermore, topical administration of a 0.4% solution of asiaticoside in punch wounds of streptozotocin-induced diabetic rats, in which healing is delayed, increased hydroxyproline content, tensile strength, and collagen content (Shukla, *et al*., 1999a). One another study was performed *in vitro* using chick chorioallantoic membrane model to investigate angiogenic activity of asiaticoside. The result showed that asiaticoside increased angiogenesis (Shukla, *et al*., 1999a). Applying 0.2% asiaticoside to the wound twice daily for 7 days led to an increase of enzymatic and non-enzymatic antioxidants as well as decrease in lipid peroxidation. This effect was suggested to explain the wound healing effect of *C. asiatica* (Shukla, *et al*., 1999b). Oral administration of 250 mg/kg *C. asiatica* aqueous extract before ethanol administration significantly inhibited gastric lesion formation in rats (Cheng and Koo, 2000). Ulcer- protective and anti-ulcer effects of *C. asiatica* extract was also demonstrated in human (อรนุช โชคชัยเจริญพร, 2540). Regarding the effect on central nervous system, asiaticoside derivatives were found to inhibit H_2O_2 induced cell death, lower intracellular free radical concentration and protect against effect of β-amyloid induced neurotoxicity (Mook-Jung, *et al*., 1999). Extract of *C. asiatica* was found to increase brain γ-aminobutyric acid (GABA) levels (Chatterjee, *et al*., 1992). Asiaticoside was shown to possess antibacterial action against *Pseudomonas pyocyaneus, Trichoderma mentagrophytes* and *Entamoeba histolytica* (Brinkhaus, *et al*., 2000). Both the alcoholic and aqueous extract of *C. asiatica* were found to exhibit antiviral action against type II *Herpes simplex* virus (HSV-2) (Zheng, 1989). Oral administration of *C. asiatica* extract in a randomized controlled trial of 87 patients with chronic venous hypertensive microangiopathy, was found to be efficacious with no

appearance of side effects (Cesarone, *et al*., 1994). This extract was also efficacious in the treatment of venous insufficiency, reducing ankle edema and foot swelling (Cesarone, *et al*., 1992). Methanolic extract of *C. asiatica* and its partially purified fractions in acetone retarded the development of Ehrlich ascites tumor cells (EAC) and Dalton's lymphoma ascites tumor cells (DLA) *in vitro.* In the *in vivo* tumor model, Swiss albino mice were transplanted with DLA subcutaneously into the right hind limb for the solid tumor development. The result showed that methanolic extracts (1,000 mg/kg) and partially purified fractions in acetone of *C. asiatica* retarded the development of tumor cells and increased life span of tumor bearing mice (Babu, *et al*., 1995). Fourteen days of giving dietary *C. asiatica* (25%) was shown to cause a significant reduction of aniline hydroxylase activity, but a significant increase of glutathione-S-transferase and UDP-glucuronyltransferase activities in rats (อนงค์ เทพสุวรรณ์, และวรรณี คูสำราญ, 2540).

Adverse reactions following the use of *C. asiatica* have been described. Allergic dermatitis has been reported after topical application of various formulation of *C. asiatica* such as creams and ointments (Eun and Lee, 1985; Izu *et al*., 1992). In an animal study, toxic doses were shown to be 40 to 50 mg/kg body weight when *C. asiatica* extract was administered intramuscularly to mice and rabbits (Brinkhaus, *et al*., 2000). Maximum tolerated dose (MTD) in mice was reported by Dhar, et al. (1968) to be 250 mg/kg/body weight when *C. asiatica* extract was given intraperitoneally.

C. asiatica is a traditional plant of which stems and leaves are used for preparing beverage, being consumed as food and taking as traditional medicine. In addition, numerous studies reported that this plant possesses several pharmacological properties indicating a high potential of this plant to be developed therapeutically. Such the pharmacological effects of this plant are wound healing effect, antiulcer effect, prevention /delaying of alzheimer disease and antitumor effects. Administration of this plant either as medicine for the mentioned diseases or as diet/beverage, repeated exposure is certainly occurred. Repeated exposure to any compounds may/may not affect hepatic drug metabolizing enzymes. If *C. asiatica* modulates hepatic drug metabolizing enzymes, administration of this plant should be awared in term of drug-drug interaction if it is taken

simultaneously with other medicines metabolized by those enzymes. Furthermore, effect of this plant on the phase I drug metabolizing enzymes, cytochrome P450 (CYP), especially the isoforms which are involved in xenobiotic bioactivation is important in an aspect of the potential effect of this plant to increase/decrease risks of other xenobiotic-induce toxicities, mutagenesis and/or carcinogenesis. Generally, CYP isoforms in family 1, 2 and 3 play an important role in biotransformation of various xenobiotic compounds to toxic metabolites, mutagens and/or carcinogens (Soucek and Gut, 1992). Inhibition of these CYP isoforms are partly a key aspect explanation for antimutagenic and anticarcinogenic potential of *C. asiatica*. Induction of these CYP isoforms, in contrast, repeated exposure of *C. asiatica* may increase risk of carcinogenesis from xenobiotics that are bioactivated by the CYP isoforms which are modulated. Therefore, this study focused on the subacute (30 days) effects of *C. asiatica* on some CYP isoforms involving in activation reactions of chemical carcinogens such as CYP1A1, CYP1A2, CYP2B1/2B2, CYP2E1 and CYP3A. Since subacute toxicity of *C. asiatica* have not yet been reported, clinical blood chemistry and hematology of rats were also determined.

Hypothesis

Subacute exposure of *C. asiatica* caused an induction and/or inhibition of hepatic CYPs as well as changes of clinical blood chemistry in rats.

Anticipated benefit from the study

A preliminary data demonstrated an induction and/or inhibition effects of *C. asiatica* on hepatic CYPs, especially CYP isoforms involved in bioactivation of drugs, chemicals, and environmental pollutants resulting in reactive metabolites. Therefore, it would be useful for considering the possibility of *C. asiatica* to increase and/or decrease risks of chemical induced toxicities, mutagenesis, and/or carcinogenesis. In addition, drug-drug interaction potential should be awared if *C. asiatica* is cocomittantly consumed with other drugs that are metabolized by these CYPs. Effects of *C. asiatica* on clinical blood chemistry would be an additional data of subacute toxicity for this plant in rats.

Study design and process

1. Preparation of *C. asiatica* ethanolic extract and chemical identification tests
2. An *ex vivo* study
    2.1 Animal dosing for 30 days
    2.2 Blood collecting
    2.3 Preparation of liver microsomes
    2.4 Determination of clinical blood chemistry
    2.5 Determination of hepatic microsomal total CYP contents and CYP activities
3. Data collecting and analysis
4. Writing a thesis

CHAPTER II

LITERATURE REVIEW

Centella asiatica

Centella asiatica (Linn.) Urban (Figure 1), a plant belonging to family Umbelliferae, is generally grown in tropical and subtropical countries including Thailand. It is also known as Bua bok, Asiatic pennywort, Pa-na-e-khaa-doh (Karen-Mae Hong Son), Phak nok (Northern) (**อรนุช โชคชัยเจริญพร**, 2540). This plant is a perennial creeping herb that has flowers during August and September. Its flowers are of light violet color. The gray to brownish-green dried *C. asiatica* has smell that is reminiscent of tobacco leaves, with a mildly bitter taste. Its leaves have long petioles arising rosette-like from a common base (the node), and the individual "leaf rosettes" (the nodes) are connected by slender aerial stolons or runners. Its leaves are thin and soft, with palmate nerves, hairless or with only a few hairs, with about 2 to 5 cm in diameter. The leaf margin is crenate or slightly lobed. The petioles are between 5 and 15 cm in length, slender and hairless or bear only a few scattered hairs. The short-pediceled umbels arise in the leaf axils. The 2 to 5 fruits of each umbel are enclosed within a pericarp comprising 1 to 2 cm-large elliptical bracts. The 2 schizocarps are attached together by narrow connecting ridges. Their surface shows a clearly reticulate pattern (Brinkhaus, *et al*.,2000).

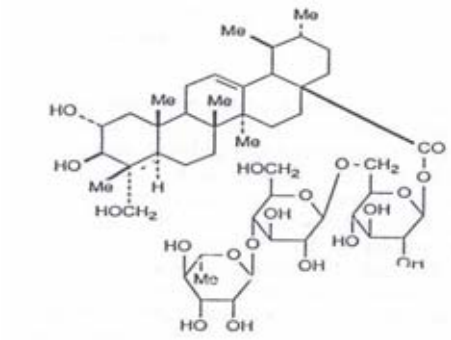
C. asiatica is usually used in Thai as traditional medicine for treatment of skin diseases, wounds, and vaginomycosis; as cardiotonic, diuretic, antidysentery, antidiarrhoeal, blood purifier, and antisnake venom (**อรนุช โชคชัยเจริญพร**,2540). Besides using as a traditional medicine, herbs of *C. asiatica* are also consumed as food and beverage.

C. asiatica contains various compounds. These compounds can be classified on the basis of their chemical structures as following (Carol, et al., 1996).

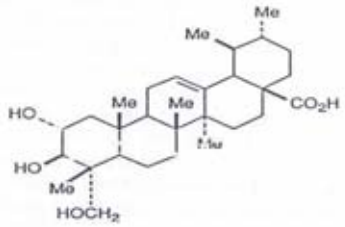
1. Flavonoid glycosides: include
   1.1 Quercetin-3-glycoside
   1.2 Kaempferol-3-glycoside
2. Free amino acids: include
   2.1 Alanine
   2.2 Serine
   2.3 Aminobutyrate
   2.4 Aspartate
   2.5 Glutamate
   2.6 Histidine
   2.7 Lysine
   2.8 Threonine
3. Terpenoids (mono and sesquiterpene): include
   3.1 α-pinene
   3.2 β-pinene
   3.3 Myrcene
   3.4 Bornyl acetate
4. Triterpenes (Figure 1) : include
   4.1 Asiaticoside
   4.2 Asiatic acid
   4.3 Braminoside
   4.4 Centoic acid
   4.5 Centellic acid
   4.6 Madecassic acid
   4.7 Madecassoside

   The chemical structures of some triterpenes were shown in figure 2.

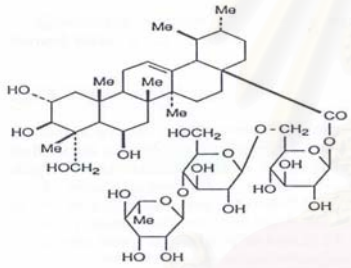
Figure 1 Herbs of *C. asiatica* (Morrisville, 2003)



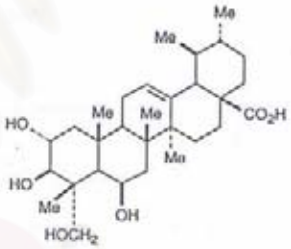
Asiaticoside



Asiatic acid



Madecassoside



Madecassic acid (Brahmic acid)

Figure 2 Chemical structures of some triterpenes in *C. asiatica* (Asean Countries, 1993)

Physiological and pharmacological effects

1. Wound healing effects

Numerous studies were performed regarding the wound healing effects of *C. asiatica*. Those included the *in vivo* experiments in animals/human and the *in vitro* experiments using human fibroblasts (Marquart, *et al*., 1990; Tenni, *et al*., 1988; Lawrence, 1967; Vogel, *et al*., 1990; Sunilkumar, *et al*., 1998; Shukla, *et al*.,1999). Topical application of *C. asiatica* extracts has been shown to be associated with accelerated wound healing in abnormal conditions of skin with a reduction in granuloma weight, and an increase in the force needed to produce rupture (rupture strength) of the wound (Vogel, *et al*., 1990). A dose-dependent increase in collagen systhesis, intracellular fibronectin content, mitotic activity of the germ layer as well as an enlargement of the kerato hyaline granules in the scar tissue were shown following treatment with topical *C. asiatica* (Marquart, et al., 1990; Tenni, *et al*., 1988; Lawrence, 1967; Sunilkumar, *et al*., 1998; Shukla, *et al*.,1999)

Marquart, *et al*. (1990) investigated the wound healing properties of the extract of *C. asiatica* (TECA, tritrated extract of *C. asiatica*) on fibroblasts obtained from human prepared and cultured *in vitro*. They found a dose-dependent increase in collagen synthesis and in the intracellular free proline content. In contrast, a simultaneous decrease in the neosynthesis of collagen was observed. The mechanism of collagen synthesis stimulation by TECA is not known.

Tenni, *et al*. (1988) investigated the biochemical changes in wound healing properties after application of the extract of *C. asiatica* (containing TTFCA; Total triterpenoid fraction of *C. asiatica*) in cultures of human skin fibroblasts. A statistically significant increase in collagen synthesis and intracellular fibrin content was detected.

Lawrence (1967) investigated the wound healing and mortality rates of mice with induced skin burns follow by an intraperitoneal therapy with asiaticoside mixture. This study revealed no significant differences in terms of wound healing and mortality rates between the mixture of asiaticosides and the reference substance (saline solution/propylene glycol). These results suggest that the asiaticoside mixture has no positive effect on normal wound healing in the skin, while in the case of abnormal skin

conditions (for example; ulcers, leprous lesions) asiaticoside mixture appears to exercise a regulatory effect.

Effect on wound healing of the major saponin containing triterpenic acids (asiaticosides, madecassoids, asiatic acid and madecassic acid) was investigated by Vogel, et al. (1990) in the connective tissue of rats. Following subcutaneous implantation of glass rods, rats were administered the four isolated triterpenic acids, either orally or subcutaneously. Three weeks after implantation, irrespectively to the form of the triterpenic acids administration, weight of the granuloma of scar tissue was seen to be dose-dependently reduced. In addition, both the rupture strength and, even more so the tensile strength, of the scar tissue were seen to be increased. The demonstrable increase in the rupture strength and tensile strength was associated with an increase in the collagen content of the scar tissue, as compared to the uninjured tissue. Increase of collagen content was considered to be the possible cause of the improvement in the mechanical properties of the scar tissue. After oral administration, both asiatic and madecassic acids were proved to have greater effects on the parameters investigated than did the glycosides. With respect to molecular weight, however, all the isolated fractions were about equally effective.

Sunilkumar, *et al*. (1998) investigated the effects of various formulations (ointment, cream and gel) of aqueous extract of *C. asiatica*, when applied topically, three times daily for 24 days on the open wounds in rats. The results showed that *C. asiatica* of all formulation increased cellular proliferation and collagen synthesis at the wound site, as evidenced by an increase in collagen content and tensile strength. The treated wounds epithelialised faster and the rate of wound contraction was higher as compared to the control wounds. The process of healing was better with gel formulation when compared to other two formulations.

Administration of a topical solution of asiaticoside (0.2%) to guinea pig punch wounds, produced a 56% increase in hydroxyproline, a 57% increase in tensile strength, increased collagen content and better epithelisation than those of the control. Similar increases were also found with the oral administration of asiaticoside (1 mg/kg) in guinea pigs with punch wounds. The topical administration of a 0.4% solution of asiaticoside in punch wounds of streptozotocin diabetic rats, in which healing is delayed, increased

hydroxyproline content, tensile strength, collagen content and epithelisation (Shukla*, et al*.,1999a). One possible explaination for the wound healing effects of *C. asiatica* was found by a study of Shukla, *et al* (1999b). In that study, application of 0.2% asiaticoside twice daily for 7 days to excision-type cutaneous wounds in rats led to an increase of enzymatic and non-enzymatic antioxidants, namely superoxide dismutase (35%), catalase (67%), glutathione peroxidase (49%), vitamin E (77%) and ascorbic acid (36%) in newly formed tissues. It also resulted in a several fold decrease in lipid peroxidation levels (69%) as measured in terms of thiobarbituric acid reactive substance.

2. Ulcer-protective and anti-ulcer effects

Two studies in animals revealed a dose-dependent significant effect of *Centella* extract on gastrointestinal ulcers, which was associated with an acceleration of healing lesions developing after administration of the extract (Chatterjee, *et al*., 1992; Cheng and Koo, 2000)

Effect of alcoholic extract of *C. asiatica* on ulcers which were induced either by application of cold or by stress in rats was investigated by Chatterjee, *et al*. (1992). The results demonstrated a dose-dependent significant anti-ulcerative effect of the *Centella* extract. This action of the plant extract was comparable with the anti-ulcerative properties of H_2 receptor blockers (famotidine) and antiepileptic agents (valproic acid).

Using a gastric *ex vivo* chamber model, gastric transmucosal potential difference was reduced by the application of 50% ethanol but *Centella* extract accelerated its recovery. Oral administration of *Centella* extract (0.05 g/kg, 0.25 g/kg and 0.50 g/kg) before ethanol administration, significantly inhibited gastric lesions formation (58% to 82% reduction) and decreased mucosal myeloperoxidase activity in a dose dependent manner. These results suggested that *Centella* extract prevented ethanol induced gastric mucosal lesions by strengthening the mucosal barrier and reducing the damaging effects of free radicals (Chen and Koo, 2000).

3.Central nervous system

It was shown that alcoholic *Centella* extract caused a dose-dependent increase of GABA level in rats. The effect of *Centella* extract and the anticonvulsant agent valproic

acid on this inhibitory transmitter in the central nervous system was inhibited by the specific GABA-antagonist bicuculline methionine. When the effect of GABA was inhibited by bicuculline methionine, no anti-ulcerative effect of either *Centella* extract or valproic acid was demonstrable (Chatterjee, *et al*.,1992).

Asiaticoside derivatives were found to inhibit or reduce H_2O_2 induced cell death and lower intracellular free radical concentration, resulting in protective effects against beta-amyloid neurotoxicity (Mook-Jung, *et al*., 1999).

4. Antimicrobial and antiviral effects

Pharmacological data regarding an antibacterial effect of *C. asiatica* against *Pseudomonas pyocyaneus, Trichoderma mentagrophytes* and *Entamoeba histolytica* were available (Tschesche and Wulff, 1965; Dhar, *et al*., 1968). Both alcoholic and aqueous extract of *C. asiatica* were also demonstrated an antiviral action against type II *Herpes simplex* virus (Zheng, 1989).

Tschesche and Wulff (1965) investigated an antimicrobial efficacy of 10 mg/ml asiaticoside in the agar diffusion tests against 7 microorganisms. An antibacterial effect was found against *Pseudomonas pyocyaneus* and *Trichoderma mentagrophytes.* Minimal inhibition concentration of asiaticoside was 1,000 μg/ml in both *Pseudomonas pyocyaneus*, and *Trichoderma mentagrophytes.* Asiaticoside exhibited no antibacterial effect against *Staphylococcus aureus*, *E. coli, Candida albicans* and *Aspergillus niger.*

An alcoholic extract of *C. asiatica* was investigated by Dhar, *et al*. (1968) for possible effects of antibacterial, antiviral, antifungal, antihelminthic, antiprotozonal, antispirochaetal using standardized test procedures. The results revealed an antibacterial effect only against *Entamoeba histolytica*.

Herbert, *et al*. (1994) investigated the *in vitro* effects of *C. asiatica* dried powder on the acid-fast membrane and the viability of the bacterium *Mycobacterium leprae*. The *Centella* powder had no effect on either the acid-fast membrane or the viability of *Mycobacterium leprae*.

Zheng (1989) investigated the antiviral efficacy of an alcoholic and an aqueous extract of *C. asiatica* against Type 2 *Herpes simplex* virus (HSV-2) in the virus-inhibition logarithm test. The results showed that the aqueous *Centella* extract develops a high

antiviral activity against the HSV-2. In contrast, the alcoholic extract was only weakly active against the HSV-2 virus.

5. Effects on cardiovascular system

Montecchio, et al. (1991) investigated the number of cells described as endothelial cells (EC) in patients with post–thrombotic syndrome (n=15), in comparison with healthy subjects (n=15). The EC were counted under the phase-contrast microscope prior to and following three-weeks of treatment with oral CATTF (*Centella asiatica* triterpenic fraction / Centellase^{R} / 3x30 mg/day). Patients with post-thrombotic syndrome initially revealed a significant increase in the EC. Following treatment with CATTF, a significant reduction of EC was demonstrated. It was conducted that the CATTF–induced reduction in the number of endothelial cells has a protective effect on the integrity of the intima of the veins.

Oral administration of 60 and 120 mg/day *C. asiatica* extract in a randomized controlled trial of 87 patients with chronic venous hypertensive microangiopathy was found to be efficacious, without side effects (Cesarone, *et al.,*1994). *C. asiatica* (60 and 120 mg/day) was found to be efficacious in a clinical trial for the treatment of venous insufficiency, reducing ankle, edema and foot swelling, as well as improving capillary filtration rate and microcirculatory parameters (Cesarone, *et al*.,1992).

Pointel, *et al*. (1987) investigated, in a randomized, multicentre, placebo-controlled double-blind study, the effect of *Centella* extract (titrated extract of *Centella asiatica* / TECA) in 94 patients with venous insufficiency. Over a period of two months, the pateints received either TECA in two different doses (60 mg/day; 120 mg/day), or a placebo. The results were evaluated both subjectively by the pateint on the basis of symptoms, and also objectively by means of plethysmograph. At both doses of TECA, significant improvements in both the subjective and plethysmographic parameters were observed. The differences in the effect of the different TECA doses were not significant, but did reveal a dose-effect relationship. Analysis of clinical tolerance revealed no statistical differences between the treatment groups.

In a double-blind, placebo-controlled study, Belcaro, *et al*. (1990a) investigated the effects of *Centella* extract (total triterpenic fraction of *C. asiatica* (TTFCA) on the capillary filtration rate (CFR) in 62 patients with venous hypertension (venous pressure: > 42 mm Hg) and in healthy subjects. In addition to the CFR measured by plethysmography,

ankle oedema and changes in the symtoms following TTFCA administered at two different doses (90 mg/day; 180 mg/day) were investigated in comparison with placebo. At the beginning of the study both the CFR and the ankle circumference of the pateints were significantly greater than the control group. At the end of the 4-week period of treatment with TTFCA, a dose-dependent significant reduction in the CFR and ankle circumference was observed. In comparison with the placebo group, the dose-dependent improvement seen in the TTFCA group was also significant.

In a subsequent study, Belcaro, *et al*. (1990b) investigated the effects of the *Centella* extract, TTCFA, on capillary permeability and the microcirculation of patients with mild (n=22) and severe (n=12) venous hypertension involving lower extremities, and in healthy subjects (n=10). The capillary permeability and the microcirculation were measured with the aid of a vacuum suction chamber-test in conjunction with a Doppler examination prior to and following 2 weeks of treatment with TTFCA at dosage of 180 mg/day or placebo. At the beginning of the treatment, the patient group had a significantly higher capillary permeability than did the healthy subjects. Two weeks of treatment with TTFCA, patients exhibited a significant reduction in capillary permeability associated with a significant improvement in the microcirculation and the clinical symptoms.

6. Antitumor effect

Researchers at the Amala Cancer Research Centre in Kerala, India, tested both a crude 80% methanolic extract of *C. asiatica* (CE) and its partially purified fractions (AF) for their antitumor activity. AF significantly inhibited the proliferation of the transformed cell lines in Ehrlich ascites tumor cells and Dalton's lymphoma ascites tumor cells with no toxic effects on normal human lymphocytes. AF was also found to inhibit the development of mouse lung fibroblast. Oral administration of both CE (1 g/kg) and AF (400 mg/kg) retarded the development of solid and ascites tumors, and increased the life span of tumor bearing mice. Tritiated thymidine, uridine and leucine incorporation assay suggest that the fraction acts directly on DNA synthesis. (Babu, *et al*.,1995)

Tepsuwan and Kusamran (1997) investigated an effect of dietary *C. asiatica* (25% of *C. asiatica* in diet for 14 days) on the level of hepatic phase I enzymes, cytochrome

P450 and its dependent monooxygenase including aniline hydroxylase (ANH) and aminopyrine-N-demethylase (AMD) as well as the capacities of S9 fractions to activate the mutagenicities of aflatoxin B1 (AFB1), benzo(a)pyrene (BP) and 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) toward *Salmonella typhimurium* and phase II (glutathione-S-transferase (GST) and UDP-glucuronosyltransferase (UGT) metabolizing enzymes in male Wistar rats. They found that dietary *C. asiatica* leaves caused a significant reduction of ANH activity (67% of the control). The capacity of S9 fractions to activate the mutagenicity of AFB1 was observed. Interestingly, feeding with diets containing *C. asiatica* markedly increased the activity of phase II enzymes in rat liver, such as the activities of GST (160% as compared to the control group) and UGT (180% as compared to the control group). From this study, *C. asiatica* inhibited some CYP isoforms (inhibited CYP2E1, which was indicated by a reduction of aniline 4-hydroxylation) , did not affect AMD activity (which represented the activity of CYP2B1, 2C11) as well as induced the important phase II detoxification enzymes (GST and UGT) while activated the mutagenicity of AFB1 (which was bioactivated by several CYPs such as CYP1A1, 2B1, 2C11, 2C12 and 3A) *in vitro*. Thus, the authors in this study suggested that using the effects of this plant on drug metabolizing enzymes performed in this study, to explain its anticarcinogenic effect was unsufficient and inconclusive.

Toxicological effects

Toxicological effects of *C. asiatica* were investigated in animals. Acute toxicity was not shown when 80% methanolic extract of *C. asiatica* was orally administered at dosage of 1g / kg body weight to normal and transplanted tumor bearing mice. No any toxic symptoms were shown even at higher concentrations (i.e. 500 mg/mice that is approximately equal to 5 g/ kg body weight) but turned out to increase body weight of the animals (Babu, *et al*., 1995). Toxic effects were shown at doses of 40 to 50 mg/kg body weight when 50% ethanolic extract was administered intramuscularly to mice or rabbit (Brinkhaus, *et al*., 2000). Following intraperitoneal administration, maximum tolerated dose (MTD) of 50% ethanolic extract of *C. asiatica* in mice was found to be 250 mg/kg/body weight (Dhar, *et al*., 1968). A study of Tepsuwan and Kusamran (1997) found that 14-day

dietary of *C. asiatica* (25%) given to male Wistar rats of weighing 60-70 g, significantly increased the capacity of S9 fraction to activate the mutagenicity of aflatoxinB1.

Adverse reactions following the use of *C. asiatica* have been described. Allergic dermatitis was reported after topical application of various creams and ointments of aqueous extract of *C. asiatica* (Eun and Lee, 1985; Izu *et al*., 1992).

Xenobiotic Metabolism

Xenobiotic metabolism is a biological process which alters many exogenous substances leading to termination or alteration of biological activity. In general, lipophilic xenobiotics are transformed to more polar and hence more readily excreatable products. The principle organ of metabolism is liver. Other tissues that display this function include gastrointestinal tract, lungs, skin, and kidneys.

Metabolism is normally divided into two phases, phase I (or functionalisation reactions) and phase II (or conjugative reactions). Phase I reaction is a preparation of drug for phase II reaction. Phase I reaction usually converts parent xenobiotic to a more polar metabolite by introducing a functional group (-OH, -NH_2, -SH etc.). Phase II reactions are usually the detoxification pathways. Chemical reactions associated with phase I and phase II metabolisms are given in Table 1.

Cytochrome P450 (CYP) genes encode for a multigene superfamily of mixed-function oxidase (MFO) responsible for phase I oxidative metabolism of a wide range of structurally diverse substrates. Substrates metabolized by P450 system are ranged from small molecular weight compounds such as methanol to large molecules such as cyclosporin A. CYPs were classed as heme-thiolate enzymes which are components of an electron transfer system in smooth endoplasmic reticulum of liver and other tissues. CYP-catalyzed reactions require CYP, NADPH-CYP reductase, and molecular oxygen. Metabolic products are often less active than the parent compounds or inactive. However, some metabolites have enhanced activities or toxic properties, including mutagenicity, teratogenicity, and carcinogenicity.

Table 1 **Reactions classed as phase I and phase II metabolism**
(Gibson and Skett, 1994)

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

CYPs that are normally known to be involved in chemical toxicities, mutagenesis, and/or carcinogenesis are in family 1, 2, and 3. Those isoforms include CYP 1A1, 2A1, 2B1, 2B2, 2C11, 2C12, 2E1, 3A1 and 3A2 in rats (Table2). CYP1A2, 2A3, 2B7, 2C9, 2E1, 3A, and 3A4 are isoforms found in humans which are involved in metabolic activation of various xenobiotics (Table2).

CYP1A subfamily comprised two members, CYP1A1 and CYP1A2. This subfamily is involved in metabolism of various procarcinogens [such as polycyclic aromatic hydrocarbon (PAHs), heterocyclic compounds, aromatic amines] to be genotoxic carcinogens (Omiecinski, *et al*., 1999). Expression of CYP1A1 occur in liver and other tissues such as lung and placenta. PAHs such as benzo[a]pyrene are CYP1A1 substrate and inducer (Gibson and Skett, 1994). The induction of CYP1A1 by xenobiotic is mediated by a ligand-activated transcription factor receptor, called aryl hydrocarbon receptor (AhR), to increase protein synthesis and gene transcription (Wen, 2002). Expression of CYP1A2 is mainly in liver. CYP1A2 is able to activate many procarcinogens including aflatoxin B1, 2-acetylaminofluorene, a number of arylamines, and food-derived aminoimidazoazarenes. Other substrates of CYP1A2 include caffeine, theophylline, phenacetin, 7-ethoxyresorufin, and R-warfarin (Levy, *et al*., 2000).

Table 2 Role of CYP isoforms involved in mutagenesis/carcinogenesis (Soucek and Gut, 1992).

CYP	Substrate → Mutagenic metabolite suggested	References
Rats		
1A1	P BP → BP-4, 5-oxide	Robertson, *et al*., 1983
	P BP-7,8-diol → B,P-7,8-diol,9,10-epox	Robertson, *et al*., 1983
	P DMBA → DMBA-3,4-diol,1,2-epoxide	Kato, 1986
	P NA → *N*-hydroxy-NA	Kawano, *et al*., 1985
	P AF → *N*-hydroxy-AF	Kawano, *et al*., 1985
	P AAT → *N*-hydroxy-AAT	Shimada and Nakamura, 1987
	P AAF → *N*-hydroxy-AAF	Shimada and Nakamura, 1987
	P MC → 1-hydroxy-MC	Shimada and Nakamura, 1987
	P AαC, MeAαC[a]	Shimada and Nakamura, 1987
	P Glu-P-1, Glu-P-2, Trp-P-1,Trp-P-2[a]	Shimada and Nakamura, 1987
	P DBA → DBA-3,4-diol	Shimada and Nakamura, 1987
	P Paracetamol → quinone-imines	Steele, *et al*., 1983
	P IQ, MeIQ, MeIQx[a]	Yamazoe, *et al*., 1984
	P MOCA[b]	Butler, *et al*., 1989
	P ABP → *N*-hydroxy-ABP	Butler, *et al*., 1989
	P PhIP → 2-hydroxyamino-PhIP	Wallin, *et al*., 1990
	P MAB → *N*-hydroxy-MAB	Kimura, *et al*., 1985
	P NA → *N*-hydroxy-NA	Hammons, *et al*., 1985
	P AFB1 → AFB1-2,3-oxide	Robertson, *et al*., 1983
	P ABP → *N*-hydroxy-ABP	Masson, *et al*., 1983
	P Aminoanthracene[b]	Shimada and Nakamura, 1987
2A1	E *N*-hydroxy-AAF[b]	Aoyama, *et al*., 1989a
	P ABP → *N*-hydroxy-ABP	Butler, *et al*., 1989
2B1	P AFB1 → AFB1-2,3-oxide	Robertson, *et al*., 1983
	P BP → BP-7,8-diol,9,10-epoxide	Kato, 1986

Table 2 Role of CYP isoforms involved in mutagenesis/carcinogenesis (cont'd).

CYP	Substrate → Mutagenic metabolite suggested	References
	M AF → *N*-hydroxy-AF	Kawajiri, *et al*., 1980
	M NDMA → *N*-hydroxy-NDMA	Kawajiri, *et al*., 1980
	M MC → 1-hydroxy-MC	Kawajiri, *et al*., 1980
	P AAF → *N*-hydroxy-AAF	Shimada and Nakamura, 1987
	P DBA → DBA-3,4-diol	Shimada and Nakamura, 1987
	P Aminoanthracene[b]	Shimada and Nakamura, 1987
	P MOCA[b]	Butler, *et al.,* 1989
	P ABP → *N*-hydroxy-ABP	Butler, *et al*., 1989
2B2	P MOCA[b]	Butler, *et al*., 1989
2C11	P AFB1 → AFB1-2,3-oxide	Shimada and Nakamura, 1987
	P MOCA[b]	Butler, *et al*., 1989
	P ABP → *N*-hydroxy-ABP	Butler, *et al*., 1989
	P PhIP → 2-hydroxyamino-PhIP	Wallin, *et al*., 1990
2C12	P AFB1 → AFB1-2,3-oxide	Shimada and Nakamura, 1987
2E1	P NDMA → nitrosamine	Levin, *et al*., 1986
3A1	M AFB1 → AFB1-2,3-oxide	Halvorson, *et al*., 1988
3A2	P SN → DHP	Williams, *et al*., 1989
Human		
1A2	E AFB1 → AFB1-2,3-oxide	Aoyama, *et al*., 1990a
	P DMeIQx[a]	Aoyama, *et al*., 1990b
	P IQ, MeIQ, MeIQx[a]	Shimada, *et al*., 1989
	P Glu-P-1, Glu-P-2, Trp-P-1,Trp-P-2[a]	Shimada, *et al*., 1989
	P AA → *N*-hydroxy-AA	Shimada, *et al*., 1989
	P AF → *N*-hydroxy-AF	Shimada, *et al*., 1989
	P AAF → *N*-hydroxy-AAF	Shimada, *et al.,* 1989
	P 2-aminoanthracene[b]	Shimada, *et al*., 1989
	P ABP → *N*-hydroxy-ABP	Shimada, *et al.,* 1989

Table 2 Role of CYP isoforms involved in mutagenesis/carcinogenesis (cont'd)

CYP	Substrate → Mutagenic metabolite suggested	References
2A3	E AFB1 → AFB1-2,3-oxide	Aoyama, *et al*., 1990a
	E BP→ BP-7,8-diol,9,10-epoxide	Crespi, *et al*., 1990
	E NDMA → N-demethylation	Crespi, *et al*., 1990
	E NDEA → N-demethylation	Crespi, *et al.,* 1990
2B7	E AFB1 → AFB1-2,3-oxide	Aoyama, *et al*., 1990a
2C9	E IQ, MeIQ[a]	Aoyama, *et al*., 1990b
2E1	M NDMA → N-demethylation	Yoo, *et al*., 1988
	M NBzMA → N-demethylation	Yoo, *et al*., 1988
	M NBuMA → N-demethylation	Yoo, *et al*., 1988
	M NDEA → N-demethylation	Yoo, *et al*., 1988
3A3	E AFB1 → AFB1-2,3-oxide	Aoyama, *et al*., 1990a
3A4	E AFB1 → AFB1-2,3-oxide	Aoyama, *et al*., 1990a
	E IQ, MeIQ, DMeIQx[a]	Aoyama, *et al*., 1990b
	P AFG1[b]	Shimada, *et al*., 1989
	P BP→ BP-7,8-diol,9,10-epoxide	Shimada, *et al*., 1989
	P 6- aminochrysene[b]	Shimada, *et al*., 1989
	P sterigmatocystin[b]	Shimada, *et al*., 1989
	P tris(DBP) → 2-bromacrolein	Shimada, *et al*., 1989

Abbreviations used: E- cell-expressed P-450, M-microsomes, P-purified P-450

Substrates: AAF-2-acetylaminofluorene, AAT-o-aminoazotoluene, ABP-4-aminobiphenyl, AαC-2-amino-9H-pyrido[2,3-b]indole, AFB1-aflatoxin B1, AF-2,-acetylfluorene, BP-benzo(a)pyrene, DBA-1,2,3,4-dibenzanthracene, DMBA-7,12-dimethylbenz(a)anthracene, DHP-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine, DmeIQx-2,-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline, Glu-P-1-2-amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole, Glu-P-2-2-aminodipyridol[1,2-a: 3',2'-d]imidazole, IQ-2-amino-3-methylimidazo[2,3-b]indole, MeIQ- 2-amino-3,5-dimethylimidazo[4,5-f]quinoline, MeIQx-2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline, MC-3-methylcholanthrene, MOCA-4,4'-

(bis)methylene chloroaniline, NA-2-naphthylamine, NbuMA-*N*-nitroso-*N*-butyl-*N*-methylamine, NbzMA- *N*-nitroso-*N*-benzyl-*N*-methylamine, NDEA- *N*-nitroso-*N*-diethylamine, NDMA-*N*-*N'*-nitrosodimethylamine, PhIP-2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, SN-senecione, tris(DBP)-tris(2,3-dibromopropyl)-phosphate, Trp-P-1-3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole, Trp-P-2-3-amino-1-methyl-5H-pyrido[4,3-b]indole.

[a] Food-derived heterocyclic aromatic amines are metabolized to highly mutagenic products via *N*-hydroxylation (Snyderwine and Battula, 1989)

[b] Data regarding mutagenic metabolite not available.

CYP2B subfamily comprises 17 different members, identified in several different species. CYP2B1 and CYP2B2 are primary members expressed in rats, whereas CYP2B6 is expressed at low levels in human liver. In rodents, enzymes in this subfamily are typically inducible by phenobarbital and other barbiturates, and are inhibited by metyrapone. CYP2B members are involved in metabolism of a variety of pharmaceutical compounds, such as amphetamines, benzodiazepines etc. CYP2B6 appears to bioactivate 6-aminochrysene and the antineoplastic drugs cyclophosphamide. Pentoxyresorufin and benzyloxyresorufin are often used as substrate probes to measure CYP2B activity *in vitro* (Omiecinski, *et. al*. 1999).

CYP2E1, the ethanol inducible CYP, is expressed in many mammalian species such as human, rat, hamster, mouse, and rabbit. The highest enzyme concentrations are found in liver. Over 70 substrates have been shown to be metabolized by CYP2E1. Most substrates are procarcinogens (benzene, nitrosamines, azoxymethan, vinyl chloride, vinyl carbamate) or toxicants (organic solvents). Only a few substrates are drugs (isoniazid, paracetamol). In human, CYP2E1 accounted for approximately 6% of total hepatic CYP. This isoform can vary up to 20-fold among individuals, probably due to induction by environmental factors. Mechanisms of CYP2E1 induction include transcriptional activation, mRNA stabilisation, translation efficiency, and enzyme stabilisation (Berthou, 2001).

CYP3A subfamily members are accounted for 30% of total CYP content in human livers (Taavitsainen, 2001). The relevant human CYP3A members include CYP3A4,

CYP3A5, and CYP3A7. CYP3A4 and CYP3A5 are expressed in liver and in intestinal mucosa. CYP3A5 is a polymorphic form and is expressed in human livers and intestines (Omiecinski, *et al*. 1999). CYP3A7 is represented in fetal liver, adult endometrium, and placenta (Taavitsainen, 2001). Enzymes in CYP3A subfamily are induced by rifampicin, barbiturates, and to a lesser extent by carbamazepine, phenytoin, and dexamethasone (Omiecinski, *et al*. 1999). These enzymes can also be inhibited by a number of compounds including azole antifungals, macrolide antibiotics, gestodene, ethynylestradiol, statins, and dihydropyridine calcium channel blockers (Zuber, *et al*. 2002). This subfamily of enzymes is responsible for metabolisms of a large and diverse group of substrates.

Mechanism of induction of CYPs (Berthou, 2001)

Induction is defined as an increase in amount and catalytic activity of CYP. Classically, definition of induction is a *de novo* synthesis of new enzyme molecules as a result of an increase transcription of its gene after stimulation by an appropriate chemical signal. An increase in enzyme activity due to activation is not usually included under the term of induction.

A considerable diversity has been depicted in the mechanisms of regulation of CYP. The most common mean of regulation is the transcriptional step. Post-transcriptional mechanisms include mRNA stabilisation and protein stabilisation protected from degradation by the substrate binding at the active site. Mechanisms of induction known to date for different CYPs are shown in Table 3.

CYP induction usually enhances detoxification; thus, under most conditions, induction is protective mechanism. Induction is likely to be advantageous in the evolution of species, allowing enhanced detoxification following exposure to xenobiotics. However, under some circumstance (like imbalance between phase I and II enzymes after induction), CYPs activate xenobiotic to carcinogenic, mutagenic, and/or cytotoxic products. Toxicity depends upon balance between phase I and II enzymes. If phase II

enzymes are depleted, xenobiotics are activated and the electrophilic intermediates react with nucleophilic cellular macromolecule.

Table 3 **Mechanisms of induction known to date for different CYPs (Berthou, 2001)**

Induction mechanism	CYPs known to be induced
Gene transcription through receptors	1A1 (cytosolic AhR), 1A2, 1B1 2A6, 2B6 (CAR), 2C8, 2C9, 2C18, 2C19 3A4, 3A5 (nuclear receptors PXR and SXR) 4A11 (PPAR ∝)
mRNA processing	1A2
mRNA stabilisation	1A1, 2E1, 3A4
Enzyme stabilisation	2E1

AhR = aryl hydrocarbon receptor
CAR = constitutive androstane receptor
PPAR = peroxisome proliferator activated receptor
PXR = pregnane (or prenenolone)-X-receptor
SXR = steroid xenobiotic receptor

For drugs that are active in their parent forms, induction may increase drug elimination and thus decrease their pharmacological effects. For prodrugs, compounds that require metabolic activation and whose effects are produced by the metabolites, enhanced pharmacodynamic effects may be expected.

Mechanism of Inhibition of CYPs

(Berthou, 2001; Gibson and Skett, 1994; Levy, *et al*., 2000; Wen, 2002)

Inhibition can be considered mostly as a post-translation of CYPs. Enzyme inhibition means a decrease of metabolism of a particular xenobiotic by another xenobiotic simutaneously present at the active site of the enzyme. Inhibition effects can take place in several ways including a destruction of pre-existing enzyme, an inhibition of enzyme synthesis, and a competition for the enzyme catalytic site. Inhibition of drug metabolism may result in either deleterious effects including drug toxicity or beneficial effects such as a modulation of CYP induced carcinogens. Thus, inhibition of CYPs is of clinical importance for both therapeutic and toxicological reasons.

Mechanism of CYPs inhibition can be divided into three categories

1. Reversible inhibition

In reversible inhibition, binding of enzymes by substrates or inhibitors can be reactivated by diluting the inhibitors. Mechanisms of reversible inhibition include: 1) competitive inhibition that involves a mutually exclusive competition between the binding of inhibitor for the catalytic site of the enzyme of interest. 2) noncompetitive inhibition, substrate and inhibitor binding to the enzyme that are not mutually exclusive. 3) uncompetitive inhibition, the inhibitor binding only to the substrate-enzyme complex. 4) Mixed-type inhibition is when an inhibitor binds either to the free enzyme or to the enzyme-substrate complex.

2. Quasi-irreversible inhibition *via* metabolic intermediate complexation

Quasi-irreversible inhibition occurs when a reactive metabolite forms a stable complex with prosthetic heme of CYP. The stable complex is called metabolic intermediate (MI) complex. The MI complex can be reversed and the catalytic activity of CYP can be restored by incubating *in vitro* with lipophilic compounds that can displace the inhibitor from the active site. However, synthesis of *de novo* enzyme is required to restore CYP activity in an *in vivo*.

3. Mechanism-base inhibition

Mechanism based inhibitors are compounds which are metabolized into a reactive intermediate by CYP. The intermediates are able to be bind with the components of CYP included a pyrrole nitrogen in prosthetic heme group, heme moiety, and apoprotein. The result is inhibiting the binding of substrate to enzyme. This inhibition is usually irreversible. So, inhibition effect is abolished by enzyme resynthesis rather than increase of inhibitor elimination.

Inhibitors bind with enzyme by two modes included uncovalent and covalent binding. The latter is called enzyme inactivation or suicide inhibition. The *in vitro* detection of mechanism-base inhibition requires a pre-incubation of liver microsomes in the presence of inhibitor and electron donor (NADPH). Then substrate is added.

CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Adult male Wistar rats of body weight between 250-300 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. Rats were housed at the Faculty of Medicine, Srinakharinwirot University and acclimatized for at least seven days prior to the experimentation. They were maintained at 25 °C on a 12-hour light/dark cycle and had free access to normal standard diet and water throughout the study. Normal standard diet was purchased from C.P. company.

The protocol of experimental animals used in this study was approved by Animal Ethic Committee of the Faculty of Pharmaceutical Science Chulalongkorn University (Appendix C)

2. Instruments

The following instruments were used in the experimentation:

Autopipets 20, 100, 200, 1000, and 5000 μL (Gilson, France)

Centrifuge (Kokusan, Japan)

Fluorescence spectrophotometer (Jasco, Japan)

Metabolic shaker bath (Heto, Denmark)

pH meter (Beckman Instruments, U.S.A.)

Potter-Elvehjem homogenizer with pestle and glass homogenizing vessel (Heidolph, Germany)

Refrigerated superspeed centrifuge (Beckman Instruments, U.S.A.)

Refrigerated ultracentrifuge (Hitachi, Japan)

Sonicator (Elma, Germany)

Spectrophotometer (Jasco, Japan)

Surgical equipments

Ultra-low temperature freezer (Forma Scientific Inc., U.S.A.)

Vortex mixer (Clay adams, U.S.A.)

3. Chemicals

These following chemicals were used in the experimentation:

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

Ammonium acetate was purchased from APS Finechem, Australia.

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

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

Glycerol was purchased from Carlo Erba, USA.

Hydrochloric acid (HCl), diethyl ether, magnesium chloride ($MgCl_2$), methanol (Gradient grade), phenol, potassium chloride (KCl), sodium chloride (NaCl), sodium hydroxide (NaOH), sulphuric acid (H_2SO_4) and trichloroacetic acid (TCA) were purchased from Merck, Germany

Sodium dithionite was purchased from Fluka Chemic, Japan

Erythromycin stearate was obtained from Siam Pharmaceutical Co. Ltd., Thailand

Methods

1. Preparation of *C. asiatica* ethanolic extract

C. asiatica were obtained from department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University. They were harvested from the areas in Bangkok and the boundary area. Their leaves and stems weighing 40 kg were cut into pieces, dried and ground into fine powder. The 4 kg herbal powder was extracted with 80% ethanol by keeping in an extraction percolater for 48 hours. The ethanolic fraction was dried under vacuum in a rotary evaporator and dried again in a vacuum desiccator. The dried ethanolic extract was weighed and ground to fine powder, kept in tightly closed and light protected container, and stored in a refrigerator until use.

2. Chemical identification tests (Asean Countries, 1993; **คณะกรรมการจัดทำตำรายา, 2545**)

Preliminary test

1. When 0.5 g of the dried ethanolic extract of *C. asiatica* was shaked vigorously with 10 mL of water, a long lasting foam is produced. This is a test for saponin.
2. Half gram of *C. asiatica* ethanolic extract was warmed with 2 mL of acetic anhydride on a water bath for 2 min. The solution was filtered and the filtrate was added carefully with 1 mL of sulfuric acid. Two layers of solutions were shown with a brownish red colour at the contacted zone of the layers. This test indicated an existence of triterpenoids in the ethanolic extract of *C. asiatica*.

Confirmatory test

One gram of *C. asiatica* ethanolic extract was warmed with 20 mL of ethanol on a water bath for 10 minutes. After the solution was filtered, the filtrate was evaporated to dryness. Four millilitre of ethanol was added and the solution was used for an identification test by thin layer chromatography (TLC). Briefly, 5 μL of the solution was spotted on a TLC

plate coated with silica gel. The plate was developed by mobile phase comprising chloroform: methanol: water (15: 7: 1) with the developing distance of 12 cm. Detection was performed by using anisaldehyde TS spraying on the TLC plate which was then warmed at 105 ^{o}C for 5 min. Identification was performed by comparing the hRf value and colour of the spots with the reference standard, asiaticoside which was spotted on the same plate.

3. An *ex vivo* study

3.1 Animal treatment

Rats were randomly divided into 3 treatment groups. Each treatment group comprised 10 rats.

3.1.1 Control group: Rats were orally administered with distilled water 1 ml/kg/day once daily for 30 days.

3.1.2 *C. asiatica* treatment group I: Rats were orally administered with *C. asiatica* at a daily dose of 250 mg/kg/day for 30 days.

3.1.3 *C. asiatica* treatment group II: Rats were orally administered with *C. asiatica* at a daily dose of 1,000 mg/kg/day for 30 days.

C. asiatica was prepared daily by dissolving 6 g of *C. asiatica* ethanolic extract with 6 mL of distilled water to make a concentration of 1,000 mg/mL of *C. asiatica* suspension and was vortexed before feeding to experimental animals.

During the treatment period, body weight of all rats, food and water consumptions were recorded every week. After thirty days of compound administration, rats were anesthetized with diethyl ether. Blood samples were collected by heart puncture. Livers were removed for preparation of microsomes.

3.2 Determination of clinical blood chemistry

Whole blood samples were used for hematological assays. The remaining blood samples were centrifuged for collecting serum samples which were used for determining various clinical blood chemistry.

3.2.1 Hematological assays

Whole blood samples were determined for complete blood count (CBC), red blood cell (RBC) morphology, RBC indices (mean corpuscular volume, MCV; mean corpuscular hemoglobin, MCH; and mean corpuscular hemoglobin concentration, MCHC), platelet count, white blood cell (WBC) count, and % differential WBC. The assays were performed by Professional Laboratory Management Corp Co., Ltd.

3.2.2 Clinical blood chemistry determinations

Serum samples were determined for various clinical blood chemistry as following: glucose, total cholesterol, triglyceride, high density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), serum creatinine (SCr), uric acid. The assays were performed by the Faculty of Allied Health Sciences, Chulalongkorn University.

Total bilirubin, direct bilirubin, sodium, potassium, and chloride. The assays were performed by Professional Laboratory Management Corp Co., Ltd.

3.3 Preparation of liver microsomes

3.3.1 Reagents

1) 0.1 M Phosphate buffer, pH 7.4

One litre of 0.1 M phosphate buffer, pH 7.4 consisted of 1.78 g of KH_2PO_4, 9.55 g of Na_2HPO_4, and 11.50 g of KCl. The solution was adjusted to pH 7.4 with 0.1 M NaOH or 0.1 M HCl.

2) 0.1 M Phosphate buffer, pH 7.4, containing 20% v/v glycerol

3) 0.9% w/v NaCl

3.3.2 Procedure

1) After removing from the body, rat livers were quickly perfused with ice-cold 0.9% w/v NaCl until the entire organ became pale.
2) The livers were rinsed with ice-cold 0.9% w/v NaCl, and blotted dry with gauzes.
3) The whole livers were weighed, cut into pieces, and homogenized with 3 volume of 0.1 M phosphate buffer, pH 7.4.
4) The liver homogenates were centrifuged at 10,000 *g* for 30 minutes at 4 ^{o}C, using refrigerated superspeed centrifuge, to pellet intact cells, cell debris, nuclei, and mitochondria.
5) The supernatants were transferred to ultracentrifuge tubes and further centrifuged at 100,000 *g* for 60 minutes at 4 ^{o}C, using refrigerated ultracentrifuge.
6) The pellets (microsomal subfractions) were resuspended with 5 mL of 0.1 M phosphate buffer, pH 7.4, containing 20% v/v glycerol. The microsomal suspensions were aliquoted, kept in microtubes, and stored at –80 ^{o}C until the time of enzyme activity assays.

3.4 Determination of protein concentrations

Liver microsomal protein concentrations were determined according to the method of Lowry, *et al*. (1951) with slight modification.

3.4.1 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

The solution was prepared freshly in a sufficient amount for all tubes in the assay (6.5 mL of the solution was required for each tube). This reagent comprised the solution of 2% w/v Na_2CO_3, 0.5M NaOH, 2% w/v sodium citrate, and 1%w/v cupric sulfate in a 100: 10: 1: 1 ratio, respectively.

3.4.2 Procedure

1) 16 x 125 mm tubes were labeled in duplicate for 7 standards (0, 50, 100, 150, 200, 250, 300 μg) and for each unknown sample.
2) The following reagents were added in μL to each standard solution tube:

Standard tube	0	50	100	150	200	250	300	μg
1 mg/mL BSA	0	50	100	150	200	250	300	μL
0.5 M NaOH	500	450	400	350	300	250	200	μL

Each tube was mixed thoroughly after addition of the reagents.

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

3.4.3 Calculations

1) The average absorbance of each standard was plotted against its amount of protein. The best fit regression line was drawn through the points. The amount of protein in each unknown sample was obtained by comparing its absorbance against the standard curve.
2) The protein concentration (mg/mL or μg/μL) in each unknown sample was obtained by dividing its amount of protein (from step 1) with the volume of microsomal sample used (i.e., 10 μL) in the reaction.

3.5 Spectral determination of total CYP contents

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

3.5.1 Reagents

1) 0.1 M Tris buffer, pH 7.4, containing 20% v/v glycerol
2) Sodium dithionite
3) Carbon monoxide

3.5.2 Procedure

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

3.5.3 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 cuvette path length of 1 cm, total CYP contents were given by:

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

3.6 Analysis of alkoxyresorufin O-dealkylation

Rate of hepatic microsomal alkoxyresorufin O-dealkylation was determined according to the methods of Burke and Mayer (1974); Burke, *et al* (1985) and Lubet, *et al*. (1985) with slight modifications. Benzyloxyresorufin and pentoxyresorufin were used as specific substrates of CYP2B1&2B2. Ethoxyresorufin and methoxyresofin were used as specific substrates of CYP1A1 and CYP1A2, respectively.

3.6.1 Reagents

1) 0.1 M Tris buffer, pH 7.4
2) 20 mM K_3PO_4, pH 7.4
3) Resorufin & alkoxyresorufins

0.5 mM Resorufin (MW 235)

1.175 mg of resorufin was dissolved with 10 mL of DMSO.

0.5 mM Benzyloxyresorufin (MW 303)

1.515 mg of benzyloxyresorufin was dissolved with 10 mL of DMSO.

0.5 mM Ethoxyresorufin (MW 241)

1.205 mg of ethoxyresorufin was dissolved with 10 mL of DMSO.

0.5 mM Methoxyresorufin (MW 227)

1.135 mg of methoxyresorufin was dissolved with 10 mL of DMSO.

0.5 mM Pentoxyresorufin (MW 283)

1.415 mg of pentoxyresorufin was dissolved with 10 mL of DMSO.

4) NADPH regenerating system

Glucose 6-phosphate dehydrogenase (G6PD), pH 7.4

G6PD was diluted to 100 units per mL with 20 mM K_3PO_4, adjusting pH to 7.4 with 0.1 M HCl or 0.1 M NaOH (10 μL contains 1 unit of G6PD).

0.5 M Glucose 6-phosphate (G6P), pH 7.4

1.41 g of glucose 6-phosphate was dissolved with 10 mL of 20 mM K_3PO_4, adjusting pH to 7.4 with 0.1 M HCl or 0.1 M NaOH (10 μL contains 5 mmoles of G6P).

0.1 M NADP, pH 7.4

0.765 g of NADP was dissolved with 10 mL of 20 mM K_3PO_4, adjusting pH to 7.4 with 0.1 M HCl or 0.1 M NaOH (10 μL contains 1 mmoles of NADP).

0.3 M $MgCl_2$, pH 7.4

609.93 mg of $MgCl_2$ was dissolved with 10 mL of 20 mM K_3PO_4, adjusting pH to 7.4 with HCl or NaOH (10 μL contains 3 mmoles of $MgCl_2$).

3.6.2 Procedure

1) Microsomes were diluted with 0.1 M Tris buffer, pH 7.4 to measure out 200 μg of protein for the 1 mL of the reaction mixture.

2) For each mL of the reaction mixture, the following reagents were added
   a. 45 μL of NADPH regenerating system comprised
      15 μL of 0.1 M NADP
      15 μL of 0.5 M G6P
      15 μL of 0.3 M $MgCl_2$
   b. 15 μL of 0.5 mM alkoxyresorufin
   c. Varied volume of diluted microsomal suspension containing 200 μg of microsomal protein
   d. 0.1 M Tris buffer, pH 7.4 qs to 985 μL.
3) Three tubes were prepared for each microsomal sample. One tube was a sample blank tube and the others were sample tubes. All tubes were preincubated in a 37 ^{o}C shaking water bath for 2 minutes.
4) The reaction was started by the addition of 15 μL of G6PD (1 unit of G6PD / 1 mL of reaction mixture volume). For a sample blank, 10 μL of 0.1 M Tris buffer, pH 7.4 was added instead of G6PD.
5) After a 5 minute incubation, the reaction was stopped by adding 1.5 mL of methanol (HPLC grade).
6) The absorbance was measured by fluorescence spectrophotometer using an excitation wavelength of 556 nm and an emission wavelength of 588 nm.
7) A resorufin standard curve was carried out using 8 concentrations of resorufin: 0.025, 0.050, 0.075, 0.1, 0.2, 0.4, 0.5, 1.0 nmol/mL.
8) The procedure was verified by varying amount of microsomal protein used in the reaction (100, 200, 300 μg of microsomal protein/ mL of the reaction mixture). The liver microsome was prepared from phenobarbital-induced rats given phenobarbital at the dosage of 80 mg/kg/day intraperitoneally for 3 days (Soucek and Gut, 1992). The reaction was performed as mentioned above using BR as a substrate. Correlation coefficient (r^2) between

amount of microsomal protein and fluorometric absorbance was 0.999 (Appendix, page 131)

3.6.3 Calculations

Rate of alkoxyresorufin O-dealkylation was calculated by dividing the amount of resorufin formed by the time of incubation (5 minutes) and an amount of microsomal protein (200 μg) used in the reaction. The units were expressed as pmol/ mg protein/ min.

3.7 Analysis of aniline 4-hydroxylation

Rate of hepatic microsomal aniline 4-hydroxylation was determined according to the method of Schenkman, *et al*. (1967). Aniline hydrochloride was used as a specific substrate of CYP2E1.

3.7.1 Reagents

1) 10 mM Aniline hydrochloride

   129.6 mg of aniline hydrochloride was dissolved with 100 mL of double distilled water. The solution was stored in a dark brown bottle.

2) 6% w/v Trichloroacetic acid

   60 g of trichloroacetic acid was dissolved with 1 L of double distilled water.

3) 20% w/v Trichloroacetic acid

   200 g of trichloroacetic acid was dissolved with 1 L of double distilled water.

4) 1% w/v Phenol

   20 g of phenol and 40 g of NaOH were dissolved with 2 L of double distilled water.

5) 1 M Na_2CO_3

212 g of anhydrous Na_2CO_3 was dissolved with 2 L of double distilled water.

6) 10 μM 4-Aminophenol

36.5 mg of 4-aminophenol was made up to 10 mL with double distilled water. Then 0.1 mL of the solution was added to 15 g of trichloroacetic acid and made up to 250 mL with double distilled water.

7) 0.1 M Tris buffer, pH 7.4

8) 0.1 M NADP

9) 0.5 M G6P

10) 100 units/mL G6PD

11) 0.3 M $MgCl_2$

3.7.2 Procedure

1) Microsomes were diluted with 0.1 M Tris buffer, pH 7.4 so as to be able to measure out 5 mg of protein for the 2 mL of the reaction mixture.

2) For each 2 mL of the reaction mixture, the following reagents were added

   a. 30 μL of NADPH regenerating system comprised

      10 μL of 0.1 M NADP

      10 μL of 0.5 M G6P

      10 μL of 0.3 M $MgCl_2$

   b. 500 μL of 10 mM aniline hydrochloride

   c. Varied volume of diluted microsomal suspension containing 5 mg of microsomal protein

   d. 0.1 M Tris buffer, pH 7.4 qs to 1,980 μL.

3) Three reaction tubes were prepared for each microsomal sample. One tube was a sample blank tube and the others were sample tubes.

4) All tubes were preincubated in a 37 ^{o}C shaking water bath for 2 minutes. The reaction was initiated by an addition of 20 μL of G6PD. For a sample blank, 20 μL of 0.1 M Tris buffer, pH 7.4 was added instead of G6PD.
5) After a 30 minutes incubation time, the reaction was stopped by adding 1 mL of ice-cold 20% w/v trichloroacetic acid and the tubes were kept on ice for 5 minutes.
6) The solution was then centrifuged at 3,000 rpm for 10 minutes.
7) After 1 mL of the supernatant was transferred to a new tube, 1 mL of 1% w/v phenol and 1 mL of 1 M Na_2CO_3 were added. The solution was mixed well by vortex mixer and kept at room temperature for 30 minutes.
8) The absorbance was measured by spectrophotometer at a wavelength of 630 nm.
9) A standard curve was carried out using 5 concentrations of 4-aminophenol standard solutions (2, 4, 6, 8, 10 μM), following the procedure from step 7 in the same manner as sample.
10) The procedure was verified by varying amount of rat microsomal protein used in the reaction (2.5, 5.0, 7.5 mg of microsomal protein/ 2 mL of the reaction mixture). The rat liver microsome was prepared and the reaction was performed as mentioned above. Correlation coefficient (r^2) between amount of microsomal protein and absorbance was 0.9985 (Appendix, page 132)

3.7.3 Calculations

Rate of aniline 4-hydroxylation was calculated by dividing amount of the product formed (4-aminophenol) by the time of incubation (30 minutes) and an amount of microsomal protein (5 mg) used in the reaction. The units were expressed as nmol/ mg protein/ min.

3.8 Analysis of erythromycin N-demethylation

Rate of hepatic microsomal erythromycin N-demethylation was determined according to the method of Nash, et al, 1953 and Friedli G., 1992 with some modifications. Erythromycin stearate was used as specific substrate of CYP3A.

3.8.1 Reagents

1) 20 mM Potassium phosphate buffer, pH 7.4

   Fifty millilitre of 20 mM KH_2PO_4 was added with 39.1 mL of 0.02 M NaOH, and diluted with distilled water qs to 200 mL. The solution was then adjusted pH to 7.4 with 0.1 M HCl or 0.1 M NaOH.

2) 20 mM KH_2PO_4

3) 10 mM Erythromycin stearate

   Erythromycin stearate 0.1018 g was dissolved and made up to 10 ml with double distilled water.

4) NADPH regenerating system comprised

   15 μL of 0.5 M G6P

   15 μL of 0.1 M NADP

   15 μL of 0.3 M $MgCl_2$

5) 100 units/mL G6PD

6) Nash's reagent

   Nash's reagent comprised 30 g of ammonium acetate, 0.4 mL of acetylacetone, 0.6 mL of glacial acetic acid, dissolving with distilled water qs to 100 mL.

7) 12.5% w/v TCA

   TCA 12.5 g was made up to 100 ml with double distilled water.

8) Formaldehyde standard (MW 30, 37% solution formalin)

3.8.2 Procedure

1) Microsomes were diluted with 20 mM potassium phosphate buffer, pH 7.4 to measure out 8 mg of protein for 1 mL of the reaction mixture.
2) For each mL of the reaction mixture, the following reagents were added
   a. 45 μL of NADPH regenerating system comprised
      15 μL of 0.1 M NADP
      15 μL of 0.5 M G6P
      15 μL of 0.3 M $MgCl_2$
   b. 100 μL of 10 mM erythromycin stearate
   c. Varied volume of diluted microsomal suspension containing 4 mg of microsomal protein
   d. 20 mM potassium phosphate buffer, pH 7.4 qs to 985 μL.

4) Two tubes were prepared for each microsomal sample. Both tubes were sample tubes. All tubes were preincubated in a 37 ^{o}C shaking water bath for 3 minutes.
5) For a sample blank tube, 20mM Potassium phosphate buffer, pH 7.4 was added instead of microsomal sample.
5) The reaction was started by an addition of 15 μL of 100 units/mL G6PD. For a sample blank tube, 20mM Potassium phosphate buffer, pH 7.4 was added instead of G6PD.
6) After a 10 minutes incubation, the reaction was stopped by adding 0.5 mL of 12.5% w/v trichloroacetic acid.
7) The solution was then centrifuged at 3,000 rpm for 10 minutes.
8) After 1 mL of the supernatant was transferred to a new tube (protected from light), 1 mL of Nash's reagent was added. The solution was mixed well by vortex mixer. Each tube was incubated in a 50 ^{o}C shaking water bath for 30 minutes.
9) The absorbance was measured by spectrophotometer at wavelength of 412 nm.

11) A formaldehyde standard curve was carried out using 1 mL of formaldehyde standard at 5 concentrations: 0.0156, 0.0313, 0.0625, 0.125, 0.250 μmol/mL with 1 ml of Nash's reagent and performed the procedure in the same maner as the sample tubes as described in 8.

12) The procedure was verified by varying amount of microsomal protein used in the reaction (2, 4, 8 mg of microsomal protein/ mL of the reaction mixture). The liver microsome was prepared from phenobarbital-induced rats given phenobarbital at the dosage of 80 mg/kg/day intraperitoneally for 3 days (Soucek and Gut, 1992). The reaction was performed as mentioned above. Correlation coefficient (r^2) between amount of microsomal protein and absorbance was 1.000 (Appendix, page 133)

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3.8.6 Calculations

Rate of erythromycin N-demethylation was calculated by dividing the amount of formaldehyde formed by the time of incubation (10 minutes) and an amount of microsomal protein (4 mg) used in the reaction. The units were expressed as nmol/ mg protein/ min.

3.9 Statistics

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

CHAPTER IV

RESULTS

1. Preparation of *C. asiatica* ethanolic extract

Four kilograms of *C. asiatica* dried powdered were used in this study. Following the extraction process, 1,000 g of the ethanolic extract were obtained. Thus, yield of *C. asiatica* ethanolic extract was 25% w/w. This was conformed the standard limitations which suggest that ethanol-soluble extraction of *C. asiatica* should not less than 15% w/w. (Asean Countries, 1993; คณะกรรมการจัดทำตำรายา, 2545).

2. Chemical identification tests

Preliminary test

The ethanolic extract of *C. asiatica* was shown to form a long lasting foam as shaking with water. As dissolving with acetic anhydride and add slowly with sulfuric acid, a green colour was developed in the solution of upper layer and a brownish red ring was formed at the contacted zone of the two layers. These were preliminary tests for saponin and triterpenoids, respectively.

Confirmatary test

Figure 3 showed the TLC chromatogram of *C. asiatica* ethanolic extract. The high retention value factor (hRf) and the colour of spots of the TLC chromatogram was shown in Table 4. Each spot possessed hRf value and colour was approximately conformable to the corresponding spot shown in the standard limitations (Table 5) (Asean Countries, 1993)

C A

Figure 3 TLC chromatogram of *C. asiatica* ethanolic extract

This figure refered to the TLC plate (coated with silica gel G) using mobile phase of chloroform: methanol: water (15: 7: 1). The plate was sprayed with anisaldehyde TS and heat at 105^{0}C for 5 minutes.

C = *C. asiatica* ethanolic extract

A = Standard (asiaticoside)

Table 4 hRf values and color of the spots shown in TLC chromatogram of *C. asiatica* ethanolic extract used in the study

Developing solvent	Spots	hRf values	Detection with Anisaldehyde TS
Chloroform : methanol : water (15 :7 :1)	1	3	Green
	2	8	Green
	3	15	Brown
	4	17	Brown
	5	28	Violet
	6	37	Green
	7*	57	Brown
	8	68	Purple
	9	72	Green
	10	77	Brown
	11	82	Brown
	12	87	Violet
	13	92	Brown
	14	95	Purple

* refered to asiaticoside

The data were recorded from the corresponding TLC chromatogram in the Figure 3

high retention value factor (hRf) value = $\frac{\text{distance from starting line to middle of spot}}{\text{distance from starting line to solvent front}} \times 100$

Table 5 hRf values of components in *C. asiatica* ethanolic extract (Asean Countries, 1993)

Developing solvent	Spots	hRf values	Detection with Anisaldehyde TS
Chloroform : methanol : water (15 :7 :1)	1	1-4	Green
	2	4-8	Purple
	3	23-28	Dark brown
	4	29-32	Light brown
	5	33-37	Blue-violet
	6	42-47	Green
	7*	55-58	Light brown
	8	59-62	Purple
	9	74-77	Light brown
	10	78-82	Light brown
	11	84-88	Violet
	12	90-93	Brown
	13	94-97	Purple

* asiaticoside

3. An *ex vivo* study

3.1 Effect of *C. asiatica* ethanolic extract on body weight, relative liver weight, and food&water consumptions

C. asiatica ethanolic extract at the doses of 250 and 1,000 mg/kg/day given orally once daily for 30 days, did not cause any effects on body weight, body weight gain and relative liver weight (Table 6). Likewise, no changes of food and water intake were observed in the *C. asiatica* treatment groups at both doses used in this experiment as compared to the control group (Figure 4). All rats were alive till the end of the experiment and exhibited no apparent signs of toxicity.

Table 6 Effect of *C. asiatica* ethanolic extract on body weight, body weight gain, and relative liver weight

	Treatment group		
	Control group	*C. asiatica* treament group I	*C. asiatica* treament group II
Initial body weight[a] (g)	353.83 ± 16.08	356.86 ± 11.87	359.72 ± 10.07
Final body weight[b] (g)	413.49 ± 11.93	400.01 ± 9.35	412.53 ± 10.03
Body weight gain (g)	59.66 ± 11.45	43.15 ± 9.66	52.81 ± 12.61
Liver weight[c] (g)	12.78 ± 0.79	12.93 ± 0.38	13.21 ± 0.43
%relative liver weight (g/100 g of body weight)	3.07 ± 0.13	3.24 ± 0.09	3.21 ± 0.10

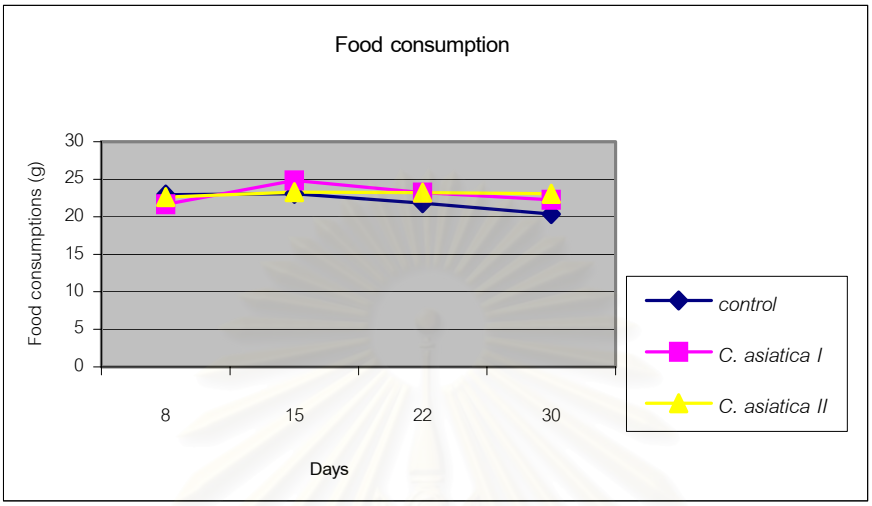
Data shown were mean ± SEM (n=10)

[a] Body weight of rat at the beginning of *C. asiatica* administration.

[b] Body weight of rat at the time of sacrifice.

[c] Liver weight at the time of sacrifice, before preparation of microsomes.

[a]



[b]

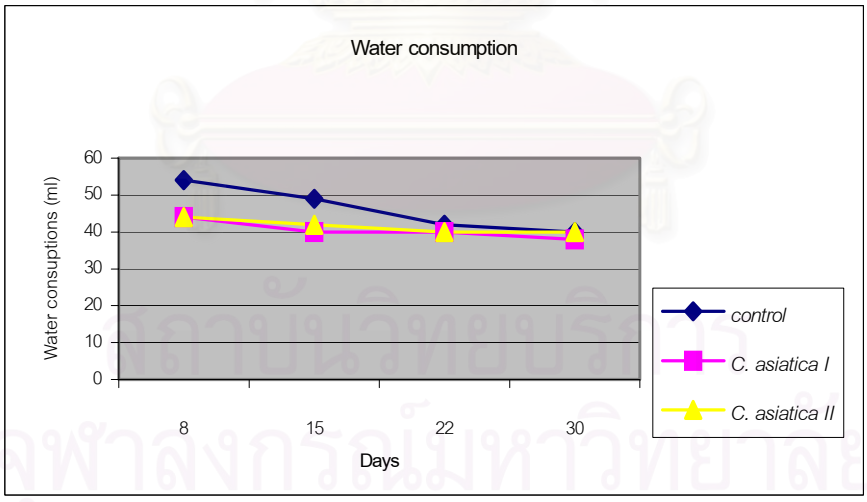


Figure 4 Effect of *C. asiatica* ethanolic extract on food [a] and water [b] consumptions

Data shown were mean ± SEM (n=10)

3.2 Effects of *C. asiatica* ethanolic extract on clinical blood chemistry

Subacute exposure (30 days) of *C. asiatica* ethanolic extract at the doses of 250 and 1,000 mg/kg/day exhibited no effects on any clinical blood chemistry in serum: glucose, total cholesterol, triglyceride, HDL-C, LDL-C, AST, ALT, ALP, total bilirubin, direct bilirubin, BUN, SCr, uric acid, sodium, potassium, and chloride (Figure 5-11).

Likewise, no effects of *C. asiatica* ethanolic extract were observed on these following blood hematological parameters as compared to the control group. These parameters included hemoglobin, hematocrit, platelet count, WBC count, RBC count, RBC indices (mean corpuscular volume; MCV, mean corpuscular hemoglobin; MCH, mean corpuscular hemoglobin concentration; MCHC) and %differential WBC (Figure 12-17).

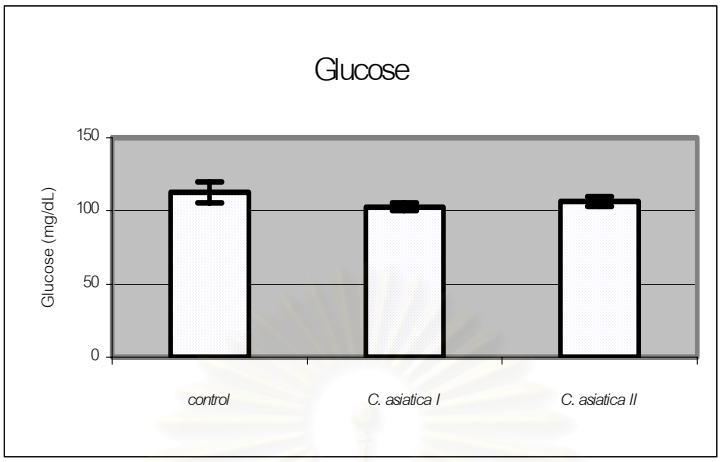


Figure 5 Subacute effect of *C. asiatica* ethanolic extract on serum glucose

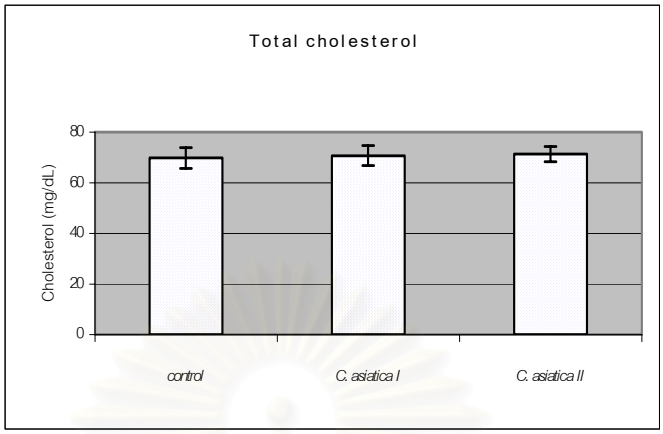
The individual bar represented mean of serum glucose level with an error bar of standard error of the mean (n=10)

Control = *C. asiatica* control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

[a]



[b]

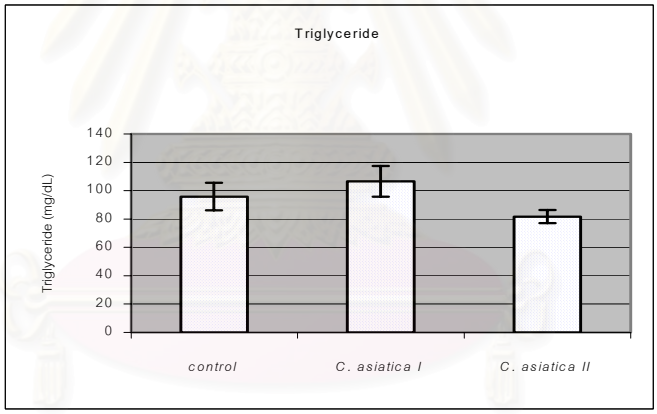


Figure 6 Subacute effect of *C. asiatica* ethanolic extract on total cholesterol [a] and triglyceride [b]

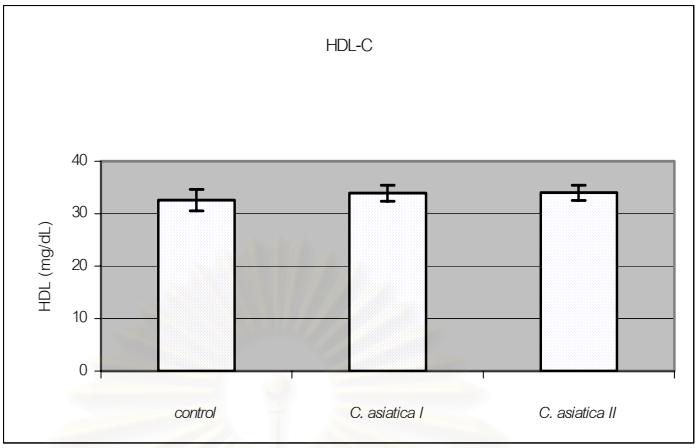
The individual bar represented mean of total cholesterol and triglyceride levels with an error bar of standard error of the mean (n= 10 except indicated).

Control = Control group (n =9)

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

[a]



[b]

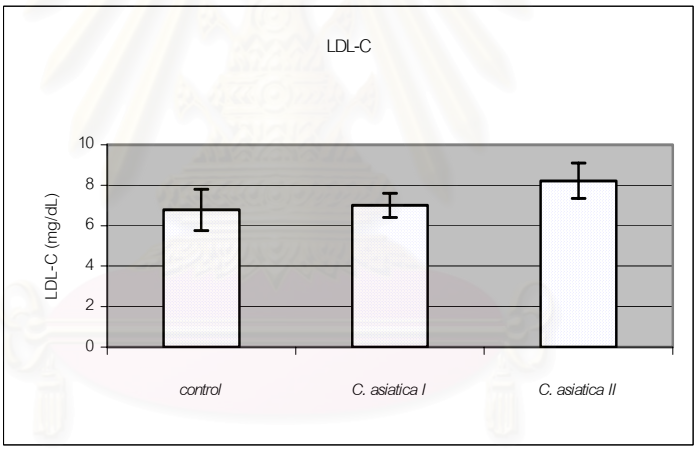


Figure 7 Subacute effect of *C. asiatica* ethanolic extract on HDL-C [a] and LDL-C [b]

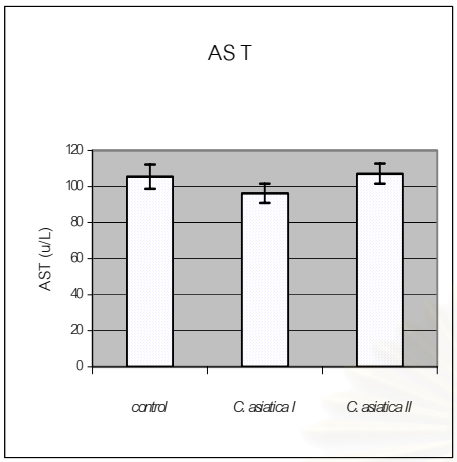
The individual bar represented mean of HDL-C and LDL-C levels with an error bar of standard error of the mean (n=10 except indicated).

Control = Control group (n=9)

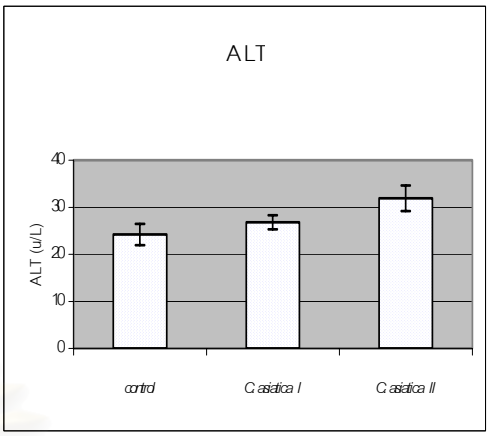
C. asiatica I = *C. asiatica* treatment group I (for LDL-C, n=9)

C. asiatica II = *C. asiatica* treatment group II (for LDL-C, n=9)

[a]



[b]



[c]

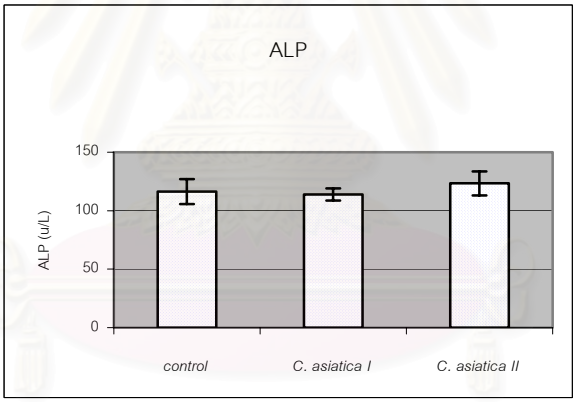


Figure 8 Subacute effect of *C. asiatica* ethanolic extract on AST [a] ALT [b] and ALP [c]

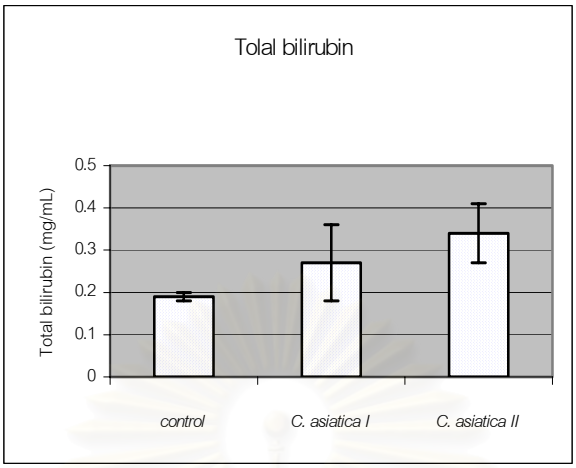
The individual bar represented mean of AST, ALT and ALP levels with an error bar of standard error of the mean (n=10 except indicated).

Control = Control group (n=9)

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II (for AST, n=9)

[a]



[b]

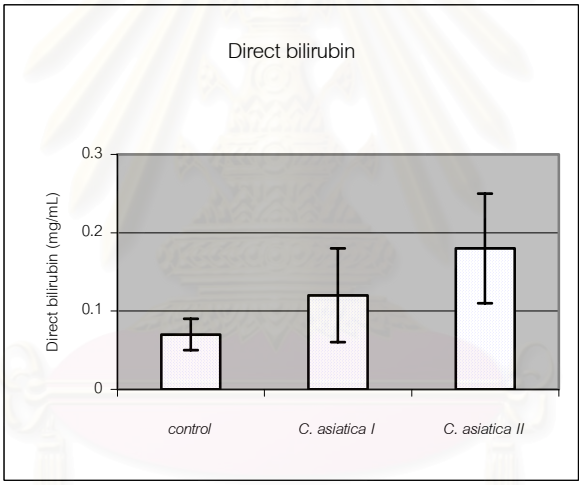


Figure 9 Subacute effect of *C. asiatica* ethanolic extract on total bilirubin [a] and direct bilirubin [b]

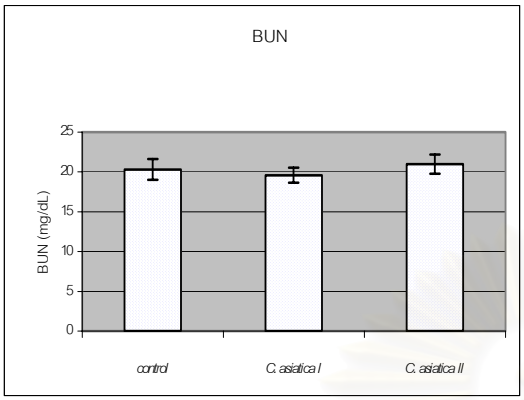
The individual bar represented mean of total bilirubin and direct bilirubin levels with an error bar of standard error of the mean (n=10 except indicated).

Control = Control group (n=9)

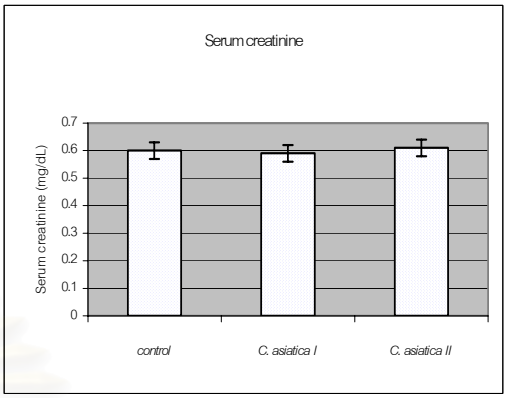
C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

[a]



[b]



[c]

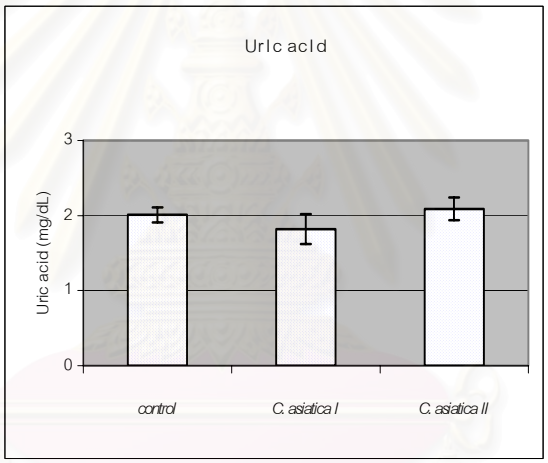


Figure 10 Subacute effect of *C. asiatica* ethanolic extract on BUN [a] Scr [b] and uric acid [c]

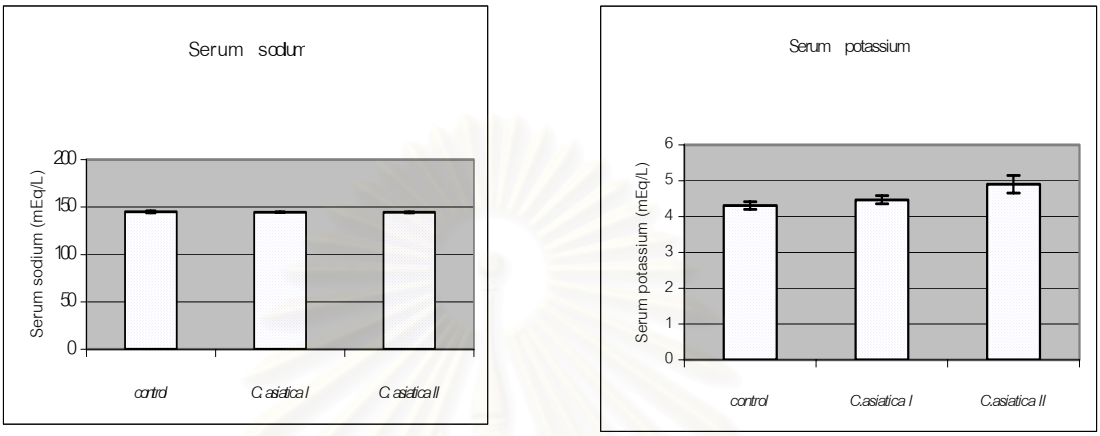
The individual bar represented mean of BUN, Scr and uric acid levels with an error bar of standard error of the mean (n=10 except indicated).

Control = Control group (n=9)

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

[a] [b]



[c]

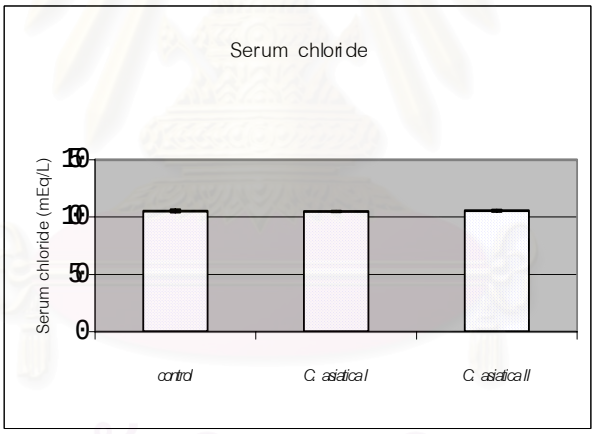


Figure 11 Subacute effect of *C. asiatica* ethanolic extract on sodium [a] potassium [b] and chloride [c]

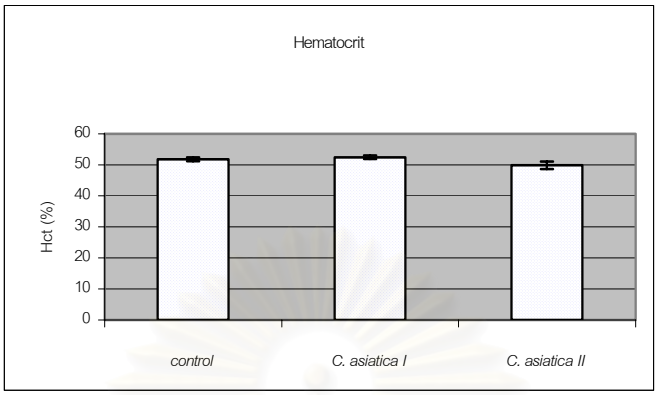
The individual bar represented mean of sodium, potassium and chloride levels with an error bar of standard error of the mean (n=10 except indicated).

Control = Control group (n=9)

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

[a]



[b]

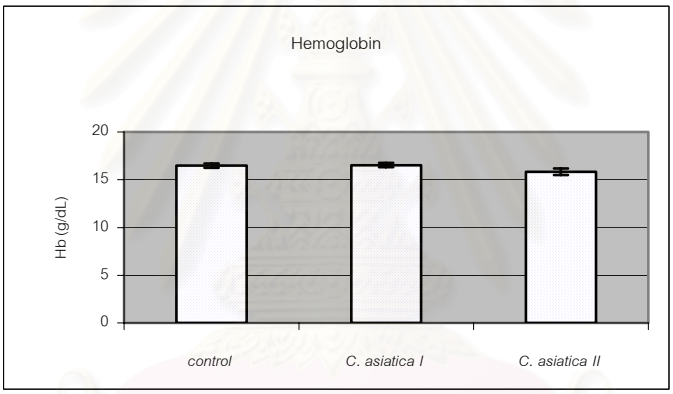


Figure 12 Subacute effect of *C. asiatica* ethanolic extract on hematocrit [a] and hemoglobin [b]

The individual bar represented mean of hematocrit, hemoglobin with an error bar of standard error of the mean (n=9).

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

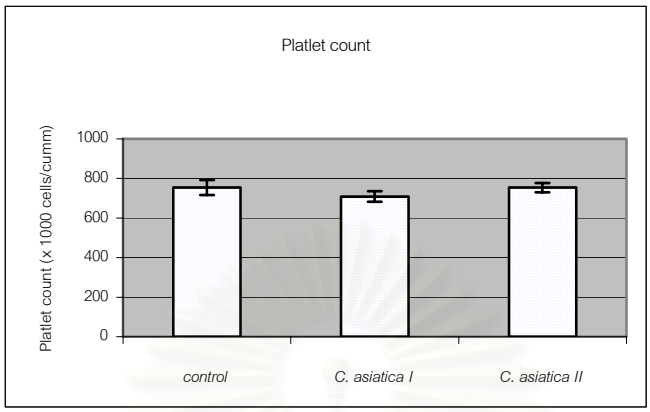


Figure 13 Subacute effect of *C. asiatica* ethanolic extract on platlet count

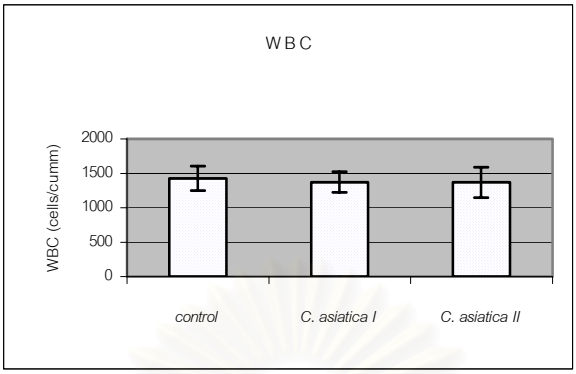
The individual bar represented mean of platlet count with an error bar of standard error of the mean (n=9).

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

[a]



[b]

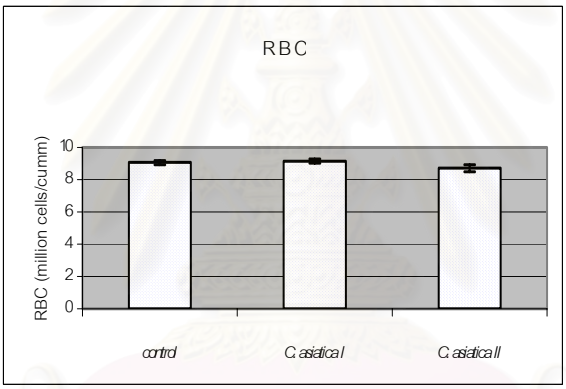


Figure 14 Subacute effect of *C. asiatica* ethanolic extract on WBC [a] and RBC [b]

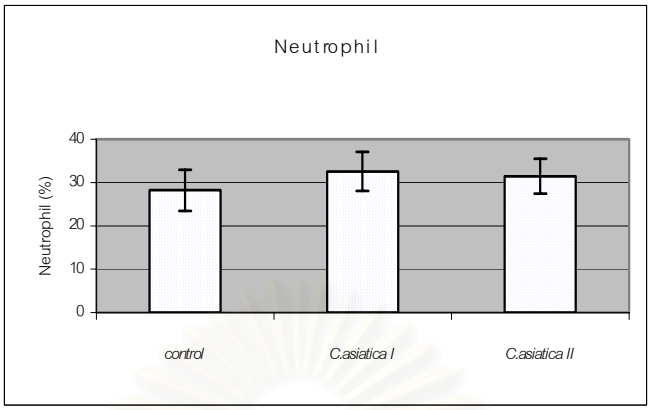
The individual bar represented mean of WBC and RBC counts with an error bar of standard error of the mean (n=9).

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

[a]



[b]

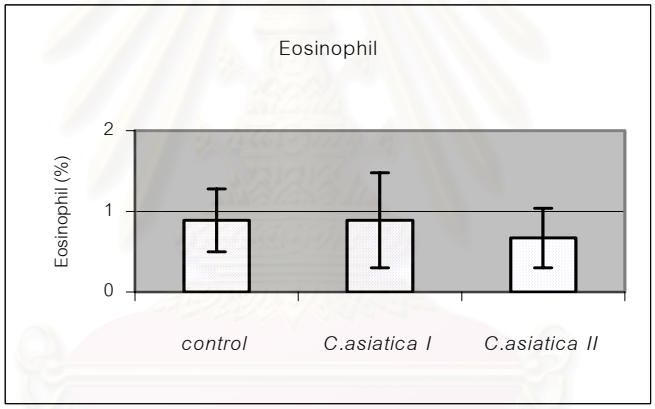


Figure 15 Subacute effect of *C. asiatica* ethanolic extract on neutrophil [a] and eosinophil [b]

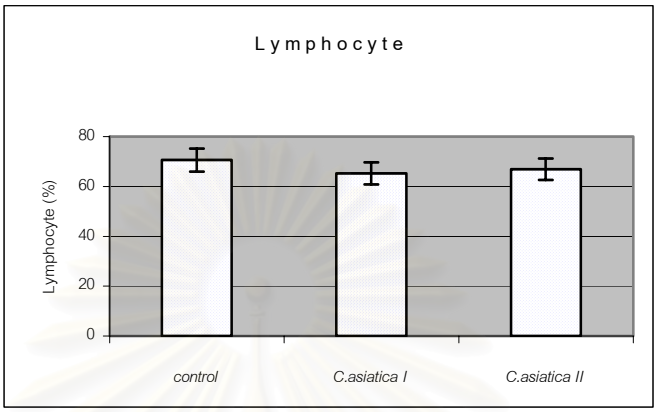
The individual bar represented mean of neutrophil and eosinophil counts with an error bar of standard error of the mean (n=9).

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

[a]



[b]

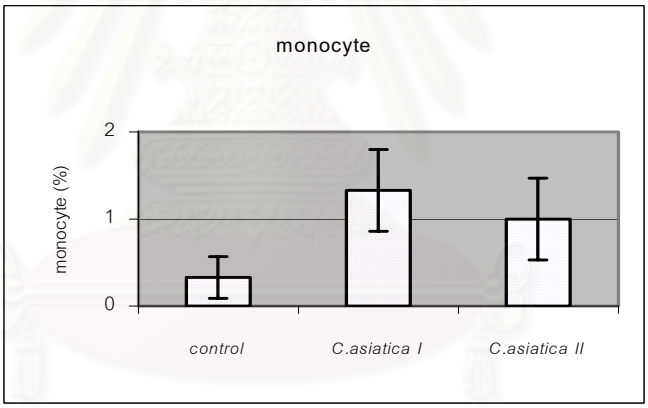


Figure 16 Subacute effect of *C. asiatica* ethanolic extract on lymphocyte [a] and monocyte [b]

The individual bar represented mean of lymphocyte and monocyte counts with an error bar of standard error of the mean (n=9).

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

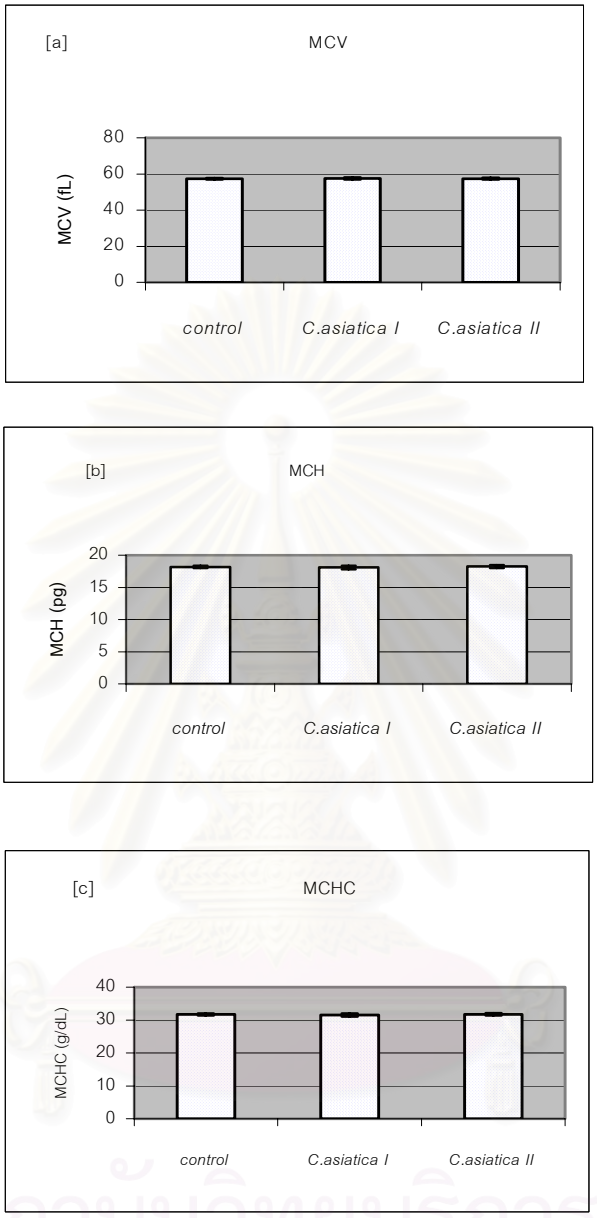


Figure 17 Subacute effect of *C. asiatica* ethanolic extract on MCV [a] MCH [b] and MCHC [c]

The individual bar represented mean of MCV, MCH and MCHC with an error bar of standard error of the mean (n=9).

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

3.3 Effect of *C. asiatica* ethanolic extract on hepatic microsomal CYPs

3.3.1 Effect of *C. asiatica* ethanolic extract on hepatic microsomal total CYP contents

C. asiatica ethanolic extract at both doses used in this study did not exhibit any significant effects on hepatic microsomal total CYP contents in rats (Figure 18).

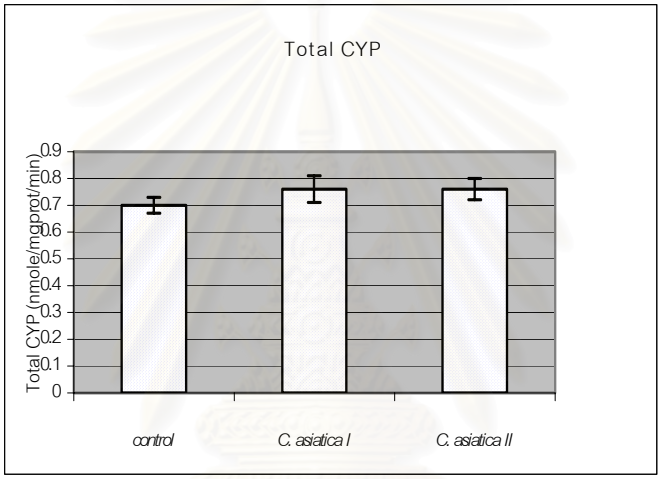


Figure 18 Effect of *C. asiatica* ethanolic extract on hepatic microsomal total CYP contents

The individual bar represented mean of hepatic microsomal total CYP content with an error bar of standard error of the mean (n=10)

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

3.3.2 Effect of *C. asiatica* ethanolic extract on hepatic microsomal alkoxyresorufin O-dealkylation

Both doses of *C. asiatica* ethanolic extract did not exhibited any significant changes on rate of ethoxyresorufin O-dealkylation (EROD; which represented the activities of CYP1A1) (Figure 19), methoxyresorufin O-dealkylation (MROD; which represented the activities of CYP1A2) (Figure 20); benzyloxy- and pentoxyresorufin O-dealkylation (BROD and PROD, respectively; which represented the activities of CYP2B1&2B2) (Figure 21, 22)

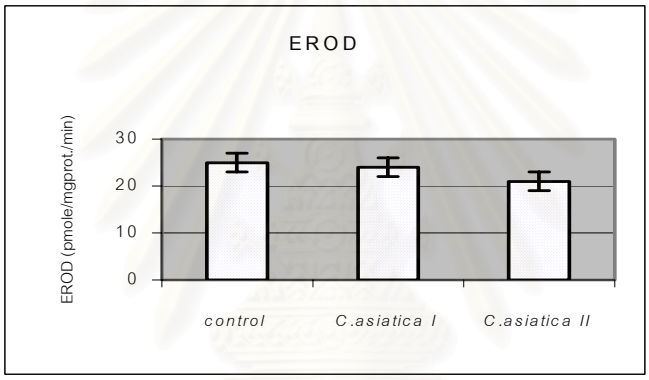


Figure 19 Effect of *C. asiatica* ethanolic extract on hepatic microsomal EROD

The individual bar represented mean of EROD with an error bar of standard error of the mean (n=10)

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

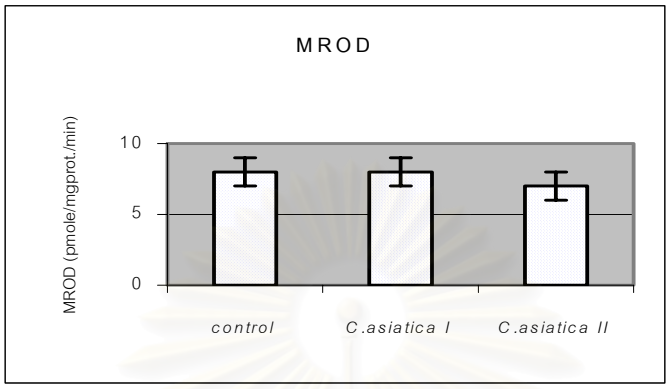


Figure 20 Effect of *C. asiatica* ethanolic extract on hepatic microsomal MROD

The individual bar represented mean of MROD with an error bar of standard error of the mean (n=10)

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

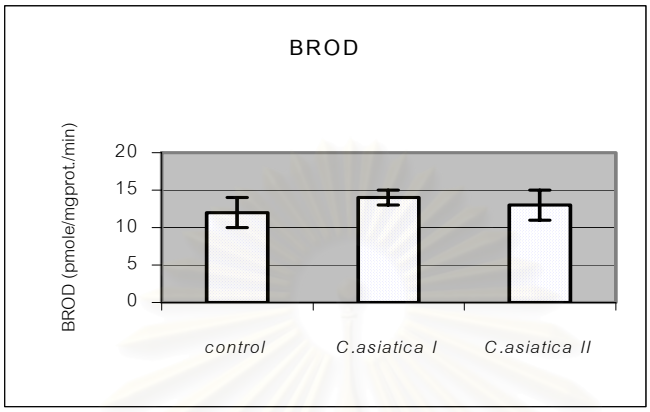


Figure 21 Effect of *C. asiatica* ethanolic extract on hepatic microsomal BROD

The individual bar represented mean of BROD with an error bar of standard error of the mean (n=10)

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

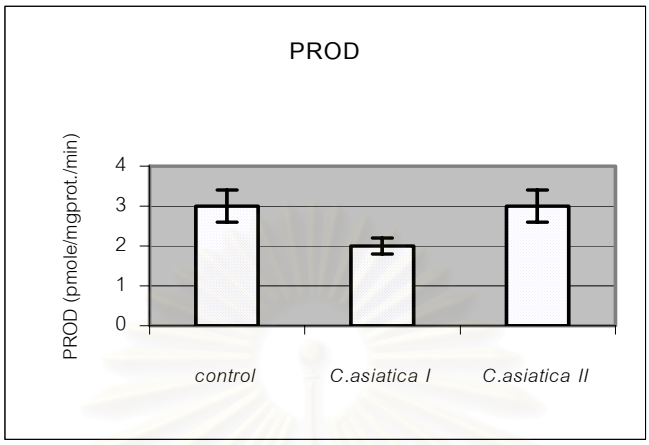


Figure 22 Effect of *C. asiatica* ethanolic extract on hepatic microsomal PROD

The individual bar represented mean of PROD with an error bar of standard error of the mean (n=10)

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

3.3.3 Effect of *C. asiatica* ethanolic extract on hepatic microsomal aniline 4-hydroxylation

Rate of aniline 4-hydroxylation, which represented the activity of CYP2E1, was not changed when *C. asiatica* ethanolic extract was given to rats at both doses in this study as compared to the control group (Figure 23).

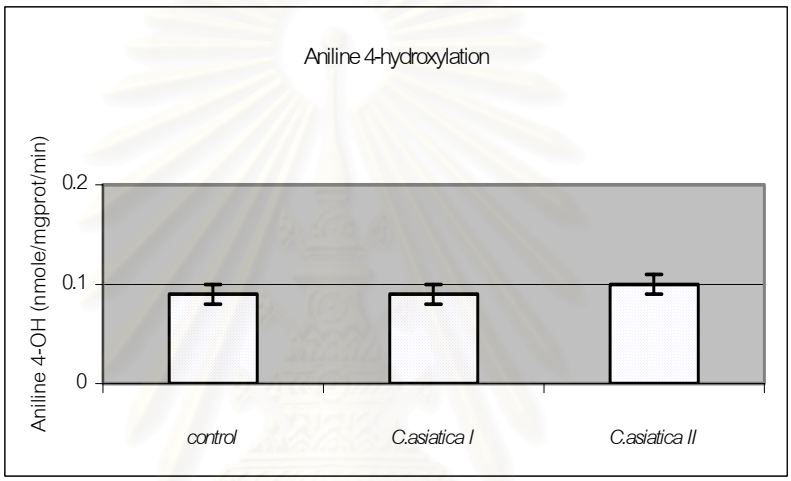


Figure 23 Effect of *C. asiatica* ethanolic extract on rate of aniline 4-hydroxylation

The individual bar represented mean of rate of aniline 4-hydroxylation with an error bar of standard error of the mean (n=10)

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

3.3.4 Effect of *C. asiatica* ethanolic extract on hepatic microsomal erythromycin N-demethylation

C. asiatica ethanolic extract at both doses used in this study did not exhibit any significant effect on the rate of erythromycin N-demethylation (which represented the activity of CYP3A) (Figure 24).

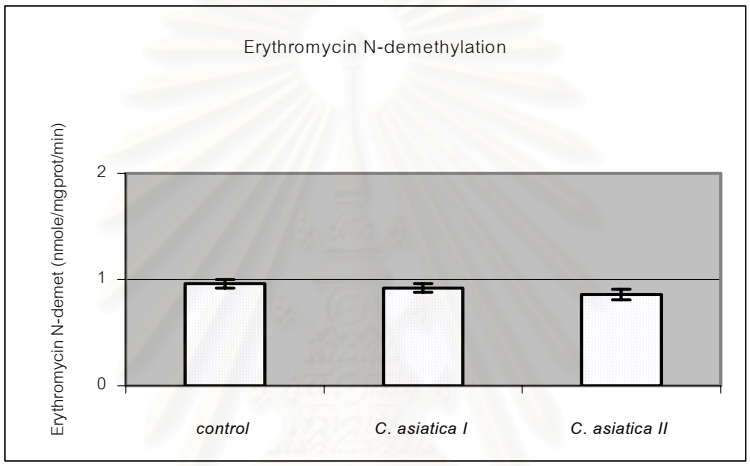


Figure 24 Effect of *C. asiatica* ethanolic extract on rate of erythromycin N-demethylation

The individual bar represented mean of rate of erythromycin N-demethylation with an error bar of standard error of the mean (n=10)

Control = Control group

C. asiatica I = *C. asiatica* treatment group I

C. asiatica II = *C. asiatica* treatment group II

CHAPTER V

DISCUSSION AND CONCLUSION

This study was performed to investigate subacute effects of *C. asiatica* ethanolic extract on hepatic CYPs and clinical blood chemistry in male Wistar rats at the doses of 250 and 1,000 mg/kg/day for 30 days. *C. asiatica* is used in folk medicine for a wide range of indications as well as consumed as daily diet and beverage. Thus, repeated exposure to this plant is likely to occur and may result in more or less modulation of hepatic drug metabolizing enzymes. This was the first report regarding the effects of *C. asiatica* ethanolic extract on hepatic CYPs particularly the isoforms that are responsible for xenobiotic bioactivation such as CYPs 1A1, 1A2, 2B1&2B2, 2E1, and 3A. Chronic exposure to this plant may induce induction of these isoforms resulting to increase risks to chemical-induced toxicities, mutagenesis, and carcinogenesis. In the other hand, antimutagenic and/or anticarcinogenic potential of this plant against many xenobiotics which are bioactivated by these CYPs, may be indicated if this plant possessed inhibition effects on these CYPs. In addition, drug-drug interactions and drug-food interactions should be concerned if *C. asiatica* is found to modulate drug metabolizing enzymes.

In this study, *C. asiatica* was extracted with 80% ethanol, the fraction which was shown to possess antitumour effect (Babu, *et al.*, 1995). Percentage yield of the extract was 25% w/w of the dried herbal *C. asiatica* which was in the range limited in the standard limitations that ethanol-soluble extractive fraction should not be less than 15% (Asean Countries, 1993 ; คณะกรรมการจัดทำตำรายา, 2545). Chemical identification tests including preliminary test and confirmatory test (Asean Countries, 1993 ; คณะกรรมการจัดทำตำรายา, 2545) were performed before the extract was given to the animals. The dosage of 250 mg/kg/day used in this study was the dosage regimen shown to be the maximum tolerated dose (MTD) in mice when *C. asiatica* ethanolic extract was administered intraperitoneally (Dhar, *et al.*, 1968). This dose was also shown to exhibit preventive effect on ethanol-induced gastric lesions in rats (Cheng and Koo, 2000). The dosage of 1,000 mg/kg/day

used in this study was also the dosage regimen shown to possess antitumour effect (Babu, *et al*., 1995).

To investigate effects of *C. asiatica* ethanolic extract on hepatic CYPs, specific substrate of the individual CYP was used and rate of specific substrate oxidation was determined to represent the corresponding CYP activity in hepatic microsomes of rats treated with the extract. ER, MR and PR&BR have been proved to be specific substrates of CYP1A1 (Burke and Mayer, 1974), CYP1A2 (Burke, *et al*., 1985) and CYP2B1&2B2 (Burke, *et al*., 1985; Lubet, *et al*., 1985), respectively. Aniline 4-hydroxylation was shown to represent CYP2E1 activity (Schenkman, *et al*., 1967) while erythromycin N-demethylation was classically used for determining CYP3A activity (Nash, 1953, Friedli, 1992).

Results from this study showed that both doses of *C. asiatica* ethanolic extract caused no changes of hepatic microsomal total CYP contents as well as the activities of CYPs 1A1, 1A2, 2B1&2B2, 2E1 and 3A. Lack of effects on total CYP contents and those isoforms tested of CYP would be an advantageous characteristic of this extract in term of risks to chemical-induced toxicities, mutagenesis, and/or carcinogenesis as well as drug-drug interaction. No induction effects on these isoform of CYPs indicated no potential increased risks of the extract on xenobiotic-induced toxicities, mutagenesis and/or carcinogenesis. Examples of xenobiotics bioactivated by CYP1A1 are benzo (a) pyrene, dimethylbenz (a) anthracene, aflatoxin B1, 6-nitrochrysene; by CYP1A2 are 2-aminofluorene, aflatoxin B1, 2-amino-3-methylimidazo[4,5]quinoline; by CYP2B1&2B2 are aflatoxin B1, benzo (a) pyrene, methylene chloroaniline; by CYP2E1 are nitrosodimethylamine, acetaminophen, benzene; and by CYP3A are aflatoxin B1, benzo (a) pyrene, 2-amino-3-methylimidazo[4,5]quinoline etc. (Soucek and Gut, 1992). No inhibition effects of *C. asiatica* ethanolic extract on these CYP isoforms, excluded an utilization of this aspect to explain the chemoprotective effects of this extract against procarcinogens bioactivated by these CYPs. No effects of this extract on these CYPs excluded the possibilities of drug-drug interaction or drug-food interaction if *C. asiatica* ethanolic extract is consumed concomittantly with other medicines that are metabolized by these CYPs. Examples of such therapeutic drugs that are metabolized by CYP1A1 are

warfarin, paracetamol; by CYP1A2 are paracetamol, theophylline, tamoxifen; by CYP2B1 are phenobarbital, diazepam; by CYP2B2 are phenobarbital, dexamethasone; by CYP2E1 are paracetamol, chlorzoxazone, by CYP3A are clarithromycin, carbamazepine, etc (Lin and Lu, 1998). However, to extrapolate this drug interaction data to human, effect of *C. asiatica* ethanolic extract should be further investigated in human such as *in vitro* study using human liver microsomes, *in vivo* clinical study, etc. Inconsistent to a previous study by Tepsuwan and Kusamran (1997), they found that 14-days dietary *C. asiatica* (25%) given to male Wistar rats 60-70 g, inhibited aniline 4-hydroxylase activity that represented the activity of CYP2E1. Differences between the previous study and this study were likely explained by these following diffences: preparation form of *C. asiatica*. In the previous study, they used crude dried plant that might have more impurities than the ethanolic extract used in this study. Secondly, the duration of exposure to *C. asiatica.* In the previous study, *C. asiatica* was given to rats fed 14 days but in this study, rats were given the ethanolic extract of *C. asiatica* fed 30 days. Third, there were diffrences of age and weight of rats used between the studies. In the previous study, they used male Wistar rats of age between 3-4 weeks with weight between 60-70 g but in this study, we used 8 weeks-male Wistar rats with weight between 250-300 g.

This study provided an additional subacute (30 days) toxicity data for *C. asiatica.* Results from this study showed that both doses of *C. asiatica* ethanolic extract demonstrated no effects on body weight, relative liver weight, food&water consumptions. Likewise, no adverse effects on many importance organs/systems were observed such as liver, kidney, blood system, some electrolytes in serum (sodium, potassium, chloride), lipid, and carbohydrate metabolism. This results was consistent to a previous study of Babu, *et al*. (1995). In that study, acute toxicity of *C. asiatica* was investigate by giving oral administration of 1 g/kg body weight of 80% methanolic extract of *C. asiatica* to normal and transplanted tumor bearing mice for 5 alternate days. The results showed that the extract did not produce any toxic symtoms even at high concentration (500 mg/mice that was equal to 5 g/kg body weight) and turned out to increase body weight of the animals. Likewise, in a previous study of Dhar, *et al*. (1968), they investigated subchronic toxicity of 50% ethanolic extract of *C. asiatica* by giving intraperitoneally to mice, the

maximum tolerated dose (MTD) was found to be 250 mg/kg body weight. In contrast, a previous study by Tepsuwan and Kusamran (1997) demonstrated that 14-days dietary *C. asiatica* (25%) giving to male Wistar rats weighing 60-70 g, significantly increased the capacity of S9 fraction to activate the mutagenicity of aflatoxinB1. Adverse reactions following the use of *C. asiatica* have been reported. Allergic contact dermatitis has been reported after the topical application of various preparation of aqueous *C. asiatica* such as cream and ointment (Eun and Lee, 1985; Izu, *et al*., 1992). Further studies should be investigated regarding the toxicity of *C. asiatica* in term of long term chronic exposure especially in clinical studies.

In conclusion, subacute effects of *C. asiatica* ethanolic extract on hepatic CYPs and blood clinical chemistry were studied in male Wistar rats. Two doses (250 and 1000 mg/kg/day) of the extract were given orally to rats for 30 days compared to a control group given distilled water in the same manner. The results showed that *C. asiatica* ethanolic extract did not cause any changes on total CYP contents and the activities of CYP1A1, CYP1A2, CYP2B1&2B2, CYP2E1, and CYP3A. In addition, *C. asiatica* ethanolic extract at both doses used in this study did not cause any harmful effects on various important organs/systems. No effects on total CYP contents and these isoforms of CYP would be an advantageous characteristic of this extract in term of risk to drug-drug interaction, drug-food interaction as well as chemical-induced toxicities, mutagenesis and/or carcinogenesis. In addition, an explanation for the chemoprotective effects of this extract against procarcinogens bioactivated by these CYPs was excluded. Further studies on the effects of this plant extract on human hepatic CYPs and human chinical blood chemistry were suggested. Effect of *C. asiatica* ethanolic extract on Phase II enzymes that might contribute to the cancer preventive effect of this plant should also be investigated.

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APPENDIX A

Table 7 Body weight of an individual rat in an control group

Rat Number	Day 0	Day 7	Day 14	Day 21	Day 28	Day 30
1	268.8	324.8	358.5	373.8	385.9	380.5
2	312.4	342.5	361.9	378.5	397	380.9
3	313.3	368	403.8	423.9	424.7	439.7
4	335.6	383.7	402.3	413	419.4	409.2
5	352.2	372.3	374.4	397.3	401.8	392.1
6	328.9	339.8	355.3	362.7	366	349.4
7	392.5	402	425.4	444.6	467.8	446.7
8	415.5	431.7	460.7	466.9	478.3	461.8
9	399.2	415	393.7	426.1	443.5	417.2
10	419.9	445.3	431.2	459.5	468.3	457.4
Mean	353.83	382.51	396.72	414.63	425.27	413.49
SEM	16.08	12.85	11.10	11.47	12.13	11.93

Unit expressed as g

Table 8 Body weight of an individual rat in *C. asiatica* treatment group I

Rat Number	Day 0	Day 7	Day 14	Day 21	Day 28	Day 30
1	303.1	351	377	389.4	398.3	381
2	330.4	358	381.3	416	432.6	411.7
3	435	448.4	453.2	451.7	449.2	428
4	339	373.7	404.5	413.3	426.6	404.8
5	331.9	349.3	374.1	380.5	367.5	386.6
6	367.5	389.8	404.5	406.7	422	400.9
7	346.5	368.8	364.3	360.4	363.3	345.8
8	344.3	357.7	379.3	386.9	394.1	374.5
9	381.7	397.7	386.5	416.2	431.3	416.5
10	389.2	411.6	429.1	452.3	465	450.3
Mean	356.86	380.60	395.38	407.34	414.99	400.01
SEM	11.87	10.01	8.78	9.33	33.46	29.58

Unit expressed as g

Table 9 Body weight of an individual rat in *C. asiatica* treatment group II

Rat Number	Day 0	Day 7	Day 14	Day 21	Day 28	Day 30
1	305.3	362	383.5	417.3	437.3	416.7
2	322.4	350.5	370.7	392.4	410.8	441.5
3	372.8	404	423.3	442.4	450.2	457.5
4	327.7	354.1	373.4	376	381.5	369.4
5	385.5	399.3	410.9	417.8	426.5	410.9
6	378	388.8	389.2	396.1	405.5	381.5
7	383.5	413	432.9	449.1	463.1	442.8
8	353.1	370.8	396.4	394.8	404.8	385.8
9	365.2	380.9	375.2	387	393	380.5
10	403.7	423.7	422.5	438.9	453	438.7
Mean	359.72	384.71	397.80	411.18	422.57	412.53
SEM	10.07	8.00	7.29	8.12	8.74	10.03

Unit expressed as g

Table 10 Liver weight of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	10.51	12.64	12.04
2	10.36	11.78	11.58
3	16.66	12.1	16.1
4	11.85	12.92	13.72
5	9.71	12.44	12.51
6	10.98	13.09	12.19
7	14.08	11.05	14.19
8	14.00	14.27	13.47
9	13.18	14.96	12.39
10	16.44	14.00	13.94
Mean	12.78	12.93	13.21
SEM	0.79	0.38	0.43

Unit expressed as g

Table 11 The one- week food consumption of an individual rat in an control group

Rat Number	Day 8	Day 15	Day 22	Day 30
1	12.00	18.00	22.00	20.00
2	20.00	18.00	18.00	18.00
3	43.00	24.00	28.00	23.00
4	26.00	24.00	22.00	28.00
5	21.00	22.00	10.00	20.00
6	20.00	21.00	20.00	20.00
7	24.00	23.00	29.00	22.00
8	24.00	28.00	22.00	11.00
9	15.00	22.00	22.00	20.00
10	22.00	28.00	22.00	20.00
Mean	22.00	22.00	22.00	20.00
SEM	2.00	1.00	2.00	1.00

Unit expressed as g

Table 12 The one- week food consumption of an individual rat in *C. asiatica* treatment group I

Rat Number	Day 8	Day 15	Day 22	Day 30
1	32.00	32.00	31.00	22.00
2	22.00	22.00	24.00	20.00
3	21.00	30.00	16.00	16.00
4	26.00	22.00	27.00	28.00
5	13.00	17.00	14.00	22.00
6	16.00	27.00	22.00	24.00
7	25.00	21.00	24.00	24.00
8	11.00	17.00	22.00	18.00
9	22.00	25.00	24.00	22.00
10	27.00	34.00	28.00	25.00
Mean	22.00	24.00	23.00	22.00
SEM	2.00	2.00	2.00	1.00

Unit expressed as g

Table 13 The one- week food consumption of an individual rat in *C. asiatica* treatment group II

Rat Number	Day 8	Day 15	Day 22	Day 30
1	32.00	32.00	31.00	22.00
2	22.00	22.00	24.00	20.00
3	30.00	28.00	23.00	26.00
4	26.00	22.00	27.00	28.00
5	13.00	17.00	14.00	22.00
6	16.00	16.00	20.00	20.00
7	25.00	21.00	24.00	24.00
8	11.00	14.00	17.00	20.00
9	22.00	25.00	24.00	22.00
10	27.00	34.00	28.00	25.00
Mean	22.00	23.00	23.00	22.00
SEM	2.00	2.00	2.00	1.00

Unit expressed as g

Table 14 The one- week water consumption of an individual rat in an control group

Rat Number	Day 8	Day 15	Day 22	Day 30
1	42.00	42.00	34.00	38.00
2	56.00	36.00	34.00	28.00
3	62.00	53.00	44.00	50.00
4	48.00	48.00	47.00	52.00
5	60.00	56.00	47.00	54.00
6	62.00	96.00	54.00	54.00
7	60.00	34.00	45.00	38.00
8	38.00	45.00	38.00	12.00
9	71.00	46.00	40.00	38.00
10	46.00	38.00	40.00	38.00
Mean	54.00	49.00	42.00	40.00
SEM	3.00	6.00	2.00	4.00

Unit expressed as mL

Table 15 The one- week water consumption of an individual rat in *C. asiatica* treatment group I

Rat Number	Day 8	Day 15	Day 22	Day 30
1	74.00	51.00	45.00	36.00
2	44.00	38.00	34.00	40.00
3	44.00	38.00	23.00	20.00
4	47.00	60.00	67.00	60.00
5	44.00	37.00	24.00	47.00
6	38.00	32.00	42.00	42.00
7	40.00	38.00	38.00	41.00
8	27.00	24.00	36.00	32.00
9	38.00	49.00	43.00	36.00
10	42.00	42.00	45.00	34.00
Mean	44.00	40.00	40.00	38.00
SEM	4.00	3.00	4.00	3.00

Unit expressed as mL

Table 16 The one- week water consumption of an individual rat in *C. asiatica* treatment group II

Rat Number	Day 8	Day 15	Day 22	Day 30
1	74.00	51.00	45.00	36.00
2	44.00	38.00	34.00	40.00
3	49.00	40.00	46.00	48.00
4	47.00	60.00	67.00	60.00
5	44.00	37.00	24.00	47.00
6	38.00	32.00	23.00	32.00
7	40.00	38.00	38.00	41.00
8	26.00	31.00	33.00	35.00
9	38.00	49.00	43.00	36.00
10	42.00	42.00	45.00	34.00
Mean	44.00	42.00	40.00	40.00
SEM	4.00	2.00	4.00	2.00

Unit expressed as mL

Table 17 Blood glucose of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	86.00	107.00	116.00
2	122.00	112.00	110.00
3	140.00	98.00	124.00
4	119.00	91.00	106.00
5	105.00	110.00	99.00
6	97.00	96.00	91.00
7	113.00	94.00	90.00
8	88.00	103.00	117.00
9	158.00	116.00	104.00
10	100.00	100.00	107.00
Mean	112.80	102.70	106.40
SEM	7.24	2.63	3.49

Unit expressed as mg/dL

Table 18 Serum total cholesterol of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	75.00	62.00
2	61.00	85.00	76.00
3	75.00	47.00	66.00
4	92.00	83.00	61.00
5	57.00	83.00	84.00
6	60.00	60.00	69.00
7	58.00	64.00	61.00
8	68.00	79.00	82.00
9	72.00	62.00	67.00
10	85.00	69.00	85.00
Mean	69.78	70.70	71.30
SEM	4.14	3.94	3.05

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 19 Serum triglyceride of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	109.00	76.00
2	88.00	109.00	83.00
3	131.00	85.00	97.00
4	53.00	93.00	87.00
5	60.00	147.00	97.00
6	110.00	93.00	77.00
7	83.00	66.00	102.00
8	134.00	88.00	64.00
9	85.00	184.00	76.00
10	118.00	92.00	57.00
Mean	95.78	106.60	81.60
SEM	9.75	10.89	4.62

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 20 Serum HDL-C of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	39.10	29.70
2	27.00	31.30	29.30
3	41.60	26.70	39.00
4	36.70	39.60	30.40
5	24.20	41.80	44.00
6	31.30	33.60	32.00
7	28.10	33.60	31.40
8	29.60	33.70	34.70
9	34.00	30.40	34.10
10	40.70	29.60	35.50
Mean	32.58	33.94	34.01
SEM	2.04	1.53	1.46

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 21 Serum LDL-C of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	5.00	8.00	7.00
3	5.00	4.00	7.00
4	14.00	9.00	7.00
5	7.00	7.00	11.00
6	5.00	7.00	7.00
7	5.00	8.00	4.00
8	4.00	8.00	9.00
9	8.00	4.00	9.00
10	8.00	8.00	13.00
Mean	6.78	7.00	8.22
SEM	1.02	0.60	0.88

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 22 Serum AST of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	126.00	99.00
2	132.00	97.00	-
3	70.00	101.00	94.00
4	104.00	88.00	89.00
5	105.00	124.00	138.00
6	135.00	97.00	116.00
7	109.00	87.00	92.00
8	103.00	86.00	126.00
9	107.00	80.00	98.00
10	84.00	76.00	112.00
Mean	105.44	96.20	107.11
SEM	6.79	5.39	5.64

Unit expressed as U/L

Missing value (-) was due to blood insufficiency and outlier

Table 23 Serum ALT of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	33.00	24.00
2	20.00	20.00	51.00
3	23.00	22.00	29.00
4	20.00	33.00	35.00
5	32.00	30.00	33.00
6	36.00	30.00	31.00
7	29.00	23.00	29.00
8	24.00	27.00	39.00
9	17.00	28.00	21.00
10	17.00	22.00	27.00
Mean	24.22	26.80	31.90
SEM	2.25	1.51	2.69

Unit expressed as U/L

Missing value (-) was due to blood insufficiency

Table 24 Serum ALP of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	113.00	96.00
2	101.00	88.00	95.00
3	199.00	122.00	187.00
4	95.00	114.00	169.00
5	99.00	122.00	145.00
6	118.00	141.00	107.00
7	107.00	130.00	111.00
8	101.00	110.00	111.00
9	111.00	107.00	115.00
10	116.00	92.00	98.00
Mean	116.33	113.90	123.40
SEM	10.66	5.10	10.24

Unit expressed as U/L

Missing value (-) was due to blood insufficiency

Table 25 Total bilirubin in serum of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	0.30	0.20
2	0.20	0.70	0.60
3	0.20	0.10	0.40
4	0.20	0.20	0.20
5	0.20	< 0.10	0.20
6	0.20	0.20	0.20
7	< 0.10	< 0.10	< 0.10
8	< 0.10	< 0.10	0.60
9	0.20	< 0.10	< 0.10
10	0.10	0.10	< 0.10
Mean	0.19	0.27	0.34
SEM	0.01	0.09	0.07

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 26 Direct bilirubin in serum of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	0.20	0.10
2	0.00	0.60	0.50
3	0.10	0.10	0.30
4	0.10	0.10	0.10
5	0.10	0.00	0.10
6	0.00	0.10	0.10
7	0.00	0.00	0.00
8	0.00	0.00	0.60
9	0.20	0.00	0.00
10	0.10	0.10	0.00
Mean	0.07	0.12	0.18
SEM	0.02	0.06	0.07

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 27 Serum BUN of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	20.40	16.60
2	18.6	22.30	21.90
3	29.6	20.00	24.50
4	20.00	20.10	16.40
5	16.80	18.50	19.00
6	22.10	20.50	19.10
7	16.50	19.70	17.90
8	18.10	20.40	28.20
9	20.60	22.40	22.40
10	20.50	11.60	23.80
Mean	20.31	19.59	20.98
SEM	1.31	0.96	1.21

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 28 SCr of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	0.50	0.50
2	0.50	0.50	0.60
3	0.50	0.70	0.50
4	0.50	0.50	0.60
5	0.50	0.50	0.80
6	0.70	0.70	0.50
7	0.70	0.60	0.60
8	0.60	0.70	0.80
9	0.70	0.60	0.60
10	0.70	0.60	0.60
Mean	0.60	0.59	0.61
SEM	0.03	0.03	0.03

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 29 Serum Uric acid of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	1.20	1.90
2	1.80	3.40	3.00
3	2.00	1.60	2.00
4	2.00	1.90	2.40
5	1.90	2.10	2.40
6	1.90	1.50	1.90
7	1.70	1.70	1.30
8	1.90	1.60	1.90
9	2.20	2.00	2.40
10	2.70	1.20	1.70
Mean	2.01	1.82	2.09
SEM	0.10	0.20	0.15

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency

Table 30 Serum sodium of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	145.00	146.00
2	143.00	144.00	143.00
3	142.00	144.00	142.00
4	142.00	144.00	146.00
5	143.00	146.00	143.00
6	142.00	143.00	143.00
7	145.00	146.00	147.00
8	148.00	145.00	145.00
9	153.00	144.00	144.00
10	146.00	145.00	145.00
Mean	144.89	144.60	144.40
SEM	1.23	0.31	0.52

Unit expressed as mEq/L

Missing value (-) was due to blood insufficiency

Table 31 Serum potassium of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	4.30	3.80
2	4.00	4.70	5.20
3	4.60	4.80	4.70
4	4.80	4.50	5.10
5	4.30	5.00	5.70
6	4.30	4.10	5.00
7	3.80	4.00	3.90
8	4.10	4.20	4.00
9	4.30	4.80	5.40
10	4.60	4.30	6.20
Mean	4.31	4.47	4.90
SEM	0.11	0.11	0.25

Unit expressed as mEq/L

Missing value (-) was due to blood insufficiency

Table 32 Serum chloride of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	107.00	104.00
2	104.00	104.00	109.00
3	102.00	105.00	104.00
4	105.00	105.00	107.00
5	103.00	106.00	104.00
6	105.00	103.00	106.00
7	105.00	106.00	106.00
8	107.00	104.00	103.00
9	113.00	104.00	106.00
10	104.00	106.00	107.00
Mean	105.33	105.00	105.60
SEM	1.07	0.39	0.58

Unit expressed as mEq/L

Missing value (-) was due to blood insufficiency

Table 33 Hct of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	52.00	54.00	49.00
3	51.00	52.00	50.00
4	50.00	50.00	50.00
5	51.00	52.00	52.00
6	53.00	55.00	52.00
7	49.00	51.00	52.00
8	52.00	51.00	41.00
9	55.00	52.00	50.00
10	53.00	55.00	53.00
Mean	51.78	52.44	49.89
SEM	0.60	0.60	1.20

Unit expressed as percent value

Missing value (-) was due to blood insufficiency

Table 34 Hb of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	16.30	16.80	15.20
3	16.30	15.70	15.80
4	16.30	16.50	16.00
5	16.50	16.30	16.60
6	17.50	17.90	17.30
7	15.30	16.10	16.40
8	17.00	17.10	13.60
9	17.10	16.00	15.60
10	16.00	16.40	16.10
Mean	16.48	16.53	15.84
SEM	0.22	0.22	0.35

Unit expressed as g/dL

Missing value (-) was due to blood insufficiency

Table 35 Platlet Count of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	719.00	763.00	783.00
3	699.00	688.00	746.00
4	912.00	702.00	772.00
5	674.00	762.00	736.00
6	712.00	874.00	886.00
7	666.00	622.00	686.00
8	934.00	650.00	668.00
9	852.00	684.00	702.00
10	626.00	628.00	802.00
Mean	754.89	708.11	753.44
SEM	37.91	26.79	22.36

Unit expressed as x 10^3 cells/cumm

Missing value (-) was due to blood insufficiency

Table 36 WBC Count of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	2,220.00	737.00	581.00
3	1,710.00	1,488.00	2060.00
4	1,854.00	1,900.00	1514.00
5	1,350.00	874.00	2160.00
6	1,636.00	1,946.00	2120.00
7	1,478.00	1,138.00	966.00
8	758.00	1,796.00	1388.00
9	1,328.00	1,396.00	1192.00
10	488.00	1,070.00	338.00
Mean	1,424.67	1,371.67	1368.78
SEM	178.05	149.04	222.55

Unit expressed as cells/cumm

Missing value (-) was due to blood insufficiency

Table 37 RBC Count of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	8.92	9.14	8.70
3	9.20	9.14	8.38
4	8.72	8.58	8.86
5	9.14	9.42	9.22
6	9.24	9.84	9.12
7	8.50	9.40	8.92
8	9.02	8.78	7.12
9	9.86	8.82	9.14
10	8.98	9.22	8.92
Mean	9.06	9.15	8.71
SEM	0.13	0.13	0.22

Unit expressed as million cells/cumm

Missing value (-) was due to blood insufficiency

Table 38 RBC Morphology of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	Micro 3+	Micro 3+	Micro 3+
3	Micro 2+	Micro 3+	Micro 2+
4	Micro 3+	Micro 3+	Micro 3+
5	Micro 3+	Micro 3+	Micro 3+
6	Micro 2+	Micro 2+	Micro 2+
7	Micro 2+	Micro 2+	Micro 2+
8	Micro 2+	Micro 2+	Micro 2+
9	Micro 3+	Micro 3+	Micro 3+
10	Micro 3+	Micro 3+	Micro 3+

Missing value (-) was due to blood insufficiency

Table 39 Neutrophil of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	20.00	41.00	27.00
3	33.00	32.00	26.00
4	24.00	35.00	39.00
5	14.00	24.00	23.00
6	50.00	57.00	40.00
7	45.00	22.00	48.00
8	40.00	44.00	46.00
9	12.00	12.00	18.00
10	16.00	26.00	16.00
Mean	28.22	32.56	31.44
SEM	4.74	4.50	4.01

Unit expressed as percent value

Missing value (-) was due to blood insufficiency

Table 40 Eosinophil of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	1.00	0	3.00
3	0	0	0
4	2.00	4.00	1.00
5	2.00	4.00	2.00
6	0	0	0
7	3.00	0	0
8	0	0	0
9	0	0	0
10	0	0	0
Mean	0.89	0.89	0.67
SEM	0.39	0.59	0.37

Unit expressed as percent value

Missing value (-) was due to blood insufficiency

Table 41 Lymphocyte of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	79.00	59.00	70.00
3	67.00	64.00	74.00
4	74.00	60.00	58.00
5	83.00	70.00	73.00
6	50.00	43.00	60.00
7	52.00	77.00	48.00
8	60.00	53.00	53.00
9	88.00	87.00	82.00
10	82.00	74.00	84.00
Mean	70.56	65.22	66.89
SEM	4.66	4.45	4.24

Unit expressed as percent value

Missing value (-) was due to blood insufficiency

Table 42 Monocyte of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	0	0	0
3	0	4.00	0
4	0	1.00	2.00
5	1.00	2.00	2.00
6	0	0	0
7	0	1.00	4.00
8	0	3.00	1.00
9	0	1.00	0
10	2.00	0	0
Mean	0.33	1.33	1.00
SEM	0.24	0.47	0.47

Unit expressed as percent value

Missing value (-) was due to blood insufficiency

Table 43 MCV of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	58.10	58.80	56.50
3	55.70	56.90	59.70
4	57.60	58.60	56.10
5	56.20	55.10	56.90
6	57.70	56.20	57.20
7	57.80	54.80	58.20
8	58.00	58.10	57.60
9	55.90	58.80	55.00
10	58.80	59.80	59.20
Mean	57.31	57.46	57.38
SEM	0.37	0.59	0.50

Unit expressed as fL

Missing value (-) was due to blood insufficiency

Table 44 MCH of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	18.20	18.40	17.50
3	17.70	17.20	18.90
4	18.70	19.20	18.10
5	18.10	17.30	18.00
6	18.90	18.20	19.00
7	18.00	17.10	18.40
8	18.80	19.50	19.20
9	17.30	18.20	17.10
10	17.80	17.80	18.00
Mean	18.17	18.10	18.24
SEM	0.18	0.28	0.23

Unit expressed as pg

Missing value (-) was due to blood insufficiency

Table 45 MCHC of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	-	-	-
2	31.30	31.20	30.90
3	31.80	30.30	31.60
4	32.50	32.80	32.30
5	32.20	31.50	31.60
6	32.70	32.30	33.20
7	31.10	31.20	31.50
8	32.50	33.60	33.20
9	31.00	31.00	31.00
10	30.30	29.80	30.50
Mean	31.71	31.52	31.76
SEM	0.28	0.40	0.32

Unit expressed as g/dL

Missing value (-) was due to blood insufficiency

Table 46 The concentration of microsomal protein of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	31.46	17.24	15.53
2	23.95	32.03	29.03
3	40.43	24.47	27.78
4	37.73	29.65	25.09
5	27.47	36.31	32.27
6	28.47	28.41	30.86
7	33.57	16.93	36.22
8	32.27	27.71	25.98
9	33.14	31.84	28.58
10	38.35	34.66	33.14
Mean	32.68	27.92	28.45
SEM	1.64	2.10	1.79

Unit expressed as mg/mL

Table 47 Hepatic microsomal total CYP content of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	0.87	1.10	0.73
2	0.83	0.85	0.91
3	0.59	0.73	0.82
4	0.58	0.79	0.93
5	0.70	0.82	0.83
6	0.72	0.76	0.75
7	0.71	0.73	0.74
8	0.64	0.65	0.65
9	0.75	0.70	0.64
10	0.58	0.46	0.59
Mean	0.70	0.76	0.76
SEM	0.03	0.05	0.04

Unit expressed as nmol/mg protein

Table 48 Hepatic microsomal EROD of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	28.00	39.00	22.00
2	32.00	24.00	32.00
3	14.00	26.00	14.00
4	18.00	26.00	21.00
5	28.00	22.00	19.00
6	34.00	19.00	27.00
7	28.00	22.00	22.00
8	22.00	22.00	18.00
9	32.00	20.00	24.00
10	14.00	20.00	20.00
Mean	25.00	24.00	22.00
SEM	2.00	2.00	2.00

Unit expressed as pmol/mg protein/min

Table 49 Hepatic microsomal MROD of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	14.00	14.00	9.00
2	12.00	10.00	8.00
3	2.00	7.00	6.00
4	8.00	10.00	4.00
5	8.00	5.00	10.00
6	10.00	10.00	8.00
7	8.00	6.00	10.00
8	4.00	6.00	6.00
9	10.00	6.00	9.00
10	4.00	4.00	4.00
Mean	8.00	8.00	7.00
SEM	1.00	1.00	1.00

Unit expressed as pmol/mg protein/min

Table 51 Hepatic microsomal BROD of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	6.00	22.00	15.00
2	12.00	13.00	20.00
3	6.00	12.00	8.00
4	6.00	16.00	10.00
5	18.00	8.00	8.00
6	22.00	14.00	20.00
7	15.00	14.00	20.00
8	12.00	12.00	8.00
9	12.00	14.00	15.00
10	8.00	10.00	8.00
Mean	12.00	14.00	13.00
SEM	2.00	1.00	2.00

Unit expressed as pmol/mg protein/min

Table 50 Hepatic microsomal PROD of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	4.00	4.00	3.00
2	3.00	2.00	5.00
3	1.00	2.00	2.00
4	2.00	4.00	3.00
5	4.00	2.00	2.00
6	4.00	3.00	6.00
7	5.00	4.00	5.00
8	4.00	2.00	2.00
9	4.00	3.00	3.00
10	2.00	2.00	2.00
Mean	3.00	2.00	3.00
SEM	0.40	0.20	0.40

Unit expressed as pmol/mg protein/min

Table 52 Hepatic microsomal aniline 4- hydroxylase activity of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	0.12	0.09	0.11
2	0.12	0.10	0.11
3	0.03	0.08	0.04
4	0.03	0.04	0.09
5	0.10	0.04	0.04
6	0.13	0.10	0.13
7	0.12	0.15	0.09
8	0.08	0.11	0.11
9	0.10	0.11	0.15
10	0.05	0.06	0.12
Mean	0.09	0.09	0.10
SEM	0.01	0.01	0.01

Unit expressed as nmol/mg protein/min

Table 53 Hepatic microsomal erythromycin N-demethylase activity of an individual rat

Rat Number	Treatment group		
	Control group	*C. asiatica* treatment group I	*C. asiatica* treatment group II
1	0.83	1.03	0.96
2	0.93	0.75	0.83
3	1.14	1.05	1.11
4	0.81	1.02	1.02
5	0.93	1.12	1.05
6	1.01	0.79	0.63
7	0.75	0.85	0.78
8	1.03	0.85	0.85
9	1.17	0.93	0.68
10	0.95	0.85	0.68
Mean	0.96	0.92	0.86
SEM	0.04	0.04	0.05

Unit expressed as nmol/mg protein/min

APPENDIX B

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

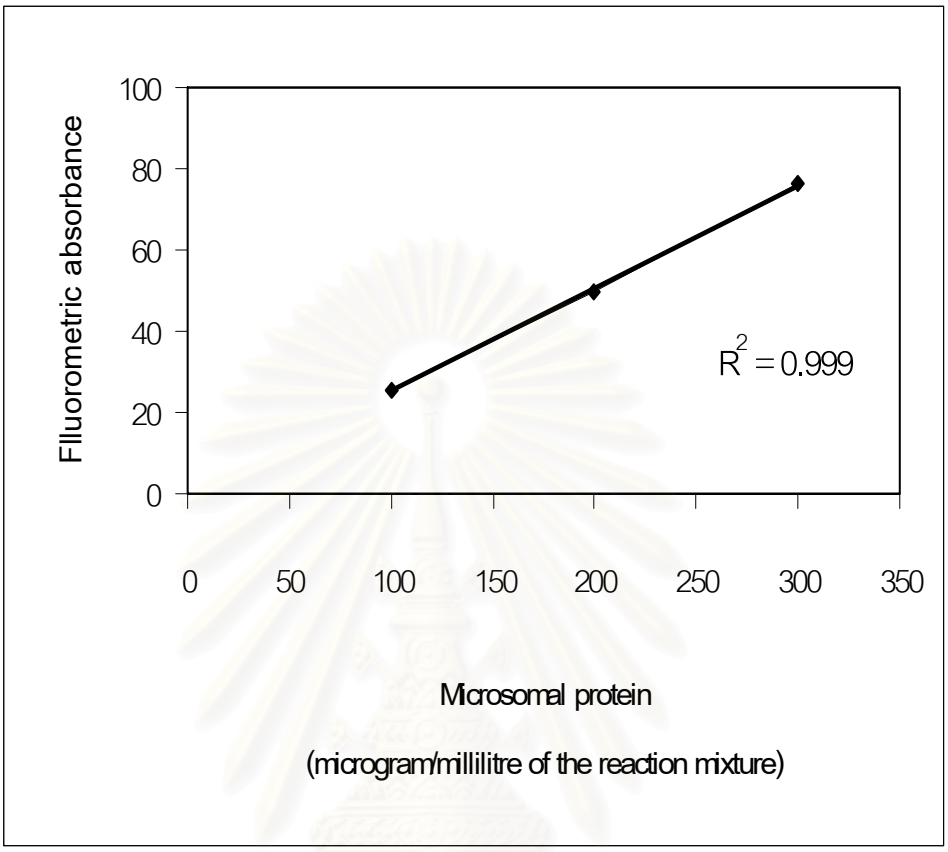


Figure 25 Verification of alkoxyresorufin O-dealkylation. Correlation between the amount of microsomal protein used in the reaction and fluorometric absorbance was shown with a correlation coefficient (r^2) of 0.999. Each point was mean of n=2 (Procedure was demonstrated in the Materials and Methods).

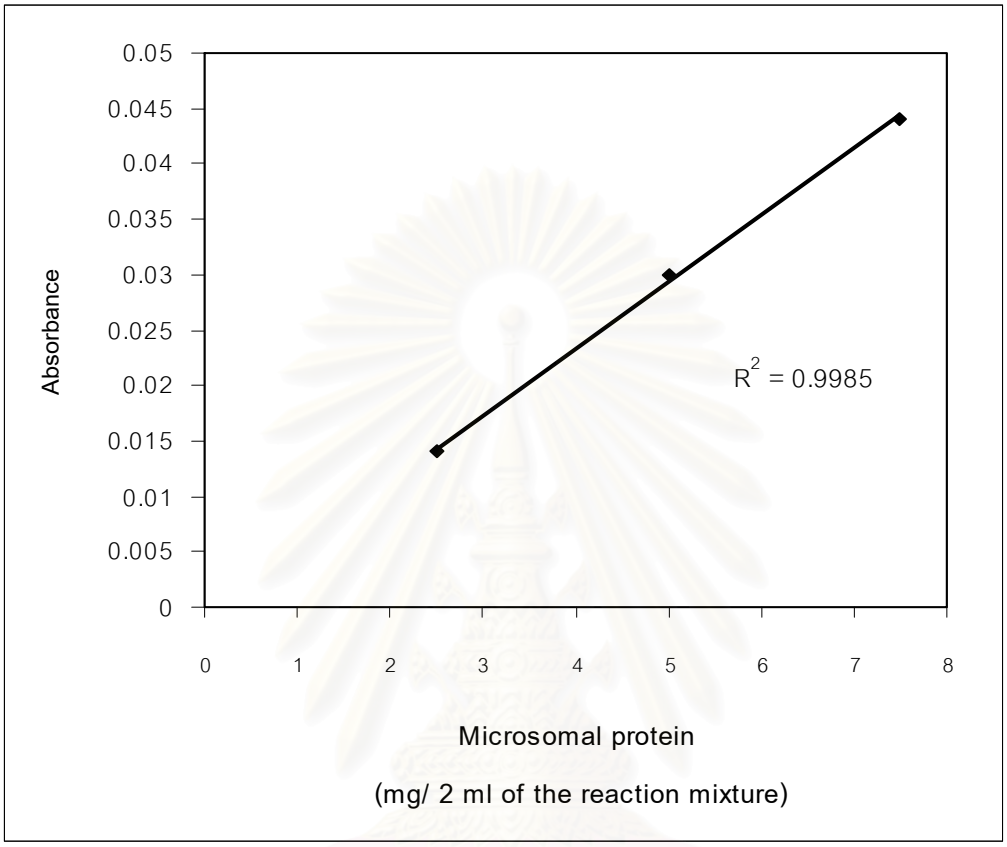


Figure 26 Verification of aniline 4-hydroxylation. Correlation between the amount of microsomal protein used in the reaction and absorbance was shown with a correlation coefficient (r^2) of 0.9985. Each point was mean of n=2 (Procedure was demonstrated in the Materials and Methods).

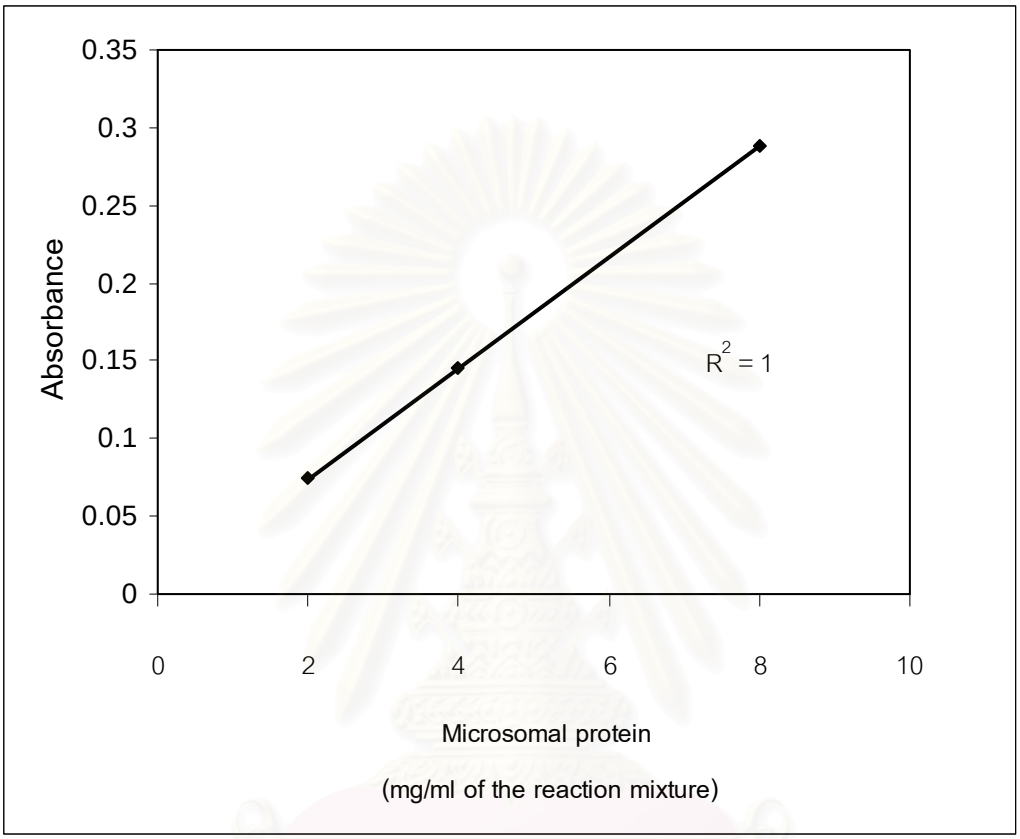
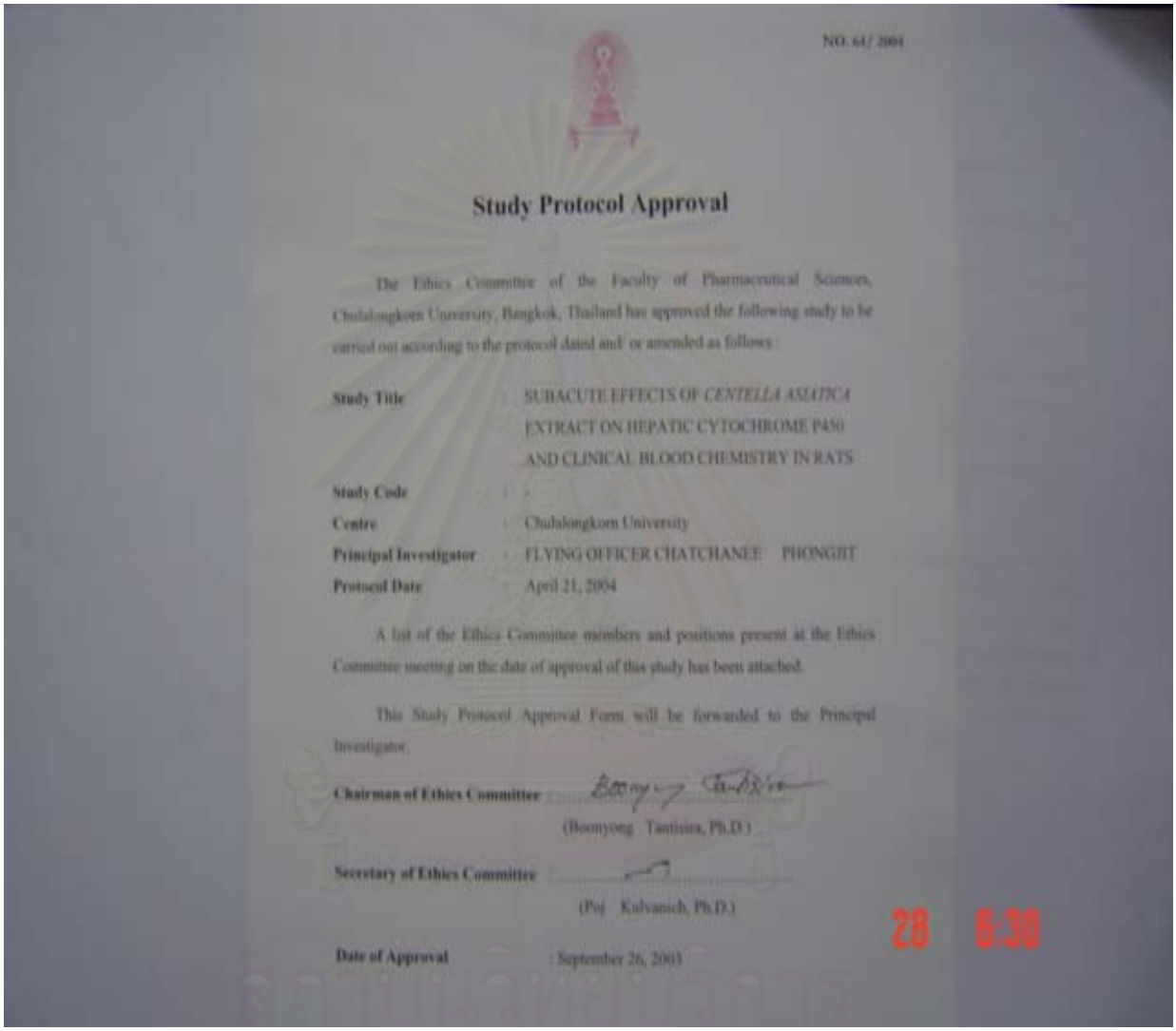


Figure 27 Verification of erythromycin N-demethylation. Correlation between the amount of microsomal protein used in the reaction and absorbance was shown with a correlation coefficient (r^2) of 1.000. Each point was mean of n=2 (Procedure was demonstrated in the Materials and Methods).

Appendix C

Figure 28 Study Protocol Approval by Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bankok, Thailand.



NO. 6A/ 2004

Study Protocol Approval

The Ethics Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand has approved the following study to be carried out according to the protocol dated and/ or amended as follows :

Study Title : SUBACUTE EFFECTS OF *CENTELLA ASIATICA* EXTRACT ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS

Study Code : -

Centre : Chulalongkorn University

Principal Investigator : FLYING OFFICER CHATCHANEE PHONGJIT

Protocol Date : April 21, 2004

A list of the Ethics Committee members and positions present at the Ethics Committee meeting on the date of approval of this study has been attached.

This Study Protocol Approval Form will be forwarded to the Principal Investigator.

Chairman of Ethics Committee .....

(Boonyong Tantisira, Ph.D.)

Secretary of Ethics Committee .....

(Puj Kulvanich, Ph.D.)

Date of Approval : September 26, 2003

28 6:30

Appendix D

Table 54 Normal Lab Values of rats (……..)

Parameters	Lab values	Units
Glucose	50-135	mg/dL
Total cholesterol	55-88	mg/dL
AST	77-110	U/L
ALT	27-42	U/L
ALP	104-160	U/L
Total bilirubin	0.2-0.55	mg/dL
BUN	15-21	mg/dL
Creatinine	0.2-0.8	mg/dL
Sodium	143-156	mEq/L
Potassium	4.6-5.9	mEq/L
Chloride	100-110	mEq/L
Hemoglobin	10.0-16.7	g/dL
Hematocrit	41.7-49.2	%
RBC Count	6.76-9.75	X 10^6 cells/cumm
MCV	50.0-77.8	FL
MCH	16.0-23.1	Pg
MCHC	28.2-34.1	g/dL
Platlet Count	685-1,436	X 10^3 cells/cumm
Neutrophil	5.3-38.1	%
Lymphocyte	56.7-93.1	%
Monocyte	0-7.7	%
Eosinophil	0-3.4	%
Basophil	0-0.4	%

VITAE

Miss Chatchanee Phongjit was born in October 6, 1978 in Bangkok, Thailand. She graduated with a Bachelor of Science in Pharmacy in 2000 from the Faculty of Pharmaceutical sciences, Chulalongkorn University, Bangkok, Thailand. After graduation, she worked as a pharmacist in Jantarubeksa Hospital, Nakhonprathom for one year and Wing 46 Hospital for one year.

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