ผลกึ่งเฉียบพลันของสารสกัดเถาบอระเพ็ดต่อเอนไซม์ไซโตโครมพี 450 และค่าเคมีคลินิกในเลือดหนูขาว

นางสาวอุ้มชู สายเพชร

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SUBACUTE EFFECTS OF *TINOSPORA CRISPA* STEM EXTRACT ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS

Miss Oomchoo Saiphet

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for the Degree of Master of Sciences in Pharmacology

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อุ้มชู สายเพชร : ผลกึ่งเฉียบพลันของสารสกัดเถาบอระเพ็ดต่อเอนไซม์ไซโตโครมพี 450 และค่าเคมีคลินิกในเลือดหนูขาว. (SUBACUTE EFFECTS OF *TINOSPORA CRISPA* STEM 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, จำนวนหน้า 123 หน้า. ISBN 974-17-3203-1.

บอระเพ็ด (*Tinospora crispa* Miers ex Hook. f. & Thomson) เป็นสมุนไพรพื้นบ้านที่นิยมใช้เป็นยาลดน้ำตาลในเลือด การศึกษานี้มุ่งศึกษาผลกึ่งเฉียบพลันของสารสกัดเถาบอระเพ็ดต่อเอนไซม์ไซโตโครม พี450 (CYPs) ในตับ และค่าเคมีคลินิกต่างๆ ในเลือดของหนูขาวเพศผู้พันธุ์วิสตาร์ โดยแบ่งหนูขาวแบบสุ่มเป็น 3 กลุ่ม กลุ่มละ 10 ตัว ดังต่อไปนี้ กลุ่มควบคุม กลุ่มที่ได้รับบอระเพ็ด I และ II ได้รับบอระเพ็ดในขนาด 250 และ 500 มิลลิกรัม/กิโลกรัม/วัน ตามลำดับโดยวิธีป้อนทางปากเป็นเวลา 30 วันติดต่อกัน เมื่อครบระยะเวลาทำให้หนูหมดความรู้สึก เก็บตัวอย่างเลือดจากหัวใจและเก็บซีรัมเพื่อตรวจค่าเคมีคลินิก นำตับมาเตรียมไมโครโซม เพื่อวัดสมรรถนะของเอนไซม์ ผลการทดลองพบว่าบอระเพ็ดทั้งสองขนาดที่ให้แก่หนูขาวในการทดลองนี้ไม่มีผลต่อน้ำหนักตัว น้ำหนักสัมพัทธ์ของตับและค่าเคมีคลินิกในเลือดดังต่อไปนี้ total cholesterol, triglyceride, AST, ALT, ALP, total bilirubin, direct bilirubin, BUN, SCr, sodium, chloride, hemoglobin, hematocrit, RBC morphology, platelet count, WBC count และ %differential WBC หนูขาวที่ได้รับบอระเพ็ดในขนาด 250 และ 500 มิลลิกรัม/กิโลกรัม/วัน มี potassium ในซีรัมต่ำกว่ากลุ่มควบคุม หนูขาวที่ได้รับบอระเพ็ดในขนาด 500 มิลลิกรัม/กิโลกรัม/วัน มี glucose ในซีรัมต่ำกว่ากลุ่มควบคุม และมี HDL-C ในซีรัมสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญ สำหรับผลต่อ CYPs บอระเพ็ดทั้งสองขนาดมีผลลดปริมาณ total CYP อย่างมีนัยสำคัญ เมื่อวัดสมรรถนะของ CYPs แต่ละ isoform พบว่าบอระเพ็ดขนาด 500 มิลลิกรัม/กิโลกรัม/วัน มีผลยับยั้งสมรรถนะของ CYP1A1 และมีผลกระตุ้นสมรรถนะของ CYP2E1 บอระเพ็ดทั้งสองขนาดไม่มีผลต่อสมรรถนะของ CYP1A2, CYP2B1&2B2 และ CYP3A ผลจากการศึกษานี้เป็นข้อมูลเบื้องต้นที่บ่งชี้ว่าบอระเพ็ดในขนาดสูงมีผลต่อสมรรถนะของ CYP1A1 และ CYP2E1 อาจเป็นผลลดหรือเพิ่มความเสี่ยงของการเกิดมะเร็ง และ/หรือ การก่อกลายพันธุ์จากสารแปลกปลอมที่ถูกเร่งปฏิกิริยาโดยเอนไซม์ทั้งสองได้

ภาควิชา เภสัชวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2545	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEYWORDS: *TINOSPORA CRISPA* / CYTOCHROME P450 / CLINICAL BLOOD CHEMISTRY / TOXICITY

OOMCHOO SAIPHET : THESIS TITLE. SUBACUTE EFFECTS OF *TINOSPORA CRISPA* STEM EXTRACT ON HEPATIC CYTOCHROME P450 AND CLINICAL BLOOD CHEMISTRY IN RATS. THESIS ADVISOR : ASST. PROF. POL. LT. COL. SOMSONG LAWANPRASERT, Ph. D. THESIS COADVISOR : ASSOC. PROF. NUANSRI NIWATTISAIWONG, 123 pp. ISBN 974-17-3203-1.

Boraphet (*Tinospora crispa* Miers ex Hook. f. & Thomson) has been used traditionally as hypoglycemic medicine. In this study, subacute effects of *T. crispa* stem extract on hepatic cytochrome P450 (CYPs) and clinical blood chemistry were investigated in male Wistar rats. Rats were randomly divided into three treatment groups as following: Control group, *T. crispa* treatment group I and II, the groups of which rats received *T. crispa* at doses of 250 and 500 mg/kg/day, respectively. Each group consisted of 10 rats. *T. crispa* was administered orally for 30 consecutive days. At the end of the treatment, animals were anesthetized. Blood samples were collected by heart puncture and serum samples were determined for clinical blood chemistry. Microsomes were prepared from livers and being used for enzyme activity assays. The results showed that *T. crispa* at both dosages given in this study did not affect body weight, relative liver weight as well as the following clinical blood chemistry: Total cholesterol, triglyceride, AST, ALT, ALP, total bilirubin, direct bilirubin, BUN, SCr, sodium, chloride, hemoglobin, hematocrit, RBC morphology, platelet count, WBC count, and %differential WBC. Rats receiving *T. crispa* at 250 and 500 mg/kg/day possessed significantly lower serum potassium than those in the control group. Rats receiving *T. crispa* of 500 mg/kg/day possessed significantly lower serum glucose but significantly higher serum HDL-C levels as compared to the control group. Regarding the effects on CYPs, *T. crispa* at both doses caused significantly decrease of total CYP contents. Determining activities of individual CYP isoform, it was found that *T. crispa* at 500 mg/kg/day caused inhibition effect on CYP1A1 but induction effect on CYP2E1. *T. crispa* at both dosages did not affect activities of CYP1A2, CYP2B1&2B2, and CYP3A. These provided a preliminary data indicating that high dose of *T. crispa* may increase and decrease risk of chemical carcinogenesis and/or mutagenesis *via* its modulating effects on the bioactivating enzymes, CYP1A1 and CYP2E1.

Department of Pharmacology	Student's signature……..
Field of study Pharmacology	Advisor's signature……..
Academic year 2002	Co-advisor's signature…….

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List of Abbreviations

2^{nd} ed	= second edition
α	= alpha
β	= beta
°C	= degree celcius
μg	= microgram
μL	= microlitre
μM	= micromolar
μmol	= micromole
ALP	= alkaline phosphatase
ALT	= alanine aminotransferase
AST	= aspartate aminotransferase
BROD	= benzyloxyresorufin O-dealkylation
BSA	= bovine serum albumin
BUN	= blood urea nitrogen
BW	= body weight
cm	= centimetre
cont'd	= continued
CYP	= cytochrome P450
dL	= decilitre
DMSO	= dimethylsulfoxide
eds.	= editors
EDTA	= ethylenediaminetetraacetic acid
EROD	= ethoxyresorufin O-dealkylation
et al.	= et alii (and others)
etc.	= et cetera (and the rest)
g	= gram
g	= gravity
G6P	= glucose 6-phosphate

List of Abbreviations (cont'd)

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
IC_{50}	= median inhibitory concentration
i.e.	= id est (that is)
i.p.	= intraperitonium
kg	= kilogram
L	= litre
LDL-C	= low density lipoprotein cholesterol
M	= molar
mg	= milligram
mg/kg	= milligram per kilogram body weight
min	= minute
mL	= millilitre
mm	= millimetre
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
T. crispa	= *Tinospora crispa*
TLC	= thin layer chromatography
Tris	= tris (hydroxymethyl) aminomethane
U	= unit
UDPGT	= uridine 5'-diphospho-glucuronyltransferase
vol.	= volume
v/v	= volume by volume
WBC	= white blood cell
w/v	= weight by volume

CHAPTER I

INTRODUCTION

Tinospora crispa Miers ex Hook. f. & Thomson called in Thai as Boraphet, is a plant in family Menispermaceae. As a traditional medicine, *T. crispa* is used as a bitter tonic, a stomachic, an appetising enhancement, an antipyretic, and a hypoglycemic agent (นันทวันและอรนุช, 2541). Parts used are root, fresh or dried stem, leaf, flower, and fruit. Chemical constituents found in *T. crispa* include alkaloids: Berberine, choline, jateorrhizine, palmatine, tembetarine, N-*trans*-feruloyl tyramine, N-*cis*-feruloyl tyramine, N-formyl annonaine, N-formyl nornuciferine, and N-acetyl nornuciferine; flavonoids: Genkwanin; lignans: Siringin and secoisolariciresinol; steroids: β-Sitosterol, γ-sitosterol, stigmasterol, cycloeucalenol, and cycloeucalenone; terpenoids: Borapetol A, B, borapetoside A, B, C, D, E, F, G, tinocrisposide, tinotufalin B, E, F, tinotufolin A, B, C, D (นันทวันและอรนุช, 2541; Cavin, *et al*., 1998; Martin, *et al*., 1996; Murakoshi, *et al*., 1993; Pachaly, *et al*., 1992; Ragasa, *et al*., 2000).

Pharmacological effects of *T. crispa* have been explored in several aspects. Regarding the *hypoglycemic effect*, water extract of *T. crispa* stem was shown to decrease blood glucose level, improve insulinemia, and oral glucose tolerance in alloxan-diabetic rats (Noor and Ashcroft, 1989). Stimulation of islets of Langerhans with an increase of insulin secretion was proposed to be the effect of this plant (Noor, *et al*., 1989). Mechanism of action was probably due to a modulation of β-cell calcium concentration without interference with intestinal glucose uptake or uptake of glucose into peripheral cells (Noor and Ashcroft, 1998). Another study showing that ethanolic extract of *T. crispa* stem at doses of 250 and 500 mg/kg/day exhibited hypoglycemic effect in diabetic-rats but had no effect on blood glucose level in normal rats (Anulakanapakorn, *et al*.,1999).

Regarding the *cardiovascular effects,* effects of the constituents in *T. crispa* on the contractility of left and right atria were studied *in vitro.* The results showed that N-formyl nornuciferine increased force of contraction of right and left rat atria. Cycloeucalenol significantly increased force of right atria but slightly decreased force on left atria. Colombin decreased force of contraction of both right and left atria whereas

cycloeucalenone had no effect on isolated rat atria. Crude hexane extract of *T. crispa* produced no change of blood pressure whereas crude chloroform and ethanol extract produced variable reduction of blood pressure (งามผ่องและประสาน, 2542).

Antimicrobial effects A study was performed to investigate the antibacterial effects of *T. crispa* (leaf and stem) ethanolic extract on *Staphylococcus aureus,* beta-*Streptococcus* gr. A, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa.* The result showed that the extract was capable to inhibit beta-*Streptococcus* gr. A (อารีรัตน์ ลออปักษา และคณะ, 2531). Another study found that an antibacterial cream containing the extract of *T. crispa* showed an antibacterial effects on *S. aureus* (Burapadaja and Bunchoo, 1999). Water extract of *T. crispa* was found to possess antifilarial effect on *Brugia malayi* (Zaridah, *et al*., 2001). In addition, crude chloroform extract of *T. crispa* was shown to possess an antimalarial effect more than the crude ethanol extract (Rahman, *et al*., 1999).

Antipyretic effect An antipyretic effect of crude water extract of *T. crispa* stem was studied in male Wistar rats. Prior to administration of the extract, fever was induced in each rat by injection of 0.6 mL typhoid vaccine. The results showed that the crude extract at doses of 300, 200, and 100 mg/kg of body weight demonstrated antipyretic effects within 1, 2, and 3 hours, respectively, after the fever induction. However, antipyretic effects of *T. crispa* was less than of aspirin (บุญเทียม คงศักดิ์ตระกูล และคณะ, 2537).

Effect on nitric oxide (NO) synthesis Water extract of *T. crispa* was shown to suppress NO synthesis *in vitro* in mouse macrophage (Yokozawa, *et al*., 1999). N-*trans*-feruloyl tyramine which was one of the active compounds found in *T. crispa,* possessed anti-NO activity with a decrease of inducible NO synthase (iNOS) (Yokozawa, *et al*., 2001). Antioxidant effects of N-*trans*-feruloyl tyramine, N-*cis*-feruloyl tyramine, and secoisolariciresinol were found to be more potent than butylhydroxytoluene (BHT), an antioxidant standard substance (Cavin, *et al*., 1998).

Toxicological study Single oral dose of 4 g/kg of *T. crispa* ethanolic extract caused no signs of acute toxicity in mice. Chronic toxicity was investigated by giving the extract orally to Wistar rats for 6 months at doses of 0.02, 0.16, and 1.28 g/kg/day. The results showed that the extract of 0.02 g/kg/day which was equivalent to the dose used in

humans, caused no significant effects on growth rate, food intake, hematology, clinical blood chemistry, and histopathology of inner organs. Rats receiving 1.28 g/kg/day of the extract or 64 fold of the dose used in humans, had significantly higher incidence of bile duct proliferation and focal liver cell hyperplasia. Moreover, higher levels of serum alkaline phosphatase, alanine aminotransferase, and creatinine were also observed in rats at this dose as compared to the control group. The authors concluded that low dose of ethanolic extract of *T. crispa* such as the dose used in humans did not produce any signs of toxicity in animal while high dose caused toxicities to liver and renal (Chavalittumrong, *et al*., 1997).

A clinical study was performed regarding the efficacy and safety of *T. crispa* on type 2 diabetic patients. Each patient was given orally 3 g/day of *T. crispa* extract for six months. The results showed that fasting blood sugar and hemoglobin A_{1c} levels were not significantly decreased after the treatment. Sixty seven percents of the patient experienced signs of hepatotoxicity as shown by an increase serum glutamic oxaloacetic transminase and serum glutamic pyruvic transminase (ชุติมา กาญจนวงศ์, 2543).

Several studies have been performed regarding the compounds found in *T. crispa*. Berberine possessed many pharmacological activities such as antibacterial, modulation of immune system, anticonvulsant, sedative, and antitumor (Bradshaw, 2002). Berberine in *Mahonia aquifolium* had potential antimutagenic activity in *Euglena gracilis* induced by acridine orange (Cernakova, *et al*., 2002). Berberine modurately inhibited cytochrome P450 (CYPs) isoform 3A4 with an IC_{50} of 5.72 mM (Budzinski, *et al*., 2000). Berberine was found to protect rat against hepatotoxicity induced by acetaminophen and carbontetrachloride. It was proposed that this effect was probably due to inhibition effect of berberine on CYPs (Janbaz and Gilani, 2000). Genkwanin was shown to inhibit human promyelocytic leukemia (HL-60) cell differentiation (Suh, *et al*., 1995). Another study found that genkwanin from a plant named *Lethedon tanansis,* demonstrated cytotoxic effect on human nasopharynx carcinoma (KB cell) (Zahir, *et al*., 1996). Secoisolaricirecinol was found to inhibit breast and prostrate cancer (Xia, *et al*., 2001). Anticancer and chemopreventive effects of the chemical constituents found in *T. crispa* might be partly

explained by antioxidant property and inhibition effects of those compounds on CYP isoforms that activated procarcinogens (Gerhauser, *et al*., 2003).

In general, 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). Modification of hepatic phase I (toxification) and phase II (detoxification) enzymes are partly a key aspect explaination for antimutagenic and/or anticarcinogenic potential of chemicals or vice versa if it possesses induction effects.

So far, few studies have been performed regarding effects of some constituents found in *T. crispa* (not yet of the crude extract of *T. crispa*) on hepatic CYPs. Therefore, the objectives of this study were primarily to investigate subacute effects of *T. crispa* stem extract on hepatic CYPs that play a key role in carcinogenic and/or mutagenic activation of many environmental chemicals such as CYPs 1A1, 1A2, 2B1, 2B2, 2E1, and CYP3A using an *ex vivo* model. Moreover, effects of *T. crispa* stem extract on clinical blood chemistry were also determined so as to investigate subacute toxicity.

Hypothesis

Subacute exposure of *T. crispa* caused an induction and/or inhibition effects on hepatic CYPs as well as changes of clinical blood chemistry in rats.

Benefit gained from the study

To obtain preliminary data demonstrated an induction and/or inhibition effects of *T. crispa* on hepatic CYPs, especially CYP isoforms involved in a bioactivation of drugs, chemicals, and environmental pollutants resulting in reactive metabolites. Therefore, it would be useful for considering the possibility of *T. crispa* to increase and/or decrease of chemical induced toxicities, mutagenesis, and/or carcinogenesis. Effects of *T. crispa* on clinical blood chemistry would be an additional data of subacute toxicity for this plant in rats.

Study design and process

1. Preparation of *T. crispa* stem 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 REVIEWS

TINOSPORA CRISPA

Tinospora crispa Miers ex Hook. f. & Thomson, known in Thai as Boraphet, is a climbing glabrous plant belonging to family Menispermaceae. *T. crispa* is commonly found in mixed deciduous forest and village hedgerrows, altitude up to 1,000 metres. It is widely distributed throughout most area in Thailand. *T. crispa* usually used in Thai traditional medicine as a bitter tonic, a stomachic, an appetising enhancement, an antipyretic, and a hypoglycemic agent (นันทวันและอรนุช, 2541). Parts used of *T. crispa* are root, fresh or dried stem, leaf, flower, and fruit.

Previous studies showed that *T. crispa* contains various compounds. These compounds can be classified on the basis of their chemical structures (Figure 1) as following.

1. Alkaloids: include
    - 1.1 Berberine
    - 1.2 Choline
    - 1.3 Jateorrhizine
    - 1.4 N-*cis*-feruloyl tyramine
    - 1.5 N-*trans*-feruloyl tyramine
    - 1.6 N-acetyl nornuciferine
    - 1.7 N-formyl nornuciferine
    - 1.8 N-formyl annonaine
    - 1.9 Palmatine
    - 1.10 Tembetarine

2. Flavonoids: include

    2.1 Genkwanin

3. Lignans: include

    3.1 Siringin

    3.2 Secoisolariciresinol

4. Steroids: include

    4.1 β-Sitosterol

    4.2 γ-Sitosterol

    4.3 Stigmasterol

    4.4 Cycloeucalenol

    4.5 Cycloeucalenone

5. Terpenoids: include

    5.1 Borapetol A

    5.2 Borapetol B

    5.3 Borapetoside A

    5.4 Borapetoside B

    5.5 Borapetoside C

    5.6 Borapetoside D

    5.7 Borapetoside E

    5.8 Borapetoside F

    5.9 Borapetoside G

    5.10 Colombin

    5.11 Tinocrisposide

    5.12 Tinotufalin B

5.13 Tinotufalin E

5.14 Tinotufalin F

5.15 Tinotufolin A

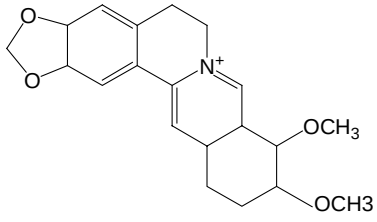
5.16 Tinotufolin B

5.17 Tinotufolin C

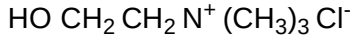
5.18 Tinotufolin D

6. Other compounds: include

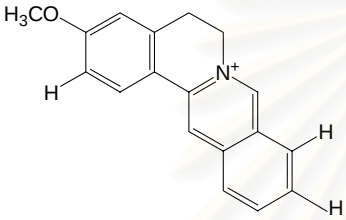
6.1 Tinotuberine



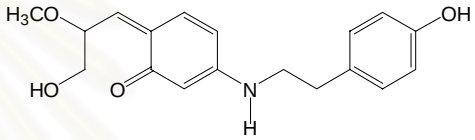
Berberine



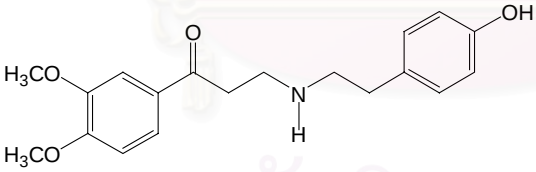
Choline



Jateorrhizine



N-*cis*-feruloyl tyramine



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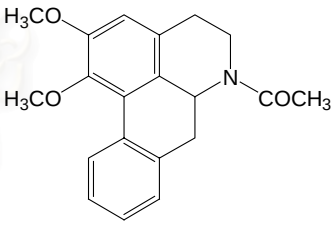
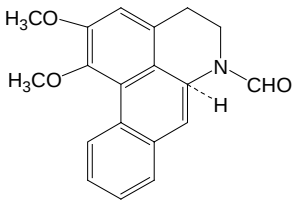
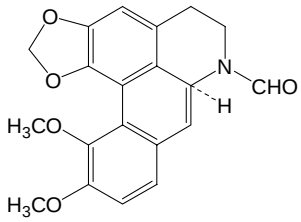


N-acetyl nornuciferine

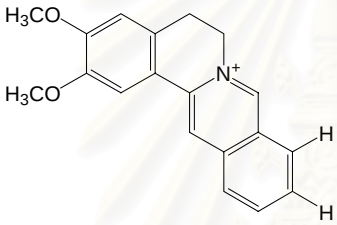
Figure 1 **Chemical structures of compounds in *T. crispa*** (งามผ่องและประสาน, 2542 Cavin, *et al*., 1998; Pathak, *et al*., 1994)



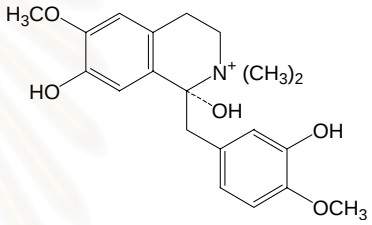
N-formyl nornuciferine



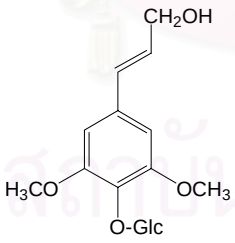
N-formyl anonaine



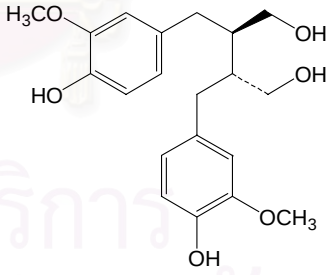
Palmatine



Tembetarine

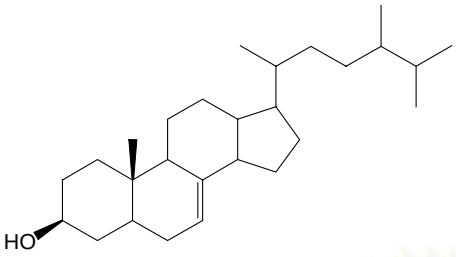


Syringin

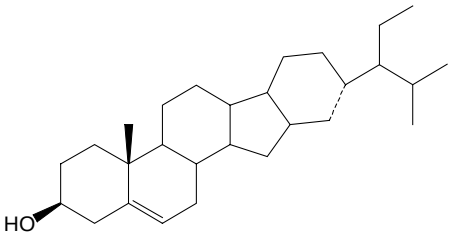


Secoisolariciresinol

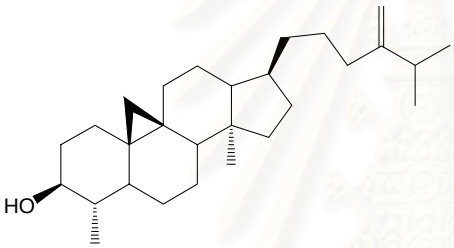
Figure 1 Chemical structures of compounds in *T. crispa* (cont'd)



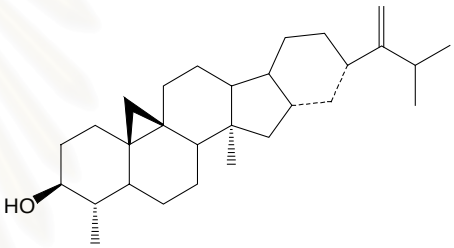
α-Sitosterol



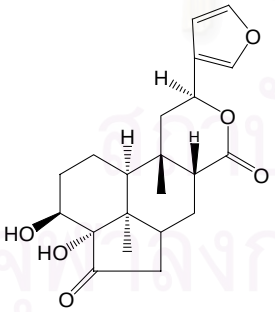
β-Sitosterol



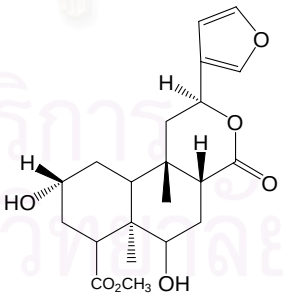
Cycloeucalenone



Cycloeucalenol

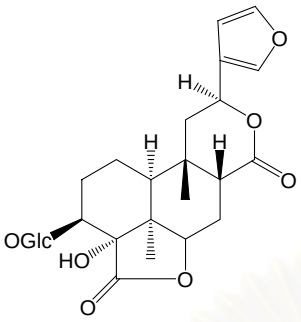


Borapetol A

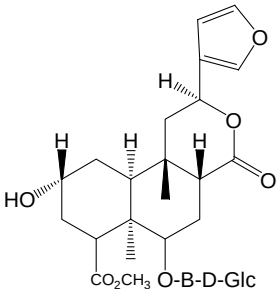


Borapetol B

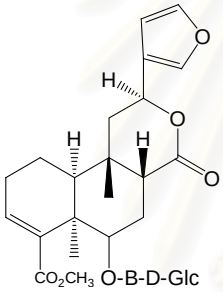
Figure 1 Chemical structures of compounds in *T. crispa* (cont'd)



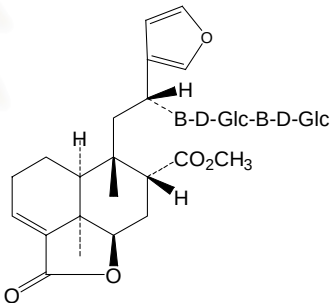
Borapetoside A



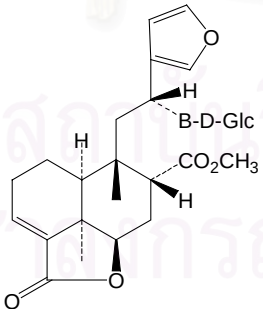
Borapetoside B



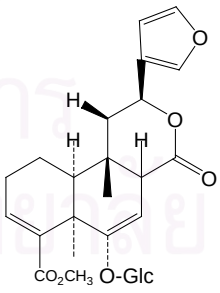
Borapetoside C



Borapetoside D

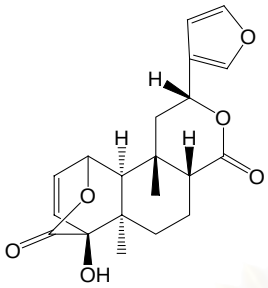


Borapetoside E

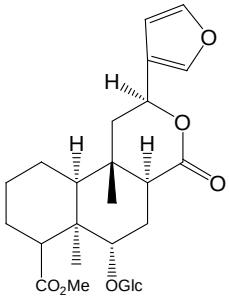


Borapetoside F

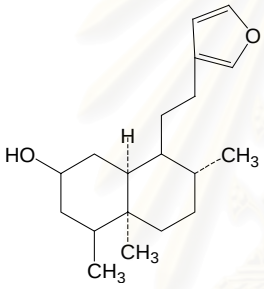
Figure 1 Chemical structures of compounds in *T. crispa* (cont'd)



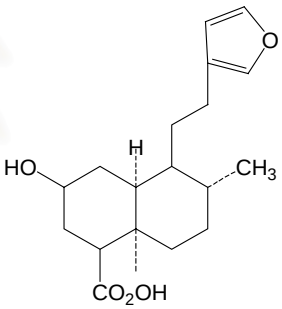
Colombin



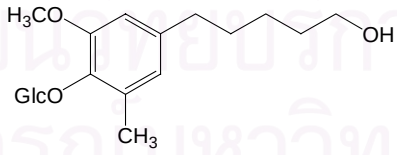
Tinocrisposide



Tinotufoline A



Tinotufoline B



Tinotuberine

Figure 1 Chemical structures of compounds in *T. crispa* (cont'd)

Pharmacological effects of *T. crispa*

1. Hypoglycemic effect

After a two-week treatment with aqueous extract (4 g/L in drinking water) of *T. crispa* stems, alloxan-diabetic rats (diabetic rats with moderately function defect of β-cells) showed an improvement of insulinemia and glucose tolerance. Intravenous administration of the extract at 50 mg/kg, into rats demonstrated an increase of plasma insulin levels. However, rats with severely diabetic condition with absolutely defect of β-cells were not found to respond to the extract. Thus, hypoglycemic activity of *T. crispa* is probably not due to extrapancreatic action (Noor and Ashcroft, 1989). Using isolated human or rat islets of Langerhans and insulin-producing insulinoma cell lines (HIT-T15 β-cells) as models to investigate an *in vitro* insulinotropic action of *T. crispa,* it was found that hypoglycemic effect of *T. crispa* was associated with an increase of insulin secretion (Noor, *et al*., 1989). A subsequent study showed that antihyperglycemic effect of *T. crispa* was not due to an interference of intestinal glucose uptake or uptake of sugar into peripheral cells. Rather, antihyperglycemic effect of this plant was probably due to a stimulation of insulin release *via* modulation of β-cell Ca^{2+} concentration (Noor and Ashcroft, 1998).

2. Antifilarial effect

In an *in vitro* study, it was revealed that *T. crispa* produced considerable antimalarial effects. Seventy-two hours exposure to methanolic extract of *T. crispa* at a concentration of 2.5 mg/mL resulted in a complete inhibition of *Plasmodium falciparum* development (Zaridah, *et al*., 2001).

3. Antioxidant and free radical scavenging properties

Thin layer chromatographic autographic assays revealed that there were three compounds in dichloromethane extract of *T. crispa* exhibiting antioxidant and free radical scavenging properties towards β-carotene and 2,2-diphenyl-1-picryhydrazyl (DPPH) radical. They were isolated and identified as N-*cis*-feruloyl tyramine, N-*trans*-feruloyl

tyramine, and secoisolaricirecinol. When tested in dilution assays on the reduction of 2,2-diphenyl-1-picryhydrazyl radical, these three compounds were proved to be more active than the synthetic antioxidant butylhydroxytoluene (BHT) (Cavin, *et al*., 1998).

4. Inhibition of nitric oxide (NO) synthesis

Inhibitory effect on NO synthesis of *T. crispa* was concentration-dependent and appeared to involve a suppression of both inducible nitric oxide synthase (iNOS) activity and NADPH-diaphorase, thus altering NO production. Since NO is one of the critical mediators in various disorders, iNOS inhibitors may have therapeutic potential. These results may explain some aspects of the multifunctional properties of *T. crispa* which have been used in various folk remedies in southeast Asia and China (Yokozawa, *et al*., 1999). N-*trans*-feruloyl tyramine was found to exhibit a strong anti-NO activity. This compound is the most active compound of *T. crispa* with respect to the suppression of NO production (Yokozawa, *et al*., 2001).

Clinical data

Hypoglycemic effect and safety of *T. crispa* were studied in thirty type 2 diabetic patients with a duration of diabetes of less than 5 years. One gram capsules of *T. crispa* extract were prescribed to be taken orally three times daily before meal for a period of six months. After the course of treatment, no side effects were observed in 10 patients (33%). In addition, fasting blood sugar and hemoglobin A_{1C} levels did not decrease significantly. Two patients withdrawn from the study because of marked symtoms of hyperglycemia. Hepatotoxic effects were found in 18 patients (67%) after 1-2 months of the treatment. SGOT rose to 212.78 $\pm$ 269.83 U/L and SGPT to 370.30 $\pm$ 398.05 U/L from the corresponding normal levels without any symptoms of hepatitis. One patient developed marked jaundice and was admitted for further treatment. In all cases, SGOT and SGPT returned to normal level within one month after *T. crispa* cessation. No significant changes of total cholesterol, triglyceride, BUN, creatinine, hematocrit, and WBC were found (ชุติมา กาญจนวงศ์, 2543).

Toxicological data

One toxicity study of ethanolic extract of *Tinospora crispa* stem was investigated in mice. The results showed that the extract at an oral dose of 4.0 g/kg, which was equivalent to 28.95 g/kg of crude dry stem, did not produce any signs of toxicity. Six-month chronic toxicity of the extract was performed in five groups of 16 Wistar rats of each sex. Water control group received 10 mL/kg/day while tragacanth control group received 10 mL/kg/day of 0.5% tragacanth suspension. Another three treatment groups were given the extract at the doses of 0.02, 0.16, and 1.28 g/kg/day which were equivalent to 0.145, 1.16, and 9.26 g/kg/day, respectively, of crude dry stem.

The results showed that body weights of female rats receiving 1.28 g/kg were significantly lower than those of the tragacanth and control groups. This might be due to the lower food intake in this group of animals. Regarding the hematological data, there was no significant changes between tragacanth control groups and all extract-treated groups in both sexes. For clinical blood chemistry, it was found that both male and female rats receiving 1.28 g/kg/day of the extract had significantly higher cholesterol levels but significantly lower glucose levels than those of the water control and tragacanth control groups. In addition, animal of both sexes receiving this dosage of the extract had significantly higher alkaline phosphatase (ALP) levels, alanine aminotransferase (ALT) levels, and relative liver weights than those of the water control and tragacanth control groups. Histopathological study indicated that male rats receiving the extract at 1.28 g/kg/day had significantly higher incidence of bile duct proliferation and focal liver cell hyperplasia than those in both control groups. These pathological findings may explain the significant increase of ALP level in this group of male rats. The authors suggested that high dose of the extract may cause hepatotoxicity that could alter both function and morphology of the liver. Male and female rats receiving the extract at 1.28 g/kg/day of the extract also had significantly higher serum creatinine levels than that of the tragacanth

control group. However, histopathological examination of the kidney showed no significant difference between the extract-treated group and both of the control groups. The authors suggested that the extract at this dose may somewhat affect kidney function but not the morphology.

Taken together, results from chronic toxicity study of ethanolic extract of *T. crispa* suggested that, due to the hepatotoxic and renal toxic potential of the extract observed in rats, prolonged use of high doses of *T. crispa* in humans should be avoided or if signs of liver or renal toxicities occur while using *T. crispa*-containing herbal medicine, the drug should be discontinued immediately (Chavalittumrong, *et al*., 1997).

Other studies related to *T. crispa*

Berberis aristata is an edible plant employed in South Asian Traditional Medicine. Its fruit are used as a tonic remedy for liver and heart. In one study, berberine was studied for its possible antihepatotoxic action in rats. Pretreatment of animals with berberine (4 mg/kg; orally twice daily for 2 days) prevented acetaminophen- or CCl_4-induced rise in serum levels of hepatic ALP and aminotransferases such as AST and ALT, suggesting of hepatoprotection. Post-treatment with three successive oral doses of berberine (4 mg/kg every 6 hours) reduced the hepatic damage induced by acetaminophen, while CCl_4-induced hepatotoxicity was not modified, suggesting a selective curative effect against acetaminophen. Pretreatment of animals with a single oral dose of berberine (4 mg/kg) induced prolongation of pentobarbital (60 mg/kg, i.p.)-induced sleeping time as well as increased strychnine (0.3 mg/kg; i.p.)-induced toxicity, suggesting of inhibitory effect on microsomal drug metabolizing enzymes, CYP (Janbaz and Gilani, 2000). Another study investigated CYP3A4 inhibitory effect of berberine *in vitro* by fluorometric plate assay. The results showed that berberine exhibited relatively moderate inhibitory ability with an IC_{50} value of 5.72 mM (Budzinski, *et al*., 2000).

XENOBIOTIC METABOLISM

Xenobiotic metabolism is a biological process which alters many 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 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 active. 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 conjugation
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, 1A2, 2B1, 2B2, 2E1, and 3A4 (Table 2).

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 Some procarcinogens and other toxins activated by CYPs

CYP	Procarcinogens and the toxins activated by CYPs	
	Reference	
1A1	Shimada, *et al*., 2001	benz[a]anthracene
		benz[a]anthracene-*trans*-1,2-diol
		benz[a]anthracene-*trans*-3,4-diol
		benz[a]anthracene-*trans*-5,6-diol
		benz[a]anthracene-*trans*-8,9-diol
		benzo[b]fluoranthene-9,10-diol
		benzo[c]phenanthrene
		benzo[c]phenanthrene-3,4-diol
		benzo[c]chrysene
		chrysene-1,2-diol
		dibenz[a,h]anthracene
		dibenzo[a,l]pyrene
		7,12-dimethylbenzo[a]anthracene
		7,12-dimethylbenzo[a]anthracene-3,4-diol
		dibenzo[a,l]pyrene-11,12 diol
		5,6-dimethyl chrysene-1,2 diol
		fluoranthene-2,3-diol
		5-methyl chrysene
		5-methyl chrysene-1,2-diol
	Soucek and Gut, 1992	Aflatoxin B_1
		benzo[a]pyrene
	Yamazaki, *et al*., 2000	1,8-dinitropyrene

Table 2 Some procarcinogens and other toxins activated by CYPs (cont'd)

CYP	Procarcinogens and the toxins activated by CYPs	
	Reference	
1A2	Oda, 2001	Aflatoxin B1
		2-amino-3-dimethylimidazole[4,5-f]quinoline
		2-amino-3,8-dimethylimidazole[4,5-f]quinoline
		3-amino-1,4-dimethyl-5h-pyrido[4,3-b]-indole
		2-amino-6-methyldipyridol[1,2-a:3,2'-d]-imidazole
		2-amino-3-methylimidazole[4,5-f]quinoline
		3-amino-1-methyl-5h-pyrido[4,3-a]-indole
	Soucek and Gut, 1992	2-acetylaminoflurene
		2-acetylaminofluorene
		2-aminoanthracene
		4-aminobiphenyl
	Turesky, *et al*., 2001	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
	Turesky, *et al*., 2002	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
2B1	Soucek and Gut, 1992	2-acetylaminofluorene
		4-aminobiphenyl
		aminoanthracene
		benzo(a)pyrene
		3-methylcholanthrene
2B2	Soucek and Gut, 1992	4,4'-(bis)methylene chloroaniline

Table 2 Some procarcinogens and other toxins activated by CYPs (cont'd)

CYP	Procarcinogens and the toxins activated by CYPs	
	Reference	
2E1	Berthou, 2001	anilline
		benzene
		pentane
		vinyl chloride
	Soucek and Gut, 1992	N-N"-nitrosodimethylamine
		N-nitroso-N-benzyl-N-methylamine
		N-nitroso-N-butyl-N-methylamine
		N-nitroso-N-dimethylamine
3A4	Oda, 2001	aflatoxin B1
		3-amino-1,4-dimethyl-5H-pyridol[4,3-b]-indole
		3-amino-1-methyl-5H-pyridol[4,3-b]-indole
	Omiecinski, *et al.*, 1998	benzo[a]pyrene-7,8-dihydrodiol
	Soucek and Gut, 1992	2-amino-3,5-dimethylimidazole[4,5-f]quinoline
		2-amino-3,8-dimethylimidazole[4,5-f]quinoxaline
		6-aminochrysene
		2-amino-3-methylimidazo[4,5-f]quinoline
		benzo[a]pyrene
		sterigmatocystin
		Tris(2,3-dibromopropyl)-phosphate

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 is 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 metabolism 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 xenobiotics 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 preincubation of liver microsomes in the presence of inhibitor and electron donor (NADPH). Then substrate is added.

CHAPTER III

MATERIALS AND METHODS

Animals

Adult male Wistar rats of body weight between 200-250 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. Rats were housed one per cage at the Faculty of Medicine, Srinakharinwirot University and acclimatised 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.

Chemicals

These following chemicals were used in the experimentation:

acetic acid, acetylacetone, 4-aminophenol, ammonium acetate, ammonium hydroxide, aniline hydrochloride, benzyloxyresorufin (BR), bovine serum albumin (BSA), carbon monoxide (CO), chloroform, cupric sulfate, diethyl ether, dimethylsulfoxide (DMSO), Dragendorff's reagent, erythromycin stearate, ethyl acetate, ethylenediaminetetraacetic acid (EDTA), ethoxyresorufin (ER), Folin&Ciocalteu's phenol reagent, glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PD), glycerol, hexane, magnesium chloride ($MgCl_2$), methanol (HPLC grade), methoxyresorufin (MR), monopotassium phosphate, nicotinamide adenine dinucleotide phosphate (NADP), pentoxyresorufin (PR), phenol, potassium chloride, potassium phosphate, resorufin, sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium citrate, sodium dithionite, sodium hydroxide (NaOH), sodium phosphate dibasic anhydrous, trichloroacetic acid, Trisma base

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.)

Methods

1. Preparation of *T. crispa* stem extract

T. crispa stems were purchased from a herbal store in Bangkok. Cut stems were dried and ground. The herbal powder was extracted with 95% ethanol in a soxhlet apparatus for 16 hours and the extract solution was dried under vacuum in a rotary evaporator and dried again in vacuum desiccator. The dried extract was ground to fine powder, kept in tightly closed and light protected container, and stored in a desiccator vacuum until use.

2. Chemical identification tests (Asean countries, 1993)

Preliminary test

1. When the dried extract powder 0.2 g was shaked virgorously with 10 mL of water, a long lasting foam is produced.
2. One gram of the dried extract powder was warmed with 10 mL of methanol on a water bath for 10 minutes. The solution was then cooled and filtered. When 1 mL of the filtrate was added with a few drops of Dragendorff's reagent, an orange precipitate was formed.

Confirmatory test

One gram of the dried extract powder was warmed with 10 mL of methanol on the water bath for 10 minutes. After the solution was filtered, the filtrate was evaporated to a volume of 2 mL. The concentrated filtrate was analysed by thin layer chromatography (TLC). Briefly, 20 μL of the filtrate was spotted on two TLC plates coated with silica gel. One plate was developed by mobile phase comprising hexane: ethyl acetate: acetic acid (75: 25: 1) and the other was developed by mobile phase comprising chloroform:

methanol: ammonium hydroxide (75: 20: 5). After an approximately developing distance of 12 cm, the TLC plate was analysed by Dragendorff's reagent and observed under ultraviolet light at wavelengh of 366 nm (Asean countries, 1993).

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: Animals were orally administered with double distilled water once daily for 30 days.

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

3.1.3 *T. crispa* treatment group II: Animals were orally administered with *T. crispa* at a daily dose of 500 mg/kg/day for 30 days.

T. crispa was prepared daily by dissolving 3 g of *T. crispa* stem extract powder with 6 mL of double distilled water to make a concentration of 500 mg/mL.

During the treatment period, body weight of all rats was recorded at every two weeks, food and water consumptions were recorded every week. Three animals were used simultaneously for each experimental period (one rat/each treatment group). 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, red blood cell (RBC) morphology, platelet count, white blood cell (WBC) count, and %differential WBC. The assays were performed by Faculty of Allied Health Sciences, Chulalongkorn University.

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), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), serum creatinine (SCr), total bilirubin, direct bilirubin, sodium, potassium, and chloride. The assays were performed by Faculty of Tropical Medicine, Mahidol University.

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 NaOH or 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 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 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 reagent was required for each tube). This reagent comprised Na_2CO_3, NaOH, sodium citrate, and cupric sulfate solutions in a 100: 10: 1: 1 ratio, respectively.

3.4.2 Procedure

1) 16 x 125 mm tubes were labelled 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 transferred 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:

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

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*. (1995) 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 DMSO qs to 10 mL.

0.5 mM Benzyloxyresorufin (MW 303)

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

0.5 mM Ethoxyresorufin (MW 241)

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

0.5 mM Methoxyresorufin (MW 227)

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

0.5 mM Pentoxyresorufin (MW 283)

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

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 HCl or 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 20 mM K_3PO_4 qs to 10 mL, adjusting pH to 7.4 with HCl or NaOH (10 μL contains 5 mmoles of G6P).

0.1 M NADP, pH 7.4

0.765 g of NADP was dissolved with 20 mM K_3PO_4 qs to 10 mL, adjusting pH to 7.4 with HCl or 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 20 mM K_3PO_4 qs to 10 mL, 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. 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. 10 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 990 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 10 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 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.

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

   93 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 2.5 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 2.5 mL.

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.

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 (1953) 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 HCl or NaOH.

2) 20 mM KH_2PO_4
3) 10 mM Erythromycin stearate
4) NADPH regenerating system comprised

   100 units/mL G6PD

   0.5 M G6P

   0.1 M NADP

   0.3 M $MgCl_2$

5) 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.

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. 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. 100 μL of 10 mM erythromycin stearate
   c. Varied volume of diluted microsomal suspension containing 8 mg of microsomal protein
   d. 20 mM potassium phosphate buffer, pH 7.4 qs to 990 μL.

3) 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.
4) The reaction was started by an addition of 10 μL of 100 units/mL G6PD.
5) After a 10 minutes incubation, the reaction was stopped by adding 0.5 mL of 12.5% w/v trichloroacetic acid.
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 (protect 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.
8) The absorbance was measured by spectrophotometer at wavelength of 412 nm.
9) Blank was prepared in the same manner as microsomal sample tubes except using 20 mM phosphate buffer, pH 7.4 instead of microsomes and G6PD.
10) A formaldehyde standard curve was carried out using 5 concentrations of formaldehyde: 0.0156, 0.0313, 0.0625, 0.125, 0.250 μmol/mL.

3.8.3 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 (8 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) was used for statistical comparisons between two groups at significant level of $p<0.05$.

CHAPTER IV

RESULTS

1. Preparation of *T. crispa* extract

2 kilograms of *T. crispa* dried stem were used in this study. Following the extraction process, 200 g of the extract were obtained. Thus, percentage yield of the extract was 10 w/w.

2. Chemical identification tests

Preliminary test

The extract of *T. crispa* was shown to form a long lasting foam as shaking with water. As warming with methanol and the solution was reacted with Dragendorff's reagent, orange precipitate was formed. These were a preliminary test for saponin and alkaloids, respectively.

Confirmatory test

Figure 2 showed TLC chromatograms of *T. crispa* stem extract using different developing mobile phases and different methods of detection. The corresponding high retention value factor (hRf) and the colour of the spots from both TLC chromatograms were shown in Table 4. The specific characteristics of spots on both TLC plates conformed those of the standard *T. crispa* stem extract mentioned in the Asean Countries (1993).

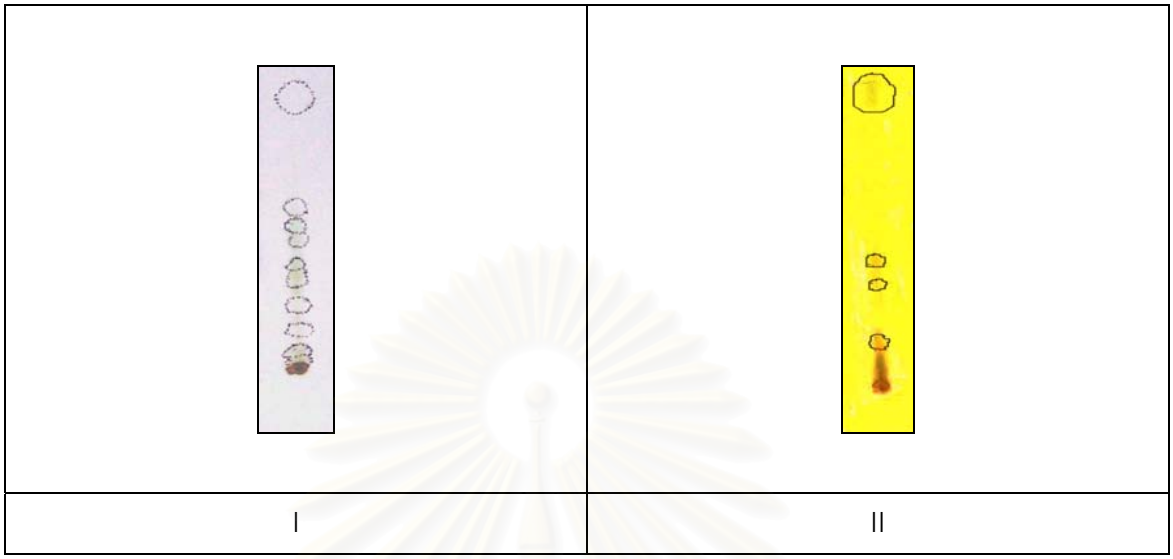


Figure 2 TLC chromatograms of *T. crispa* stem extract

I = referred to the TLC plate using
mobile phase of hexane: ethyl acetate: acetic acid (75: 25: 1) and
obtained under UV light at wavelength 366 nm

II = referred to the TLC plate using
mobile phase of chloroform: methanol: ammonium hydroxide
(75: 20: 5) and detected by Dragendorff's reagent

[I]

Developing solvent	Spots	hRf values	Colour (using UV detection)
Hexane:	1	2	blue
ethyl acetate:	2	5	red
acetic acid	3	12	blue
(75: 25: 1)	4	18	blue
	5	25	red
	6	30	red
	7	43	red
	8	49	red
	9	57	blue
	10	92	yellow

[II]

Developing solvent	Spots	hRf values	Colour (using Dragendorff's reagent)
Chloroform:	1	16	red
methanol:	2	36	red
ammonium	3	48	red
hydroxide	4	93	green
(75: 20: 5)			

Table 4 **hRf values and colour of the spots shown in TLC chromatograms of *T. crispa* stem extract**

The data were recorded from the corresponding TLC chromatograms in Figure 6

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

3. An *ex vivo* study

3.1 Effects of *T. crispa* stem extract on body weights, relative liver weights, and food&water consumptions

T. crispa stem extract demonstrated no effects on body weights and relative liver weight (Table 5). Likewise, no changes of food and water intake were observed in the *T. crispa* treatment groups at both doses used in this experiment as compared to the control group (Figure 3). All rats were alive till the end of the experiment and exhibited no apparent signs of toxicity.

Table 5 Effects of *T. crispa* stem extract on body weight, body weight gain, and relative liver weight

	Treatment group		
	Control group	*T. crispa* treament group I	*T. crispa* treament group II
Initial body weight (g)	217.1 ± 5.16	230.3 ± 9.22	226.5 ± 8.66
Final body weight (g)	366.0 ± 5.69	378.8 ± 5.40	365.2 ± 9.44
Body weight gain (g)	148.9 ± 8.38	148.5 ± 6.91	138.7 ± 6.96
Liver weight (g)	11.97 ± 0.43	12.14 ± 0.59	11.93 ± 0.52
%relative liver weight (g/100 g of body weight)	3.13 ± 0.15	3.20 ± 0.13	3.26 ± 0.13

Data shown were mean ± SEM (n=10)

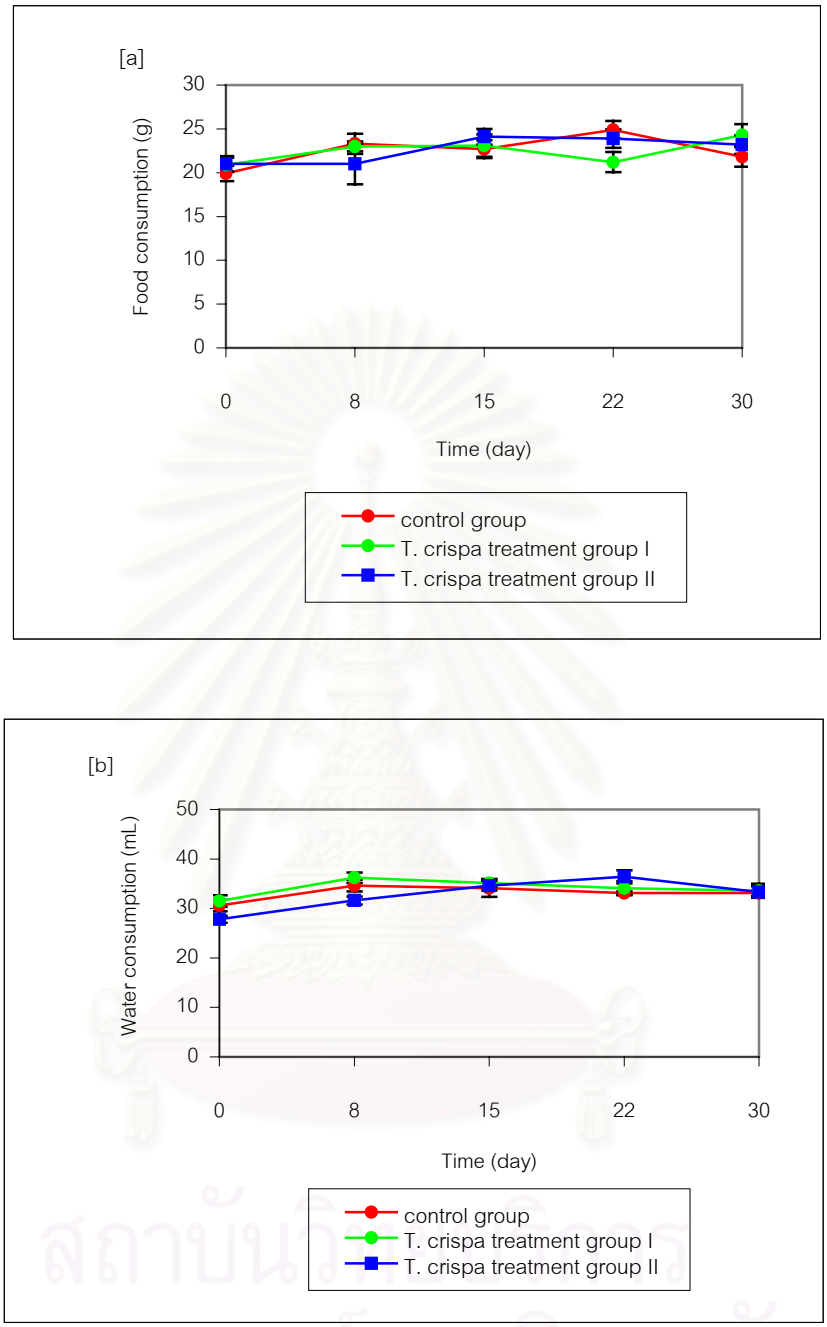


Figure 3 Effects of *T. crispa* stem extract on food [a] and water [b] consumptions
Data shown were mean ± SEM (n=10)

3.2 Effects of *T. crispa* stem extract on clinical blood chemistry

Subacute exposure (30 days) of *T. crispa* at the doses of 250 mg/kg/day and 500 mg/kg/day exhibited no effects on any hematological parameters as compared to the control group. These parameters included hemoglobin, hematocrit, platelet count, WBC count, and %differential WBC (Figure 10-12). Likewise, no effect of *T. crispa* stem extract on these following clinical blood chemistry: Lipid parameters included total cholesterol and triglyceride, AST, ALT, ALP, total bilirubin, direct bilirubin, BUN, SCr, sodium, potassium, and chloride (Figure 5-9). The exception was observed in *T. crispa* treatment group II (500 mg/kg/day) in which the animals possessed significantly lower blood glucose levels (Figure 4) but higher HDL-C levels as compared to those of the animals in control group (Figure 5).

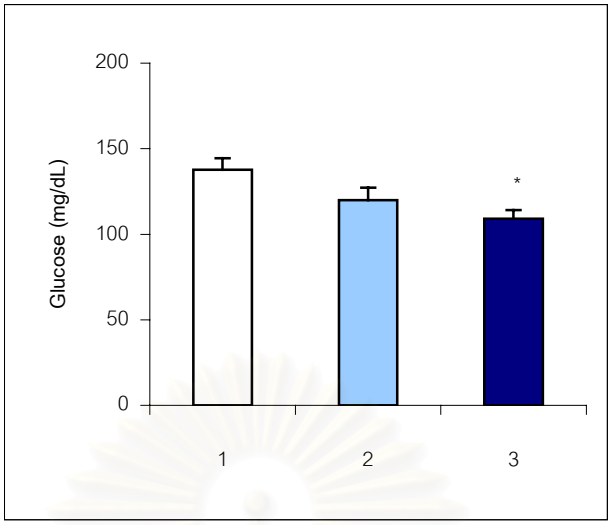


Figure 4 **Subacute effect of *T. crispa* stem extract on serum glucose**

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

* *P*<0.05; *T. crispa* treatment group vs. control group

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

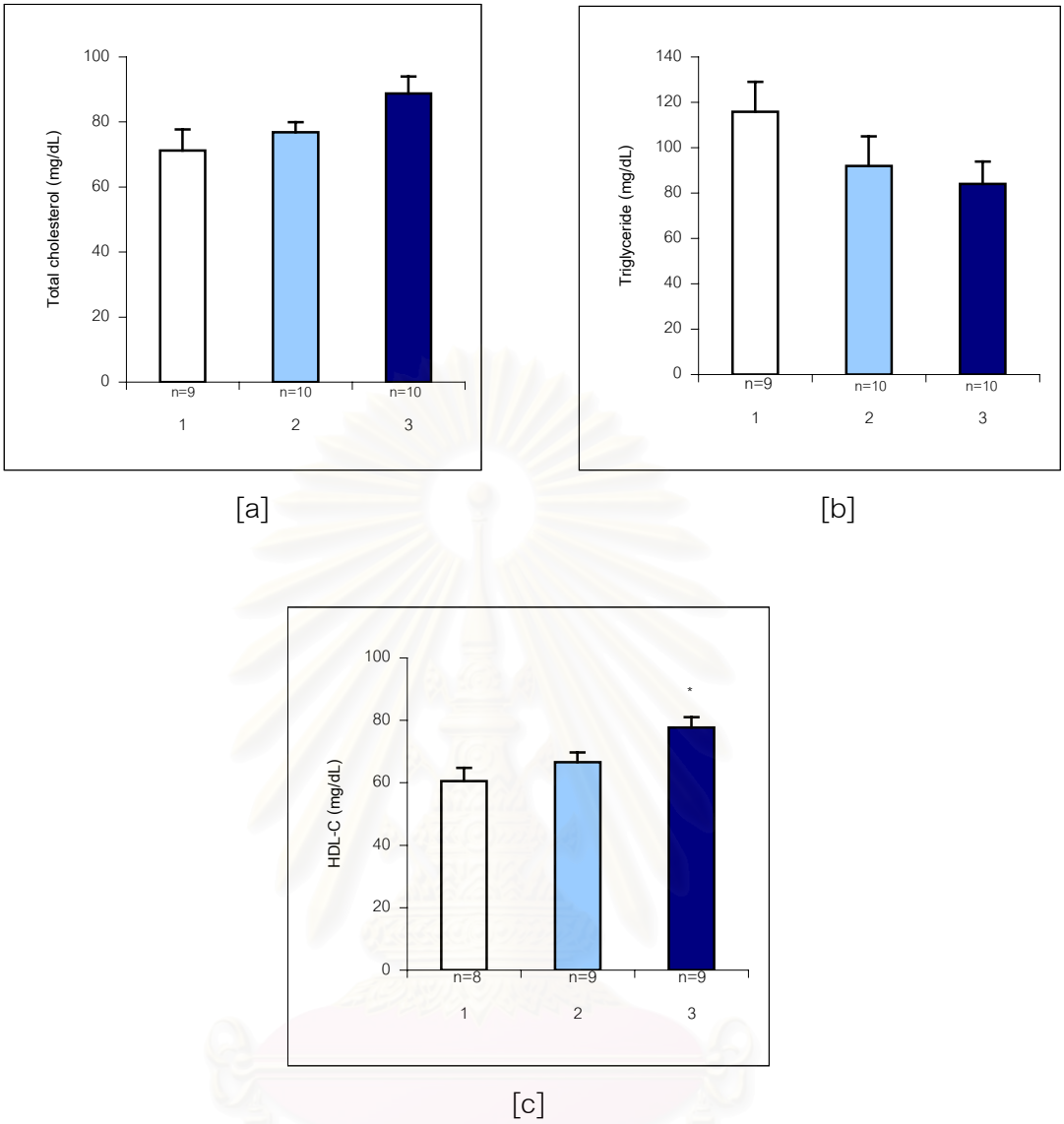


Figure 5 Subacute effects of *T. crispa* stem extract on serum total cholesterol [a] triglyceride [b] and HDL-C [c]

The individual bar represented mean of serum total cholesterol, triglyceride, and HDL-C levels with an error bar of standard error of the mean

* $P<0.05$; *T. crispa* treatment group vs. control group

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

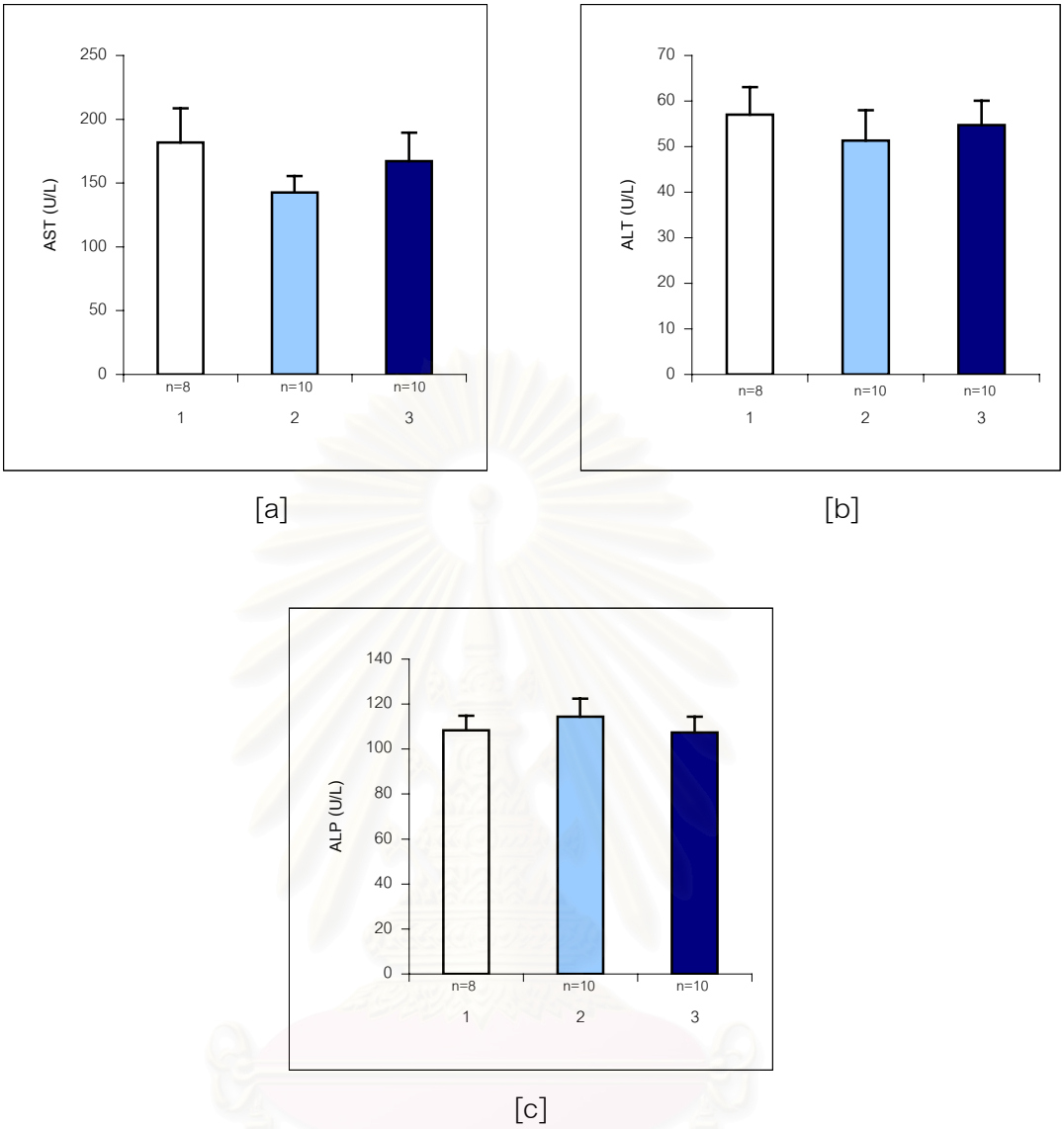


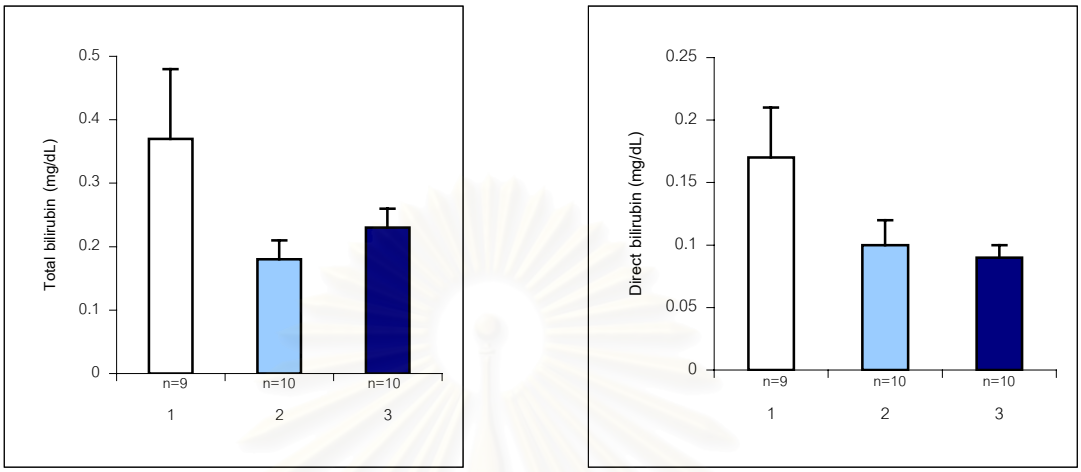
Figure 6 **Subacute effects of *T. crispa* stem extract on serum AST [a] ALT [b] and ALP [c]**

The individual bar represented mean of serum AST, ALT, and ALP levels with an error bar of standard error of the mean

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II



[a] [b]

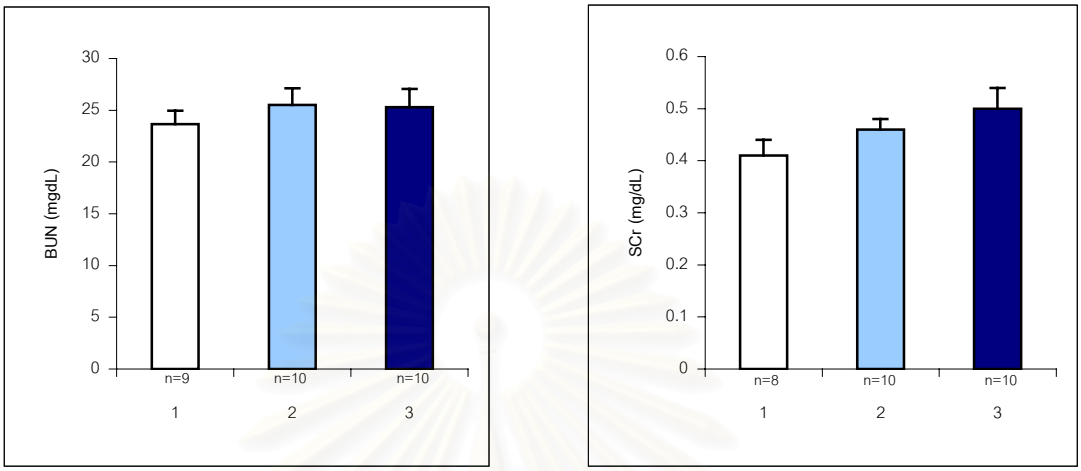
Figure 7 **Subacute effects of *T. crispa* stem extract on serum total bilirubin [a] and direct bilirubin [b]**

The individual bar represented mean of serum total bilirubin and direct bilirubin levels with an error bar of standard error of the mean

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II



[a] [b]

Figure 8 **Subacute effects of *T. crispa* stem extract on BUN [a] and SCr [b]**

The individual bar represented mean of BUN and SCr levels with an error bar of standard error of the mean

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

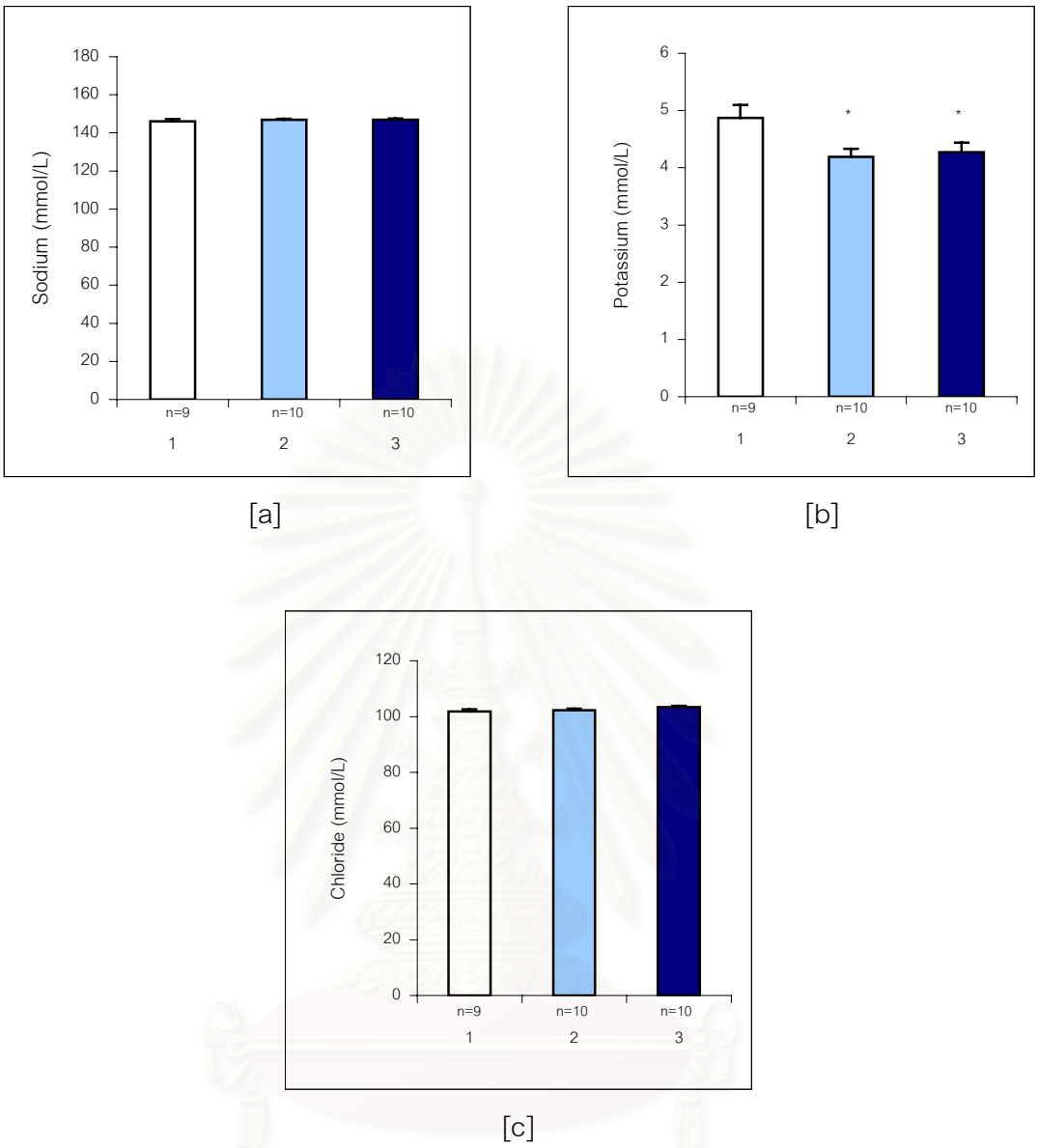


Figure 9 Subacute effects of *T. crispa* stem extract on serum sodium [a] potassium [b] and chloride [c]

The individual bar represented mean of serum sodium, potassium, and chloride levels with an error bar of standard error of the mean

* *P*<0.05; *T. crispa* treatment group vs. control group

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

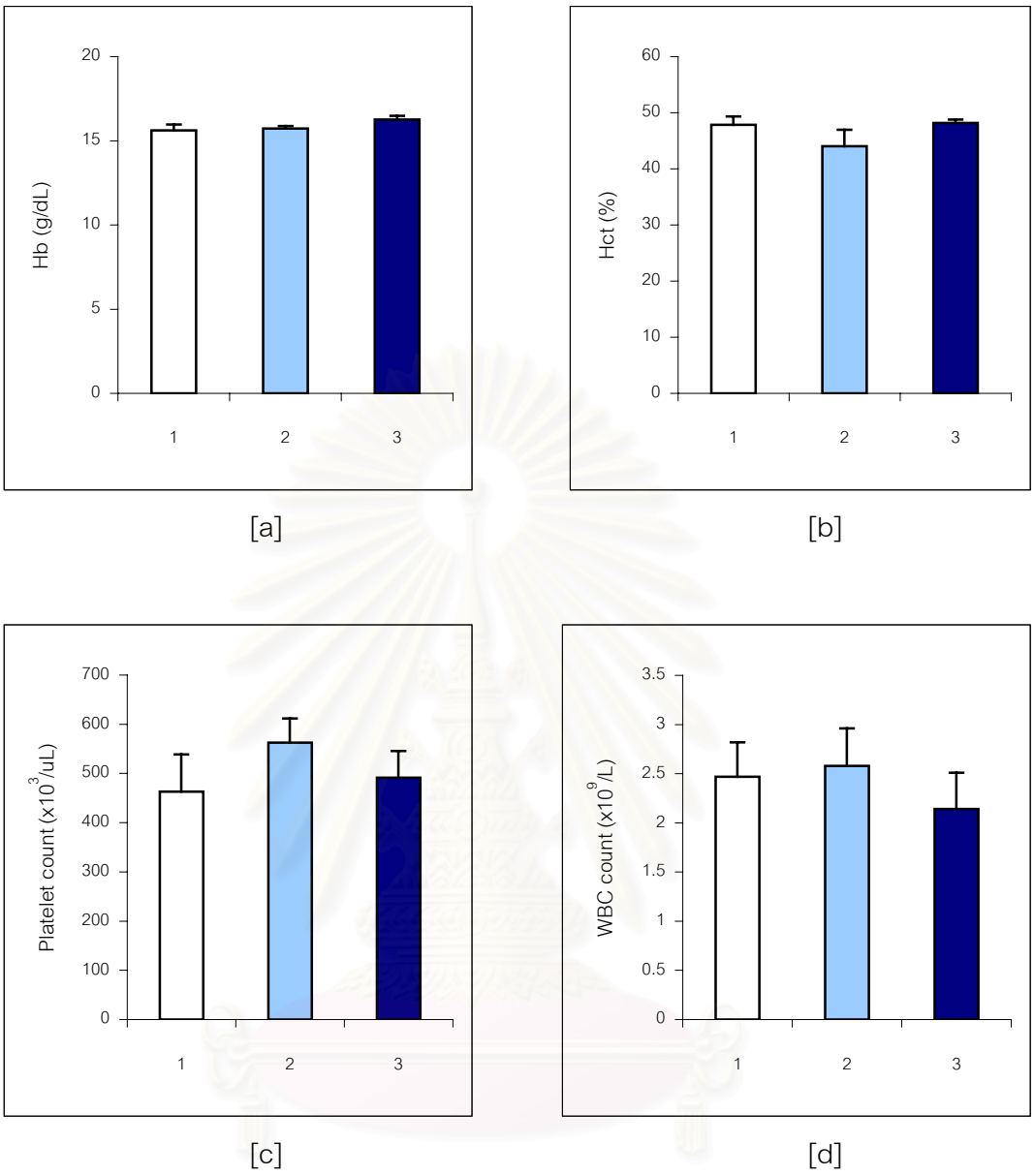


Figure 10 **Subacute effects of *T. crispa* stem extract on Hb [a] Hct [b] platelet count [c] and WBC count [d]**

The individual bar represented mean of Hb, Hct, platelet count, and WBC count levels with an error bar of standard error of the mean (n=10)

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

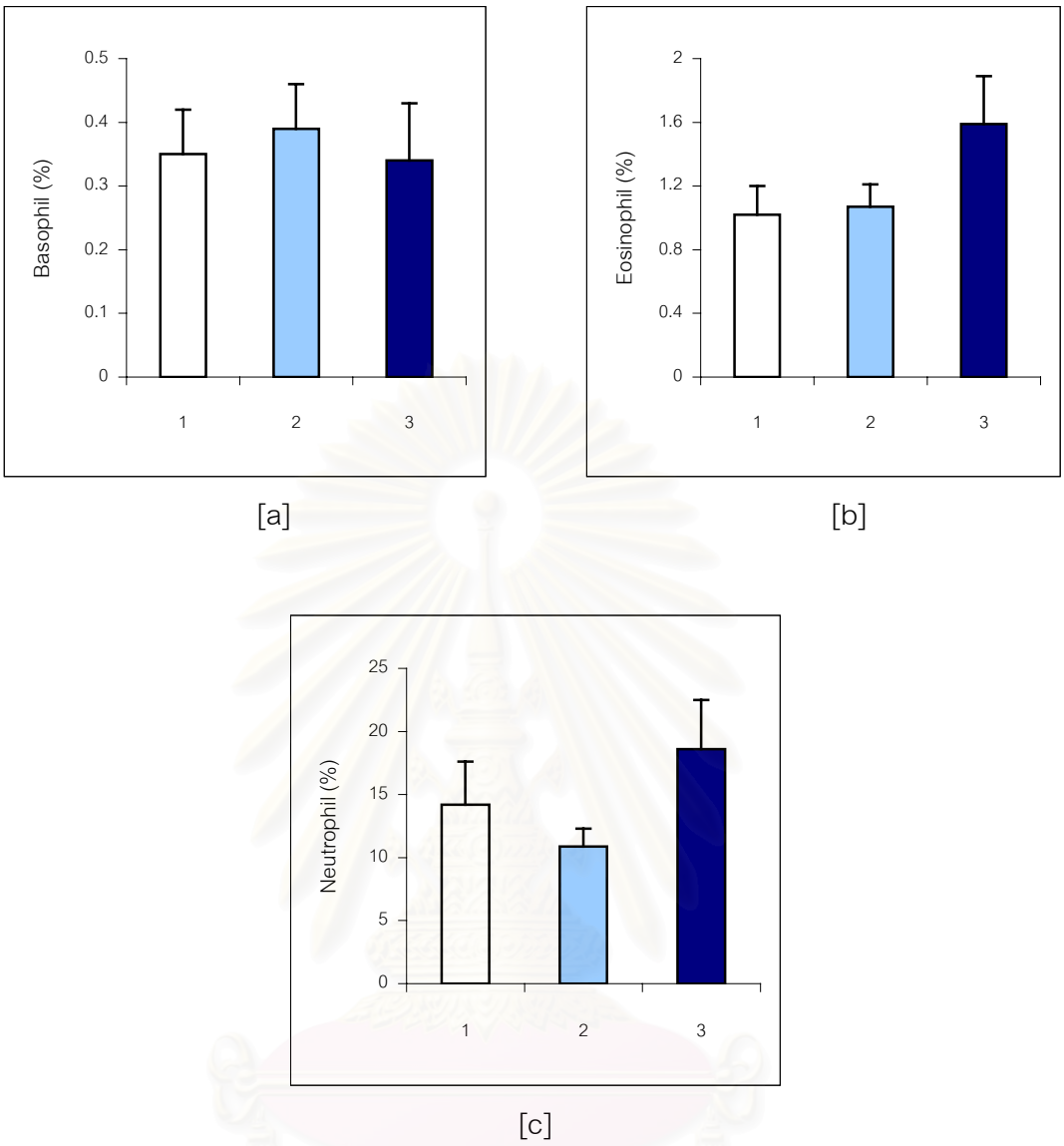


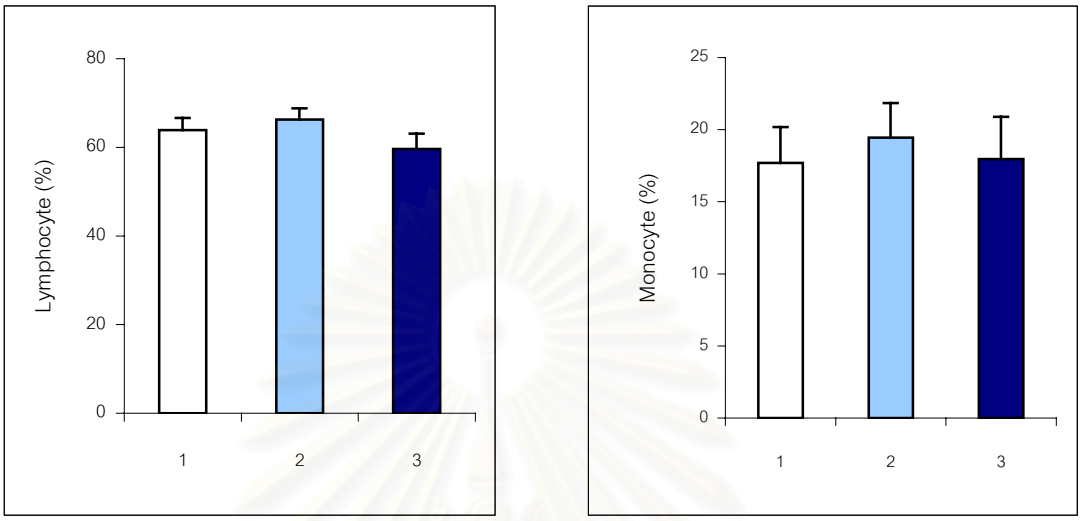
Figure 11 Subacute effects of *T. crispa* stem extract on basophil [a] eosinophil [b] and neutrophil [c]

The individual bar represented mean basophil, eosinophil, and neutrophil levels with an error bar of standard error of the mean (n=10)

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II



[a] [b]

Figure 12 **Subacute effects of *T. crispa* stem extract on lymphocyte [a] and monocyte [b]**

The individual bar represented mean of on lymphocyte and monocyte levels with an error bar of standard error of the mean (n=10)

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

3.3 Effect of *T. crispa* stem extract on hepatic microsomal CYPs

3.3.1 Effect of *T. crispa* stem extract on hepatic microsomal total CYP contents

T. crispa stem extract significantly decreased hepatic microsomal total CYP contents in rats receiving either 250 mg/kg/day or 500 mg/kg/day of the extract as compared to those in rats of the control group (Figure 13).

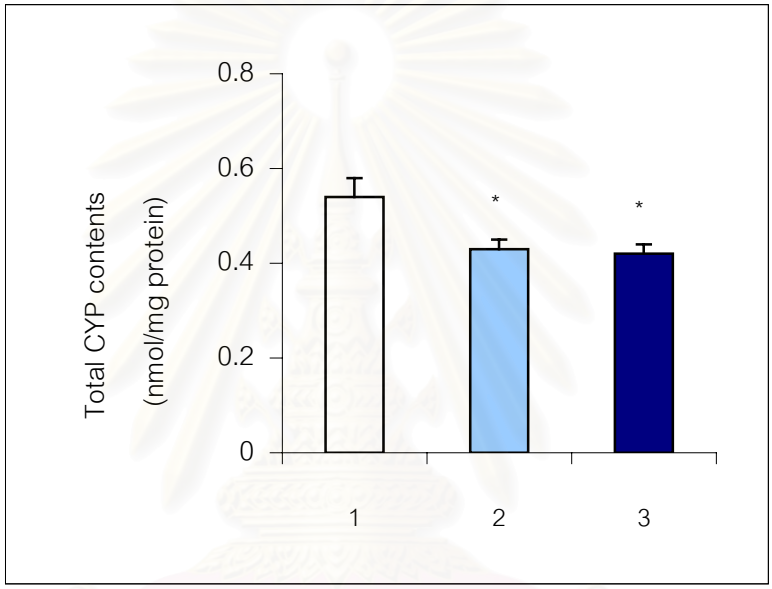


Figure 13 **Effect of *T. crispa* stem extract on hepatic microsomal total CYP contents**

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

* $p<0.05$; *T. crispa* treatment group vs. control group

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

3.3.2 Effect of *T. crispa* stem extract on hepatic microsomal alkoxyresorufin O-dealkylation

T. crispa stem extract did not significant affect rate of ethoxyresorufin O-dealkylation (EROD; which represented the activities of CYP1A1) as giving at 250 mg/kg/day. A significant decrease of this enzyme was observed when *T. crispa* was given at 500 mg/kg/day (Figure 14). Both doses of *T. crispa* did not exhibited any significant changes on rate of methoxyresorufin O-dealkylation (MROD; which represented the activities of CYP1A2) (Figure 15); benzyloxy- and pentoxyresorufin O-dealkylation (BROD and PROD, respectively; which represented the activities of CYP2B1&2B2) (Figure 16,17).

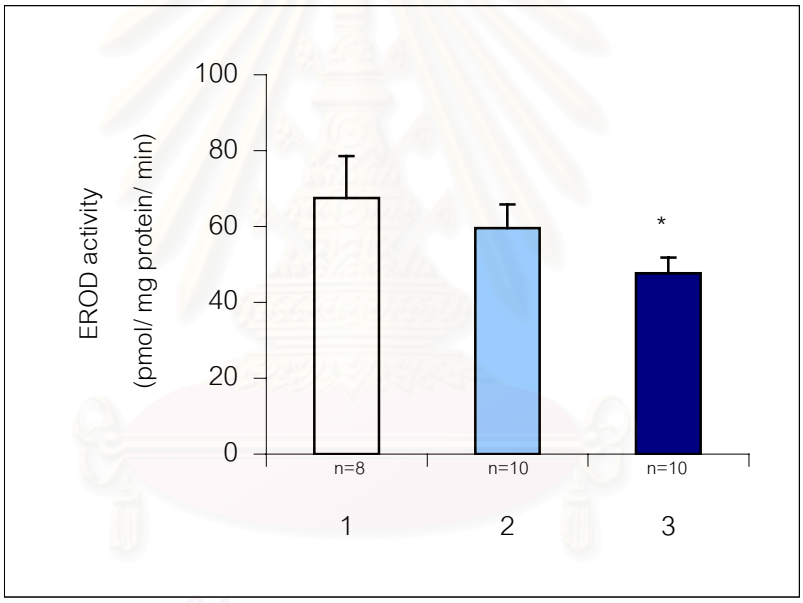


Figure 14 **Effect of *T. crispa* stem extract on hepatic microsomal EROD activity**

The individual bar represented mean of EROD activity with an error bar of standard error of the mean

* $p<0.05$; *T. crispa* treatment group vs. control group

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

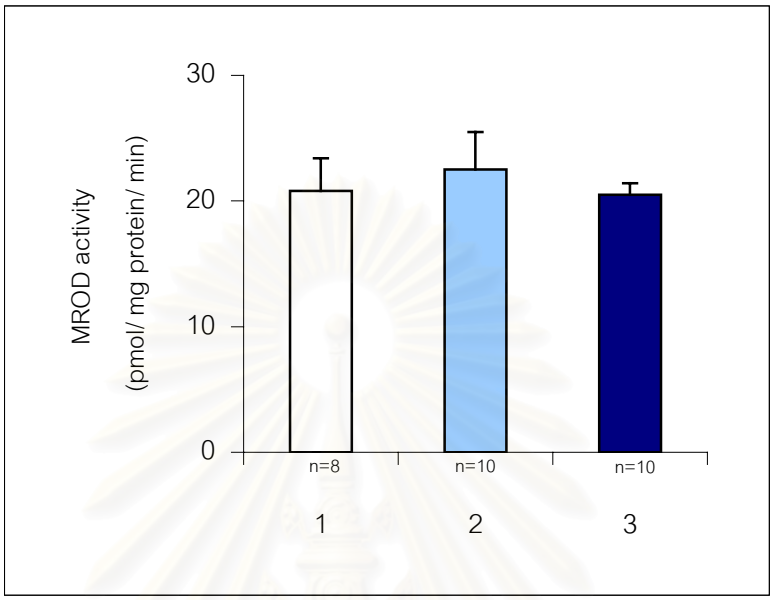


Figure 15 Effect of *T. crispa* stem extract on hepatic microsomal MROD activity

The individual bar represented mean of MROD activity with an error bar of standard error of the mean

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

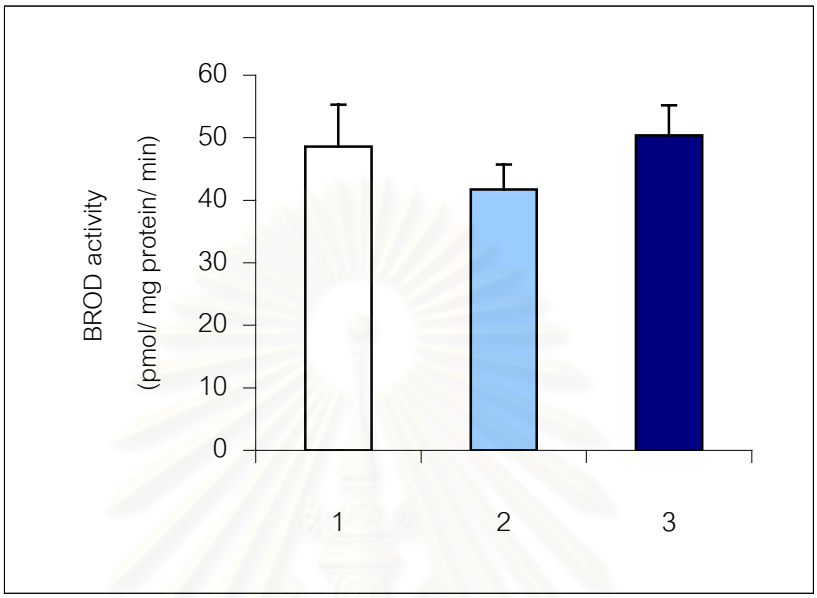


Figure 16 Effect of *T. crispa* stem extract on hepatic microsomal BROD activity

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

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

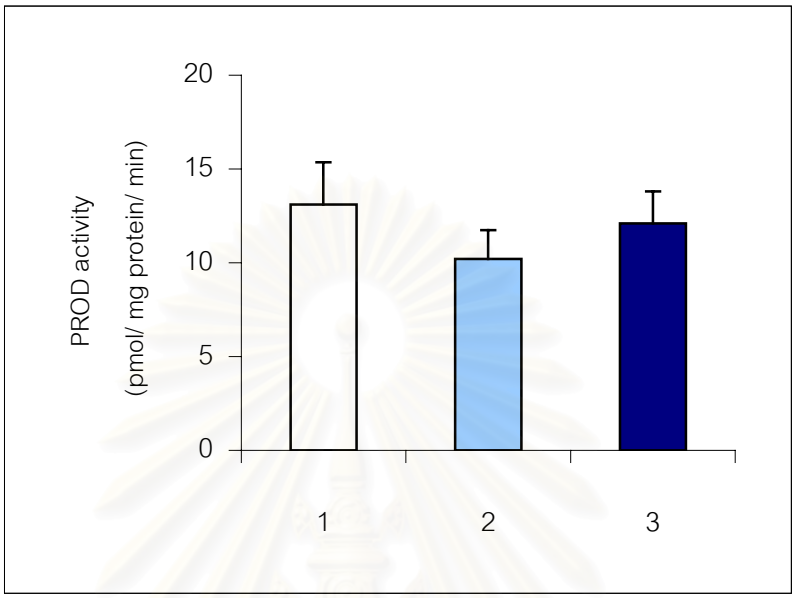


Figure 17 Effects of *T. crispa* stem extract on hepatic microsomal PROD activity

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

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

3.3.3 Effect of *T. crispa* stem extract on hepatic microsomal aniline 4-hydroxylase activity

Rate of aniline 4-hydroxylation, which represented the activity of CYP2E1, was not changed when *T. crispa* stem extract was given to rats at 250 mg/kg/day. A significant increase of CYP2E1 activity was observed when the extract was given at 500 mg/kg/day as compared to the control group (Figure 18).

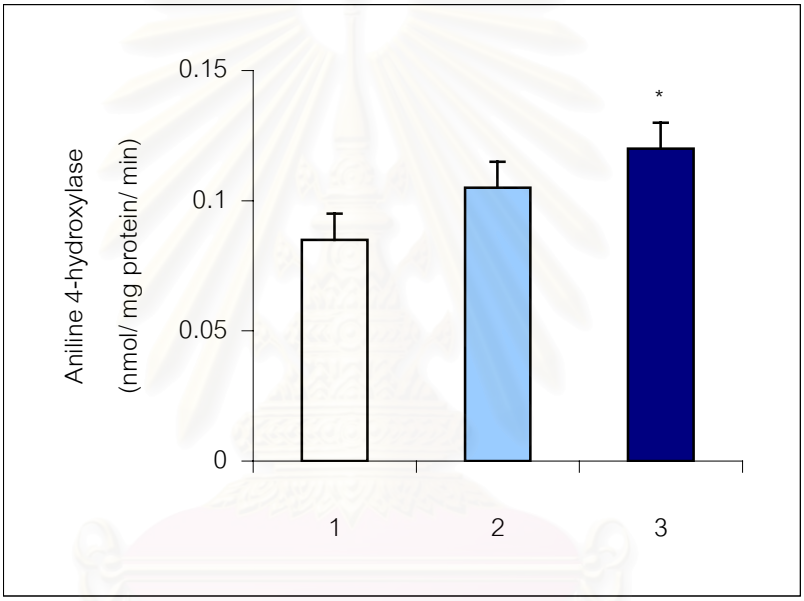


Figure 18 **Effect of *T. crispa* stem extract on rate of aniline 4-hydroxylase activity**

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

* $p<0.05$; *T. crispa* treatment group vs. control group

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* treatment group II

3.3.4 Effect of *T. crispa* stem extract on hepatic microsomal erythromycin N-demethylase activity

T. crispa stem extract at both doses used in this study did not exhibit any significant effects on the rate of erythromycin N-demethylation (which represented the activities of CYP3A) (Figure 19).

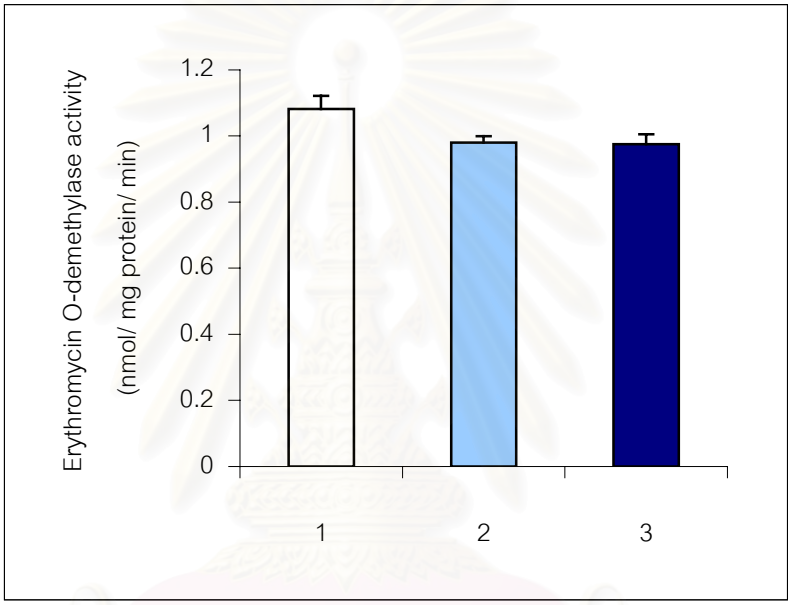


Figure 19 **Effect of *T. crispa* stem extract on rate of erythromycin N-demethylase activity**

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

1 = Control group

2 = *T. crispa* treatment group I

3 = *T. crispa* Atreatment group II

CHAPTER V

DISCUSSION AND CONCLUSION

This study was performed to investigate subacute effects of *T. crispa* stem extract on hepatic CYPs and clinical blood chemistry. Male Wistar rats were given orally the ethanolic extract of *T. crispa* stem at doses of 250 and 500 mg/kg/day for 30 days. This was the first report regarding the effects of *T. crispa* on hepatic CYPs. Since stem of *T. crispa* is traditionally used as hypoglycemic agent, repeated exposure of this plant is likely occurred and may result in more or less modulation of hepatic drug metabolizing enzymes. This study focused on the effect of this plant on hepatic CYPs particularly the isoforms that are responsible for xenobiotic bioactivation such as CYPs 1A1, 1A2, 2B1&2B2, 2E1, and 3A. Induction of these isoforms indicated the potential risks to chemical-induced toxicities, mutagenesis, and carcinogenesis of this plant if patients received chronically. In the other hand, antimutagenic and/or anticarcinogenic potential may be indicated if this plant possessed inhibition effects on these CYPs. In addition, diabetic patients are normally polydrug therapy, modulation of hepatic CYPs by *T. crispa* stem should be concerned in term of drug-drug interaction.

In this study, *T. crispa* stem was extracted with 95% ethanol, the fraction which was shown to possess hypoglycemic effect (Anulakanapakorn, *et al*., 1999). Percentage yield of the extract was 10% w/w of the dried *T. crispa* stem which was in the range limited in Standard of Asean Herbal Medicine (Ethanol-soluble extractive yield should not be less than 5%). Chemical identification tests included preliminary test and confirmatory test were performed (Asean countries, 1993) before the extract was given to the animals. Two doses (250 and 500 mg/kg/day) used in this study were the dosage regimen, shown to possess hypoglycemic effect (Anulakanapakorn, *et al*., 1999).

Results from this study showed that both doses of *T. crispa* stem extract caused a significant reduction of total CYP contents. When an individual CYP activity was determined, significant inhibition of only CYP1A1 and induction of CYP2E1 were observed

following 500 mg/kg/day dosage of the extract administration. No changes of CYPs 1A2, 2B1&2B2, and 3A were observed at both doses of *T. crispa* stem extract. No effects on these isoforms 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. However, administration of this extract at high dose should be concerned regarding the modulation effect on CYP1A1 and CYP2E1.

Inhibition of phase I enzymes was considered a logical strategy in chemoprevention, which was especially beneficial in early stages of carcinogenesis (Gerhauser, *et al*., 2003). Inhibition effect of *T. crispa* stem extract on CYP1A1 activity would be an advantageous effect in term of a decrease risk of chemical-induced toxicities, mutagenesis, and/or carcinogenesis by any substances that are bioactivated by this CYP isoform such as polycyclic aromatic hydrocarbons, etc (Gibson and Skett, 1994; Palackal, *et al*., 2001; Kondraganti, *et al*., 2003). In contrast, induction of CYP2E1 possibly caused an opposite effect resulting in a potentiation of toxic and/or carcinogenic effects of several compounds such as aniline, p-nitrophenol, N-nitrosodimethylamine (Arinc, *et al*., 2000), acetaminophen (Sumioka, *et al*., 2001), etc.

In addition, induction and inhibition of CYP are often involved to drug-drug interactions resulting in metabolic alteration of drugs given concurrently (Na-Bangchang and Wernsdorfer, 2001). *T. crispa* stem extract at high dose demonstrated an inhibition effect on CYP1A1 activity. This effect might lead to accumulation and toxicity of coadministrative drug detoxified by this CYP isoform such as theophylline, a substrate of CYP1A1 (Levy, *et al*., 2000). Induction effect of *T. crispa* stem extract on CYP2E1 activity might cause a reduction of blood level and clinical effect of drugs metabolized by this CYP isoform such as acetaminophen, some halogenated anesthesias i.e. enflurane, isoflurane, halothane, methoxyflurane, etc (Raucy, *et al*., 1989; Berthou, 2001). Inconsistent results between total CYP contents and individual CYP activity shown in this study may be because an inhibition effect occurred on other CYP isoforms that were not determined in this study.

One previous study of Chavalittumrong, *et al*. (1997) was performed regarding acute and chronic (6 months) toxicity studies of *T. crispa* stem extract in mice and Wistar rats, respectively. Thus, this study provided an additional subacute toxicity data for this extract. Results from this study showed that both doses of *T. crispa* stem 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 (AST, ALT, ALP), kidney (BUN, SCr), blood system (hemoglobin, hematocrit, platelet count, WBC count, and %differential WBC) and some electrolytes (sodium, chloride) in serum following both doses of the extract administration. High dose of the extract (500 mg/kg/day) caused an increase of serum HDL-C and a decrease of serum glucose whereas low dose of the extract (250 mg/kg/day) did not affect these parameters. Both doses of the extract caused a decrease of serum potassium. These results were mostly consistent to the chronic toxicity data reported previously by Chavalittumrong, *et al*. (1997) despite somewhat difference of dosages given to the animals.

In the chronic toxicity study of *T. crispa* stem extract by Chavalittumrong, *et al*. (1997), 16 male Wistar rats of each group were given ethanolic extract of *T. crispa* stem at doses of 20, 160, and 1280 mg/kg/day for 6 months. The results showed that *T. crispa* stem extract did not affect on body weight, food consumption, hematological parameters including hematocrit, WBC count, platelet count and %differential WBC in rats of all three treatment groups. However, some different effects on clinical blood chemistry were shown when different doses of the extract were given to the animals. An increase of SCr was observed in rats of all three treatment groups. Dose-related increase of serum total cholesterol was observed at 160 and 1280 mg/kg/day of the extract. An increase of ALP but a decrease of serum glucose was observed in rats given the extract at 1280 mg/kg/day. These slightly differences between the results of Chavalittumrong, *et al*. (1997) and this current results were probably influenced by many factors such as the different source of plant, procedure of the extraction, duration of the animal treatment, doses of the extract administration, ect.

Advantageous effects were observed at the dose of 500 mg/kg/day such as significant increase of serum HDL-C and decrease of serum glucose. The latter result

supported the hypoglycemic indication of *T. crispa* stem extract (Noor, *et al*., 1989; Noor and Ashcort, 1989; Noor and Ashcort, 1998). Type 2 diabetic patients were commonly possess low levels of HDL-C and high risk of coronary heart disease. Elevation of serum HDL-C would attenuate risk of coronary heart disease via removal of cholesterol from peripheral cells to liver for excretion in bile (Gotto, 2002). This was an interesting effect of *T. crispa* stem extract that it possessed hypoglycemic effect simultaneously with an advantageous effect against coronary heart disease in diabetic patients.

Serum potassium was significantly decreased at both doses of the extract. This effect may be related to an increase of insulin secretion, the proposed mechanism that *T. crispa* stem extracted exerted its hypoglycemic effect (Noor, *et al*., 1989; Noor and Ashcort, 1989; Noor and Ashcort, 1998). However, this effect may be due to other mechanisms such as an increase of potassium excretion by kidney (Chatworthy, 2001). This would be clarified by measuring potassium concentration in urines of rats in *T. crispa* treatment group comparing to those of rats in the control group. Administration of *T. crispa* stem extract should be concerned in patients receiving coadministrative drugs which normally possess hypokalemic effect such as loop diuretics, thiazides, some antibiotics (carbenicillin, gentamicin, amphotericin B) (Chatworthy, 2001), etc.

In conclusion, *T. crispa* stem extract given orally to male Wistar rats at doses of 250 and 500 mg/kg/day for 30 days, did not cause any effects on CYP 1A2, 2B1&2B2, and 3A. Significant inhibition on CYP1A1 and induction on CYP2E1 were observed following 500 mg/kg/day of *T. crispa* stem extract. No adverse effects on many important organs/systems were observed at both doses of the extract. Significant increase of HDL-C and decrease of serum glucose were shown at 500 mg/kg/day. Serum potassium was significantly decreased at both doses of the extract. Further studies on the effects of *T. crispa* stem extract on human hepatic CYPs and phase II metabolizing enzymes were suggested. Mechanism by which this extract inhibit CYP1A1 and induce CYP2E1 should also be clarified.

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APPENDIX

Table 6 Body weight of an individual rat in control group

Rat number	Day 0	Day 15	Day 30
1	206	297	384
2	192	302	367
3	195	291	362
4	207	318	393
5	201	312	368
6	206	316	360
7	252	312	350
8	246	299	351
9	246	333	388
10	220	340	337
Mean	217.1	312.0	366.0
SEM	7.17	4.96	5.69

Unit expressed as g

Table 7 Body weight of an individual rat in *T. crispa* treatment group I

Rat number	Day 0	Day 15	Day 30
1	203	295	389
2	208	312	367
3	196	301	366
4	211	320	380
5	201	302	344
6	245	343	390
7	256	344	387
8	252	324	388
9	256	328	372
10	275	348	405
Mean	230.3	321.7	378.8
SEM	9.22	6.07	5.4

Unit expressed as g

Table 8 Body weight of an individual rat in *T. crispa* treatment group II

Rat number	Day 0	Day 15	Day 30
1	197	272	341
2	203	312	343
3	191	290	358
4	212	305	365
5	205	280	315
6	249	329	362
7	254	326	363
8	249	335	386
9	264	353	409
10	241	352	410
Mean	226.5	315.4	365.2
SEM	8.66	9.03	9.44

Unit expressed as g

Table 9 Liver weight of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	10.61	9.86	9.78
2	10.84	10.63	11.18
3	10.38	10.78	10.79
4	11.54	11.91	10.86
5	10.82	9.97	10.31
6	14.13	14.80	14.39
7	10.37	13.41	11.74
8	13.03	14.87	15.61
9	12.64	12.89	12.62
10	11.60	12.24	11.92
Mean	**11.97**	**12.14**	**11.93**
SEM	**0.43**	**0.59**	**0.52**

Unit expressed as g

Table 10 The one-week food comsumption of an individual rat in control group

Rat number	Day 0	Day 8	Day 15	Day 22	Day 30
1	20	23	25	30	26
2	19	25	28	27	20
3	17	22	21	24	26
4	17	29	27	25	21
5	22	17	20	20	20
6	22	21	25	25	26
7	19	24	20	21	17
8	19	24	20	25	20
9	26	28	22	27	20
10	18	20	19	-	-
Mean	19.9	23.3	22.7	24.9	21.8
SEM	0.88	1.14	1.03	1.02	1.12

Unit expressed as g

Table 11 The one-week food comsumption of an individual rat in *T. crispa* treatment group I

Rat number	Day 0	Day 8	Day 15	Day 22	Day 30
1	19	23	21	17	25
2	19	26	24	26	27
3	19	22	19	23	22
4	21	23	29	27	22
5	20	22	23	18	17
6	24	25	29	19	28
7	23	22	25	21	26
8	17	25	22	20	26
9	22	21	16	17	20
10	25	21	23	24	30
Mean	20.9	23	23.1	21.2	24.3
SEM	0.81	0.56	1.28	1.15	1.26

Unit expressed as g

Table 12 The one-week food comsumption of an individual rat in *T. crispa* treatment group II

Rat number	Day 0	Day 8	Day 15	Day 22	Day 30
1	18	17	23	19	22
2	21	28	21	25	27
3	19	24	20	31	30
4	20	21	23	26	24
5	19	22	24	23	22
6	25	23	29	24	24
7	20	23	24	23	19
8	23	27	23	21	24
9	19	2	27	26	20
10	26	23	27	21	20
Mean	21	21	24.1	23.9	23.2
SEM	0.87	2.32	0.89	1.07	1.07

Unit expressed as g

Table 13 The one-week water comsumption of an individual rat in control group

Rat number	Day 0	Day 8	Day 15	Day 22	Day 30
1	29	30	29	31	35
2	25	33	30	28	29
3	27	32	35	35	33
4	33	40	43	39	37
5	31	30	38	37	41
6	34	35	32	40	39
7	29	36	29	30	31
8	30	34	28	41	34
9	38	41	42	35	33
10	30	35	35	-	-
Mean	**30.6**	**34.6**	**34.1**	**33.1**	**33.1**
SEM	**1.17**	**1.17**	**1.74**	**0.32**	**0.58**

Unit expressed as mL

Table 14 The one-week water comsumption of an individual rat in *T. crispa* treatment group I

Rat number	Day 0	Day 8	Day 15	Day 22	Day 30
1	30	33	35	37	28
2	30	35	36	28	31
3	32	34	40	39	35
4	28	34	39	42	40
5	27	38	33	32	32
6	36	34	32	34	30
7	29	34	32	33	30
8	38	42	35	36	40
9	30	36	35	28	32
10	35	42	34	32	38
Mean	31.5	36.2	35.1	34.1	33.6
SEM	1.16	1.06	0.85	1.43	1.39

Unit expressed as mL

Table 15 The one-week water comsumption of an individual rat in *T. crispa* treatment group II

Rat number	Day 0	Day 8	Day 15	Day 22	Day 30
1	30	31	36	44	41
2	31	28	38	37	35
3	29	37	31	40	37
4	27	34	39	32	32
5	25	31	33	34	29
6	27	31	35	41	31
7	30	32	38	37	30
8	29	32	34	35	30
9	25	30	31	31	34
10	25	30	31	33	34
Mean	27.8	31.6	34.6	36.4	33.3
SEM	0.73	0.78	0.98	1.33	1.17

Unit expressed as mL

Table 16 Serum glucose of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	136	109	90
2	151	92	94
3	118	121	109
4	121	111	88
5	106	92	102
6	151	138	137
7	156	147	117
8	130	101	113
9	130	130	113
10	178	158	128
Mean	137.7	119.90	109.10
SEM	6.76	7.25	5.07

Unit expressed as mg/dL

Table 17 Serum total cholesterol of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	39	75	63
2	59	73	85
3	64	80	72
4	83	67	87
5	54	64	79
6	94	84	83
7	-	80	103
8	88	73	90
9	65	73	114
10	95	99	112
Mean	**71.22**	**76.80**	**88.80**
SEM	**6.54**	**3.11**	**5.24**

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency.

Table 18 Serum triglyceride of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	117	39	45
2	47	77	71
3	84	64	72
4	166	110	73
5	96	65	57
6	145	91	118
7	-	75	58
8	159	134	112
9	92	83	91
10	137	182	143
Mean	**115.89**	**92.00**	**84.00**
SEM	**13.12**	**12.96**	**9.91**

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency.

Table 19 Serum HDL-C of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	64	-	-
2	49	59	73
3	53	67	64
4	75	59	79
5	43	57	68
6	79	74	68
7	-	71	88
8	67	63	79
9	54	62	92
10	-	87	88
Mean	60.50	66.56	77.67
SEM	4.26	3.19	3.37

Unit expressed as mg/dL

Missing values (-) were due to blood insufficiency.

Table 20 Serum AST of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	142	161	234
2	287	199	262
3	183	189	109
4	118	147	287
5	242	139	179
6	117	91	150
7	-	83	138
8	272	184	99
9	93	110	101
10	-	122	113
Mean	181.75	142.50	167.20
SEM	26.92	12.97	22.23

Unit expressed as U/L

Missing values (-) were due to blood insufficiency.

Table 21 Serum ALT of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	69	36	41
2	49	37	60
3	63	30	52
4	69	54	45
5	38	25	44
6	85	57	28
7	-	80	69
8	36	59	55
9	47	90	64
10	-	45	89
Mean	**57.00**	**51.30**	**54.70**
SEM	**6.08**	**6.70**	**5.40**

Unit expressed as U/L

Missing values (-) were due to blood insufficiency.

Table 22 Serum ALP of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	92	99	98
2	87	109	105
3	108	104	90
4	95	85	108
5	100	97	108
6	120	156	122
7	-	126	91
8	138	160	162
9	127	102	86
10	-	106	104
Mean	108.38	114.40	107.40
SEM	6.46	7.97	6.94

Unit expressed as U/L

Missing values (-) were due to blood insufficiency.

Table 23 Serum total bilirubin of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	0.14	0.1	0.18
2	0.15	0.08	0.38
3	0.27	0.13	0.13
4	0.2	0.2	0.4
5	0.67	0.12	0.19
6	0.14	0.14	0.19
7	-	0.15	0.15
8	0.51	0.34	0.18
9	0.11	0.19	0.22
10	1.12	0.36	0.26
Mean	**0.37**	**0.18**	**0.23**
SEM	**0.11**	**0.03**	**0.03**

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency.

Table 24 Serum direct bilirubin of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	0.08	0.09	0.1
2	0.12	0.07	0.16
3	0.14	0.09	0.06
4	0.06	0.07	0.12
5	0.19	0.06	0.08
6	0.1	0.08	0.1
7	-	0.07	0.05
8	0.29	0.21	0.07
9	0.06	0.07	0.08
10	0.46	0.16	0.1
Mean	**0.17**	**0.10**	**0.09**
SEM	**0.04**	**0.02**	**0.01**

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency.

Table 25 BUN of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	19	22	23
2	24	19	21
3	22	19	19
4	20	22	23
5	20	24	25
6	28	29	31
7	-	26	18
8	28	33	33
9	23	29	27
10	29	32	33
Mean	23.67	25.50	25.30
SEM	1.28	1.61	1.75

Unit expressed as mg/dL

Missing value (-) was due to blood insufficiency.

Table 26 SCr of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	0.4	0.4	0.8
2	0.3	0.5	0.4
3	0.4	0.5	0.4
4	0.4	0.6	0.4
5	0.4	0.5	0.6
6	0.6	0.4	0.4
7	-	0.5	0.6
8	0.4	0.4	0.4
9	0.4	0.4	0.5
10	-	0.4	0.5
Mean	**0.41**	**0.46**	**0.50**
SEM	**0.03**	**0.02**	**0.04**

Unit expressed as mg/dL

Missing values (-) were due to blood insufficiency.

Table 27 Serum sodium of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	144	148	146
2	149	150	146
3	154	148	151
4	146	145	146
5	143	145	147
6	145	146	149
7	-	147	148
8	144	146	143
9	147	147	148
10	142	147	145
Mean	146.00	146.90	146.90
SEM	1.22	0.48	0.71

Unit expressed as mmol/L

Missing value (-) was due to blood insufficiency.

Table 28 Serum potassium of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	4	4.5	4.6
2	6	3.5	5.2
3	5.2	4.5	3.3
4	5.6	4.1	4.8
5	4.5	3.5	4.1
6	4.4	4.4	3.9
7	-	4.4	4
8	5	4.3	4
9	4.1	3.7	4.1
10	5	5.0	4.7
Mean	**4.87**	**4.19**	**4.27**
SEM	**0.23**	**0.14**	**0.17**

Unit expressed as mmol/L

Missing value (-) was due to blood insufficiency.

Table 29 Serum chloride of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	100	101	103
2	97	102	103
3	104	105	103
4	103	100	103
5	104	104	105
6	104	105	106
7	-	101	104
8	103	102	104
9	101	101	102
10	100	102	101
Mean	101.78	102.30	103.40
SEM	0.81	0.56	0.45

Unit expressed as mmol/L

Missing value (-) was due to blood insufficiency.

Table 30 Hb of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	15.5	15.3	16.5
2	16.6	16.2	16.1
3	15.9	16.1	16.1
4	15.6	15.8	16.2
5	15.2	15.1	16.4
6	15.9	15.2	15.7
7	16.7	16.2	16.6
8	15.6	16.4	15.7
9	16.3	15.6	18
10	12.8	15.4	15.3
Mean	**15.61**	**15.73**	**16.26**
SEM	**0.35**	**0.15**	**0.23**

Unit expressed as g/L

Table 31 Hct of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	46.9	46.2	49.8
2	58.2	47.1	46.5
3	48.5	46.6	47.5
4	46.4	46.3	48.5
5	44.5	45.1	49.4
6	48.7	46.2	47.2
7	49.3	17.7	49.3
8	49.1	49.8	47.8
9	47.9	46.4	51.1
10	38.8	48.8	44.5
Mean	47.83	44.02	48.16
SEM	1.52	2.96	0.60

Unit expressed as percent value

Table 32 RBC morphology of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	N	N	N
2	N	N	N
3	N	N	N
4	N	N	N
5	N	N	N
6	N	N	N
7	N	N	N
8	N	N	N
9	N	N	N
10	N	N	N

N = normal

Table 33 Platelet count of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	639	587	565
2	644	548	179
3	657	595	640
4	615	270	605
5	449	686	433
6	659	696	622
7	132	483	574
8	583	350	212
9	130	642	628
10	126	767	455
Mean	**463.40**	**562.40**	**491.30**
SEM	**75.39**	**49.46**	**54.10**

Unit expressed as $x10^3$ cells/μL

Table 34 WBC count of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	2.25	1.35	0.41
2	0.77	2.03	2.3
3	4.02	4.74	1.5
4	3.43	1.79	4.4
5	3.29	2.01	1.34
6	2.14	3.44	3.3
7	1.85	3.31	1.94
8	2.00	0.88	1.61
9	3.75	2.4	1.91
10	1.15	3.81	2.7
Mean	**2.47**	**2.58**	**2.14**
SEM	**0.35**	**0.38**	**0.37**

Unit expressed as $x10^9$ cells/μL

Table 35 Basophil of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	0.2	0.6	0.9
2	0.3	0.5	0.3
3	0.3	0.6	0.2
4	0.8	0.1	0.7
5	0.5	0.1	0.3
6	0.2	0.2	0.4
7	0.1	0.4	0.2
8	0.6	0.7	0
9	0.1	0.2	0.1
10	0.4	0.5	0.3
Mean	**0.35**	**0.39**	**0.34**
SEM	**0.07**	**0.07**	**0.09**

Unit expressed as percent value

Table 36 Eosinophil of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* reatment group I	*T. crispa* reatment group II
1	0.9	1.3	1.3
2	1	1.4	4.2
3	0.7	0.8	1
4	0.6	1.7	1.1
5	2	0.3	1.3
6	0.7	1.4	1.3
7	1.7	1.2	1.7
8	0.3	1.3	1.6
9	0.6	0.7	0.9
10	1.7	0.6	1.5
Mean	**1.02**	**1.07**	**1.59**
SEM	**0.18**	**0.14**	**0.30**

Unit expressed as percent value

Table 37 Neutrophil of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	7.9	10.2	21.7
2	7.9	11.9	45.6
3	4.9	6.7	10.9
4	9.9	20.7	31.3
5	18.2	8.8	12.8
6	6.9	5.3	6.7
7	21.3	9.7	11.8
8	4.6	16.2	14.1
9	38.7	9.8	7.1
10	21.7	9.5	24.1
Mean	**14.20**	**10.88**	**18.61**
SEM	**3.42**	**1.43**	**3.89**

Unit expressed as percent value

Table 38 Lymphoctye of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	59.9	60.9	51.3
2	54.9	53.3	34.9
3	77.8	75.9	56.7
4	69.1	63.7	59.7
5	55.6	66.6	56.3
6	74	66.8	66.9
7	68.8	79.9	60
8	66.6	62.2	74.2
9	50.2	61.9	69.3
10	61.6	71.8	66.6
Mean	63.85	66.30	59.59
SEM	2.81	2.47	3.51

Unit expressed as percent value

Table 39 Monocyte of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	28.4	23	22
2	31.1	30.6	13.6
3	13.4	15.4	30.3
4	17	12.3	3.3
5	18.8	24.1	26.3
6	16.4	26.1	24.4
7	6.9	5.8	24.2
8	23	16.5	8.6
9	9.4	24.9	20.4
10	12.6	15.9	6.5
Mean	**17.70**	**19.46**	**17.96**
SEM	**2.48**	**2.38**	**2.94**

Unit expressed as percent value

Table 40 Microsomal protein concentration of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	46.46	36.20	36.75
2	45.65	38.64	41.84
3	31.81	33.63	32.61
4	54.73	38.72	36.79
5	40.10	44.09	43.69
6	45.35	55.70	38.48
7	44.32	46.96	36.96
8	36.77	40.90	40.25
9	52.21	43.82	40.95
10	44.92	41.45	36.21
Mean	**44.23**	**42.01**	**38.45**
SEM	**2.13**	**1.96**	**1.03**

Unit expressed as mg/mL

Table 41 Hepatic microsomal total CYP content of an individual rat

Rat number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	0.70	0.48	0.41
2	0.56	0.56	0.48
3	0.64	0.43	0.37
4	0.59	0.45	0.39
5	0.66	0.42	0.39
6	0.39	0.32	0.29
7	0.65	0.37	0.46
8	0.45	0.36	0.45
9	0.47	0.52	0.45
10	0.39	0.41	0.48
Mean	**0.55**	**0.43**	**0.42**
SEM	**0.04**	**0.02**	**0.02**

Unit expressed as nmol/ mg protein

Table 42 Hepatic microsomal EROD activity of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	66	51	49
2	80	66	28
3	-	98	55
4	-	53	38
5	106	71	54
6	56	28	42
7	42	50	31
8	84	39	58
9	50	72	51
10	67	68	71
Mean	**67.5**	**59.6**	**47.7**
SEM	**11.1**	**6.2**	**4.1**

Unit expressed as pmol/ mg protein/ min

Missing values (-) were due to outliers.

Table 43 Hepatic microsomal MROD activity of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	24	21	23
2	16	28	19
3	-	44	24
4	-	24	22
5	36	20	23
6	18	14	20
7	18	12	20
8	23	20	22
9	12	14	15
10	18	28	17
Mean	**20.8**	**22.5**	**20.5**
SEM	**2.6**	**3.0**	**0.9**

Unit expressed as pmol/ mg protein/ min

Missing values (-) were due to outliers.

Table 44 Hepatic microsomal BROD activity of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	60	45	65
2	44	55	59
3	82	51	45
4	62	56	60
5	79	43	44
6	40	50	69
7	40	29	29
8	28	16	23
9	30	33	58
10	21	39	52
Mean	48.6	41.7	50.4
SEM	6.7	4.0	4.8

Unit expressed as pmol/ mg protein/ min

Table 45 Hepatic microsomal PROD activity of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	10	9	11
2	8	13	10
3	26	19	24
4	22	12	10
5	21	16	12
6	8	10	16
7	12	7	6
8	10	3	6
9	6	6	10
10	8	7	16
Mean	13.1	10.2	12.1
SEM	2.3	1.5	1.7

Unit expressed as pmol/ mg protein/ min

Table 46 Hepatic microsomal aniline 4-hydroxylase activity of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	0.077	0.113	0.115
2	0.113	0.131	0.167
3	0.138	0.135	0.184
4	0.068	0.108	0.127
5	0.107	0.139	0.089
6	0.058	0.071	0.077
7	0.082	0.108	0.142
8	0.094	0.075	0.112
9	0.06	0.092	0.097
10	0.051	0.073	0.085
Mean	**0.085**	**0.105**	**0.120**
SEM	**0.01**	**0.01**	**0.01**

Unit expressed as nmol/ mg protein/ min

Table 47 Hepatic microsomal erythromycin N-demethylase activity of an individual rat

Rat Number	Treatment group		
	Control group	*T. crispa* treatment group I	*T. crispa* treatment group II
1	0.900	0.810	0.855
2	0.945	0.930	0.870
3	1.260	1.020	1.140
4	1.035	0.975	0.990
5	1.170	0.960	1.050
6	1.095	1.005	1.065
7	1.185	1.080	1.035
8	1.185	1.080	1.050
9	0.945	0.960	0.855
10	1.095	0.975	0.840
Mean	**1.082**	**0.980**	**0.975**
SEM	**0.04**	**0.02**	**0.03**

Unit expressed as nmol/ mg protein/ min

VITAE

Miss Oomchoo Saiphet was born in August 11, 1975 in Khon Kaen, Thailand. She graduated with a Bachelor of Sciences in Pharmacy in 1998 from the Faculty of Pharmacy, Khon Kaen University, Khon Kaen, Thailand. After graduation, she worked as a pharmacist in Kaedam Hospital, Mahasarakham for one year and Sirindhorn College of Public Health Khon Kaen for two years.