ผลของสารสกัดว่านชักมดลูกต่อเอนไซม์ที่เกี่ยวข้องกับเมแทบอลิซึมของยาในเฟส 2 ในตับหนูขาว

นางสาวนีรนาท จิวาภรณ์คุปต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EFFECTS OF *CURCUMA COMOSA* EXTRACTS ON PHASE II DRUG METABOLIZING ENZYMES IN RAT LIVER

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Thesis Title	EFFECTS OF *CURCUMA COMOSA* EXTRACTS ON PHASE II DRUG METABOLIZING ENZYMES IN RAT LIVER
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ลีนวัฒน์ ศรีสุนาครัว : ผลกระทบจากการเปลี่ยนแปลงการออกแบบแผ่นโลหะเคลือบกระเบื้องต่อความต้านทานการแตกหักของพอร์ซเลน. (THE EFFECT OF VARIOUS METAL FRAMEWORK DESIGNS OF PORCELAINFUSED TO METAL DISC ON FRACTURE RESISTANCE OF PORCELAIN) อ. ที่ปรึกษา: รศ.ทพ. ภาณุพงศ์ วงศ์ไทย, 85 หน้า.

การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาถึงการแตกหักของพอร์ซเลนบนชิ้นงานตัวอย่างที่มีการออกแบบโลหะ พอร์ซเลนและตำแหน่งจุดกดแตกต่างกัน ทำการทดสอบชิ้นงาน 8 กลุ่ม โดยมีการออกแบบโลหะรองรับพอร์ซเลนดังต่อไปนี้ ชิ้นงานแผ่นแบนหนา 0.3 มม.สม่ำเสมอ(กลุ่มที่1), ชิ้นงานตรงกลางบาง 0.1 มม.(กลุ่มที่2), ชิ้นงานโลหะรองรับเป็นทรงกรวยมุมแหลมส่วนหนาสุด 2.3 มม.(กลุ่มที่3), ชิ้นงานโลหะรองรับนูนเป็นมุมมนส่วนหนาสุด 2.3 มม.(กลุ่มที่4), ชิ้นงานรูปขั้นบันไดจุดกดตรงรอยต่อ(กลุ่มที่5), ชิ้นงานรูปขั้นบันไดจุดกดตรงกลาง(กลุ่มที่6), ชิ้นงานรูปขั้นบันไดจุดกดตรงขอบ(กลุ่มที่7), ชิ้นงานรูปขั้นบันไดจุดกดเป็นมุมเฉียง30องศา(กลุ่มที่8) โดยชิ้นงานโลหะเคลือบกระเบื้องขนาด 7 x 9 มม.^{2} ทุกกลุ่มๆละ 10 ชิ้น ได้เตรียมขึ้นตามคำแนะนำของบริษัทผู้ผลิต นำชิ้นงานที่ได้มายึดบนแป้นทองเหลืองด้วยซิงค์ฟอสเฟตซีเมนต์ จากนั้นนำไปแช่ในน้ำกลั่น 37 องศา เซลเซียส 24 ชั่วโมง นำชิ้นงานที่ได้มาทดสอบด้วยแรงอัดในแนวดิ่งจนพอร์ซเลนแตก ด้วยเครื่องทดสอบสากลรุ่น Instron 8872 ที่มีหัวกดรูปทรงกลมขนาดเส้นผ่านศูนย์กลาง 3 ม.ม. ความเร็วหัวกด 1 มม./ นาที ผลการทดลองพบว่าค่าเฉลี่ยและค่าเบี่ยงเบนมาตรฐาน (X $\pm$ S.D.) ของแต่ละกลุ่มมีค่าดังนี้ 1)2506.03 $\pm$257.98N 2)2027.07 $\pm$180.74N 3)2101.12 $\pm$101.55N 4)2117.12 $\pm$125.49N 5)1461.20 $\pm$139.20N 6)2092.40 $\pm$113.79N 7)791.64 $\pm$ 87.96N 8)1062.38 $\pm$187.83N เมื่อนำข้อมูลไปวิเคราะห์โดยใช้สถิติวิเคราะห์ความแปรปรวนแบบทางเดียวและการทดสอบการเปรียบเทียบเชิงซ้อนชนิดแทมเฮน ได้ผลดังนี้ ชิ้นงานกลุ่มที่1 มีค่าเฉลี่ยแรงอัดสูงสุดที่ทำให้พอร์ซเลนเกิดการแตกหักแตกมากที่สุด และมากกว่ากลุ่มอื่นๆอย่างมีนัยสำคัญทางสถิติ($p<0.05$) พบความแตกต่างอย่างมีนัยสำคัญทางสถิติในกลุ่มที่ 5, 7 และ8 ซึ่งเป็นชิ้นงานรูปขั้นบันไดที่มีตำแหน่งจุดกดแตกต่างกัน ($p<0.05$) ส่วนในชิ้นงานกลุ่มที่ 2, 3, 4 และ6 ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($p\geq0.05$) จากข้อมูลข้างต้นทำให้สรุปได้ว่า ความหนาที่ไม่สม่ำเสมอของโลหะที่รองรับพอร์ซเลนรวมทั้งตำแหน่ง และทิศทางของแรงกด มีผลต่อความต้านทานการแตกหักของชิ้นงานโลหะเคลือบกระเบื้อง

ภาควิชา.....ทันตกรรมประดิษฐ์.....ลายมือชื่อนิสิต.....

สาขาวิชา.....ทันตกรรมประดิษฐ์.....ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2550.....

4876122332 : MAJOR PROSTHODONTICS

KEY WORD: FRACTURE OF PORCELAIN / METAL CERAMIC RESTORATION / THICKNESS OF METAL / THICKNESS OF PORCELAIN

LEENAWAT SRISUNAKRUA: THE EFFECT OF VARIOUS METAL FRAMEWORK DESIGNS OF PORCELAINFUSED TO METAL DISC ON FRACTURE RESISTANCE OF PORCELAIN. THESIS ADVISOR : ASSOC. PROF. PANUPONG WONGTHAI, 85 pp.

The objective of this study was to evaluate the fracture resistance of ceramometal samples on different metal designs and location of load. Eight different metal designs were as follows: 0.3 mm uniform thickness of metal (group 1), concave specimen of 0.1 mm at the thinnest point (group2), cone-shaped with sharp apex of 2.3 mm at the thickest point (group 3), convex specimen of 2.3 mm at the thickest point (group 4), ladder-shaped with loading point at the step (group 5), ladder-shaped with loading point at the center (group 6), ladder-shaped with loading point at the porcelain thickest point (group 7) and ladder-shaped with 30° loading (group 8). Ten specimens, 7 x 9 mm^2, of each group were fabricated according to the manufacturer's recommendations. The specimen was luted to the brass specimen holder with zinc phosphate cement and stored in 37°C for 24 hour prior to the test. The test was performed on a universal testing machine (Instron 8872) with crosshead speed of 1 mm/min using metal sphere loading point diameter of 3 mm under compressive load. The maximum load prior to fracture means $\pm$ S.D. of each group was as follows: 1)2506.03 $\pm$ 257.98 N 2)2027.07 $\pm$ 180.74 N 3)2101.12 $\pm$ 101.55 N 4)2117.12 $\pm$ 125.49 N 5) 1461.20$\pm$ 139.20 N 6)2092.40 $\pm$ 113.79 N 7)791.64 $\pm$ 87.96 N 8)1062.38 $\pm$ 187.83 N. One-way ANOVA and Tamhane multiple comparisons revealed that group 1 had the greatest fracture resistance over the others ($p<0.05$). There were significant differences among different loading point on same metal design of group 5,7 and 8 ($p<0.05$). Group 2, 3, 4 and 6 were not different at $p\geq0.05$. It was concluded that the non-uniform thickness of metal supporting porcelain, position and direction of load affected the fracture resistance of porcelain fused to metal specimen.

Department…….Prosthodontics……..Student's Signature. ……

Field of Study…..Prosthodontics……..……..Advisor's Signature:……

Academic Year…….2007 ……

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LIST OF ABBREVIATIONS

β = beta
^{o}C = degree celcius
µg = microgram
µl = microlitre
µmol = micromole
µM = micromolar
α = alpha
A = Alpha
Ach = acetylcholine
AhR = aryl hydrocarbon receptor
AhRR = aryl hydrocarbon receptor repressor
ALP = alkaline phosphatase
ALT = alanine aminotransferase
APS = adenosine-5'-phosphosulfate
ARE = antioxidant response element
Arnt = AhR nuclear translocator
ASP = Angelica sinensis polysaccharides
AST = aspartate aminotransferase
ATP = adenosine triphosphate
AzMC = 7-azido-4-methylcoumarin
BFR = bile flow rate
BHA = 1,4-dialkylphenol
BMD = bone mineral density
BR = benzyloxyresorufin
BROD = benzyloxyresorufin *o*-dealkylation
BSA = bovine serum albumin
BUN = blood urea nitrogen
BW = body weight
Ca^{2+} = calcium ion
CAR = constitutive androstane receptor

CBC = complete blood count

CCl_4 = carbon tetrachloride

CDNB = 1-chloro-2,4-dinitrobenzene

CE = catechol estrogen

cm = centimeter

CN = Crigler Najjar syndrome

CV = coefficient of variation

CVD = cardiovascular diseases

CYP = cytochrome P450

Cys = cysteine

DCPIP = 2,6-dichlorophenol-indophenol

DEN = diethylnitrosamine

DHEA = dehydroepiandrosterone

DMSO = dimethylsulfoxide

E_2 = estradiol

EDTA = ethylene diamine tetra acetic acid

EE = ethinyl estradiol

EpRE = electrophilic response element

ER = ethoxyresorufin

EROD = ethoxyresorufin *o*-dealkylation

e.g. = example gratia

et al. = et alii (and other)

FAD = flavin adenine dinucleotide

FMN = flavin mononucleotide

g = gram

g = gravity

GH = growth hormone

Gly = glycine

Glu = glutamine

GRIP-1 = glucocorticoid-interacting receptor protein 1

GSH = glutathione reduced form

GSSG = glutathione oxidized form

GST = glutathione *S*-transferase

GST-P = GST *pi*

Hb	=	hemoglobin
Hct	=	hematocrit
HDL	=	high density lipoprotein
HDL-C	=	high density lipoprotein cholesterol
hPXR	=	human pregnan X receptor
HRT	=	hormone replacement therapy
5-HT	=	5-hydroxytryptamine
IC_{50}	=	median inhibitory concentration
i.e.	=	id est (that is)
IFN	=	interferon
IL-1β	=	interleukin-1β
i.p.	=	intraperitonium
IU	=	international unit
kg	=	kilogram
L	=	liter
LD_{50}	=	median lethal dose
LDL	=	low density lipoprotein
LDL-C	=	low density lipoprotein cholesterol
LPS	=	lipopolysaccharide
M	=	molar
MAP	=	mitogen-activated protein
3-MC	=	3-methylcholanthrene
mEq	=	milliequivalent
min	=	minute
mg	=	milligram
mg/kg	=	milligram per kilogram body weight
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
mmloe	=	millimole
MCV	=	mean corpuscular volume
MCH	=	mean corpuscular hemoglobin
MCHC	=	mean corpuscular hemoglobin concentration

MeIQX = 2-amino-3,8-dimethylimidazol[4,5-*f*]-quinoxaline
MFO = mixed-function oxidase
MR = methoxyresorufin
MROD = methoxyresorufin *o*-dealkylation
MTT = 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide
4-MU = 4-methylumbelliferone
MW = molecular weight
NADP = nicotinamide adenine dinucleotide phosphate
NADPH = nicotinamide adenine dinucleotide phosphate (reduced form)
NAT = *N*-acetyltransferase
NBC = *p*-nitrobenzylchloride
NF-κB = nuclear factor kappa B
2-NP = 2-nitropropane
nm = nanometer
nmol = nanomole
NNK = 4-(*Ψ*methylnitrosamine)-1-(3-pyridiyl)-1-butanone
NRH = dihydronicotinamide riboside
NQO1 = NAD(P)H quinone oxidoreductase 1
NQO2 = NAD(P)H quinone oxidoreductase 2
NQO3 = NAD(P)H quinone oxidoreductase 3
NQO4 = NAD(P)H quinone oxidoreductase 4
NQOR = NAD(P)H quinone oxidoreductase
p = para
PAH = polycyclic aromatic hydrocarbon
PAPS = 3'-phosphoadenosine-5'-phosphosulfate
PCBs = polychlorinated biphenyls
$PGF_{2\alpha}$ = prostaglandin F_2 alpha
pH = potential of hydrogen
PhIP = 2-amino-1-methyl-6-phenylimidazo[4,5-6]pyridine
PMA = phorbol-12-myristate-13-acetate
pmol = picomole
ppm = part per million
PR = pentoxyresorufin

PROD = pentoxyresorufin *o*-dealkylation
PXR = pregnan X receptor
QR = quinone reductase
RAR = retinoic acid receptor
RBC = red blood cell
r^2 = coefficient of determination
r.p.m. = revolution per minute
S = Sigma
SCr = serum creatinine
SEM = smooth endoplasmic reticulum
SRC-1 = steroid receptor coactivator-1
SULT = sulfotransferase
T = Theta
TCA = trichloroacetic acid
TCDD = 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TG = triglyceride
THA = 2,4,6-trihydroacetophenone
TL = thermolabile
TNF-α = tumor necrosis factor-α
Tris = tris (hydroxymethyl) aminomethane
TS = thermostable
U = unit
UDPGA = UDP-glucuronic acid
UDPGT = UDP-glucuronosyltransferase
v/v = volume by volume
w/v = weight by volume
w/w = weight by weight
WBC = white blood cell
XRE = xenobiotic response element

CHAPTER I

INTRODUCTION

Herbal medicines have received great attention as an alternative to clinical therapy, and the demand for these remedies has currently increased. However, it has been pointed out that commonly used herbal medicines cause some herb-drug interactions. The proposed interactions occur in aspects of pharmacokinetics and pharmacodynamics of drugs; absorption in the small intestine, metabolism in the intestine and liver, distribution to target organs, transport across cell membrane, binding to specific receptors. Among these interactions, induction and inhibition of hepatic drug metabolizing enzymes by herbal medicines or dietary compounds have been investigated (Sugiyama et al., 2004). *Curcuma comosa* Roxb. is a well-known herb belonging to Zingiberaceae family, known locally as "Waan chak motluk". On the basis of pharmacological use, hexane extract from its rhizome has demonstrated to possess uterotrophic effect, estrogenic activity (Piyachaturawat et al., 1995) and the ethanolic extract from the rhizome markedly reduced uterine contraction induced by oxytocin and some agents (Sawasdipanich, 1994). In addition to the mentioned uterotrophic and estrogenic effect, another interesting biological effect of *C. comosa* is choleretic activity. Ethyl acetate extract has been reported to stimulate bile secretion with increased biliary cholesterol secretion leading to a decrease in plasma cholesterol in normal rats (Piyachaturawat et al., 1996). The active compound isolated from the ethyl acetate extract was identified to phloracetophenone (Suksamrarn et al., 1997). The ethyl acetate extract has been shown to effectively reduce plasma triglyceride and cholesterol. This effect was associated with increased biliary secretion of bile acid, decrease secretion of cholesterol and phospholipids, and lowerd bile lithogenic index (Piyachaturawat et al., 1998, 1999). These interesting properties of *C. comosa* render the researchers to develop this plant for using in alternative medicinal therapies. Among the various steps of research and development, metabolism and the interaction with other medicines are almost without exception the most important. In phase I metabolism, a polar functional group is introduced into the molecule rendering it a suitable substrate for phase II metabolism. Phase II metabolism consists of conjugation of phase I metabolites or xenobiotics that already

possess suitable functional groups, with water-soluble endogenous substrates such as sugars, amino acids, sulfate or glutathione (Hodgson et al., 2007). Metabolism can result in either detoxication or activation, the latter being the formation of products that are more reactive than the parent compound. Generally, CYP is an important enzyme of phase I drug metabolism. CYP in families 1, 2 and 3 plays a significant role in xenobiotic (drugs, chemicals, pollutants, etc.) metabolism. CYP isoforms that play a key role in activating xenobiotics to toxic metabolites include CYPs 1A1, 2B1/2B2, 2C11/2C12, 2E1 and 3A1/3A2 in rats as well as CYP 1A1/1A2, 2B6, 2E1, and 3A4 in humans (Coleman et al., 2005)

Major phase II reactions include glucuronidation, sulfation, conjugation with glutathione. The transfer of UDP-glucuronic acid (UDPGA) to an aglycone is catalysed by a family of enzymes generally designated as UDP-glucuronosyl-transferase (UDPGT). These ubiquitous microsomal enzymes are presented in the membrane of endoplasmic reticulum. Glucuronide formation is quantitatively the most important form of conjugation for drugs and endogenous compounds and can occur with alcohols, phenols, hydroxylamines, carboxylic acids, amines, sulfonamides and thiols. *O*-Glucuronides formed from phenols, alcohol and carboxylic acids. Carboxylic acids forming 'ester' glucuronides and others 'ether' glucuronides. *N*-Glucuronides can be formed from amines (mainly aromatic), amides and sulfonamides. It has also been suggested that tertiary amines can form glucuronides giving quarternary nitrogen conjugates. *N*-Glucuronides may form spontaneously, without the presence of enzymes. Thiols group can react with UDPGA in the presence of UDPGT to yield *S*-glucuronides. Direct attachment of glucuronic acid to the carbon skeleton of the drugs has also been reported (i.e. *C*-glucuronidation). However, it is possible to produce compounds with more potent activity than parent analog, such as morphine-6-glucuronide (Collier et al., 2000). UDPGT isoforms include UDPGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B7, 2B10, 2B15, 2B17, 2B28. The substrates that form active glucuronides are bilirubin, estradiol (3-OH), all-*trans*-retinoic acid, morphine (3-OH), NSAIDs, *p*-nitrophenol, 4-methyl-umbelliferone. Numerous compounds were shown to induce glucuronidation activities, polychlorinated, biphenyls, *β*-naphthoflavone, phenobarbital, 3-methyl-cholanthrene (Miners et al., 1987). Inhibition of glucuronidation would direct the metabolism of benzo(*a*)pyrene-7,8-dihydrodiol (James et al., 2004).

Sulfation is a major conjugation pathway for phenols but can also occur for alcohols, amines and to a lesser extent, thiols. As with sugar conjugation, an energy-rich donor is required, in this case 3'-phosphoadenosine-5'-phosphosulfate (PAPS). These reactions occur in the cytosol. Sulfation occurs by interaction of the drug and PAPS in the presence of the sulfotransferase enzyme (SULT). Various forms of enzyme have been classified by their preferred substrates, molecular and gene structure such as phenol sulfotransferase, alcohol sulfotransferase, steroid sulfotransferase and arylamine sulfotransferase. The metabolic activation of the carcinogenic arylmethanols, such as 7-hydroxymethylchrysene was achieved with the major liver hydroxysteroid sulfotransferase (Ogura et al., 1994). 1-Hydroxymethyl pyrene is activated to a potent mutagen by hydroxysteroid sulfotransferase. Isoprenaline, dimetranidazole, estrone, paracetamol and 2-naphthol are substrates for SULT. In several studies, it was shown that progesterone, medrogestone, nomegestrol acetate, promegestone or tibolone can stimulate the SULT activity. Polychlorobiphenylols were reported as potent inhibitors of estrogen sulfotransferase, and 3-hydroxy(*a*)pyrene sulfotransferase. 4-Chlorobiphenyl-3'-ol was the most potent inhibitor of sulfotransferase (Wang et al., 2005).

Glutathione is recognised as a protective compound within the body for the removal of potentially toxic electrophilic compounds (Gibson et al., 2001). Many drugs either are, or can be, metabolised by phase I reactions to strong electrophiles, and these can react with glutathione to form non-toxic conjugates. (The list of compounds conjugated to glutathione include epoxides, haloalkenes, nitroalkenes, alkenes and aromatic halo-and nitro-compounds). The enzymes catalyzing the above reactions are the glutathione *S*-transferase (GST) which are located in the cytosol of liver. The nomenclature system has been confusing, an unifying nomenclature for the soluble human transferases has been suggested and classified into gene family: Alpha (A), Mu (M), Pi (P), Sigma (S), Theta (T), Zeta, Omega and Kappa and reflect their subunit composits. Trichloroethylene, dihaloalkenyl, 1,1-difluoroalkyl and polychlorinated alkenes are bioactivated through glutathione conjugation (Bladeren et al., 2000). 1,2-Dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene, 4-nitro-pyridine-*N*-oxide, *p*-nitrobenzyl chloride, *p*-nitrophenethyl bromide, 1,2-naphthalene oxide, bromosulfophthalein, 1-menaphthyl sulfate, and *trans*-4-phenyl-3-buten-2-one were used as substrates for GST (David et al,. 1983; Wallin et al., 1988). 4-Nonyl-phenol, 3-carbinol, 17β-estradiol, TCDD, 3-methylcholanthrene and polycyclic

aromatic hydrocarbons were marked increased GST activity (Baars et al., 1978; Clapper et al., 1998; Gibson et al., 2001). Equine estrogens, bromosulphophthalein, tridiphane(2-(3,5-dichlorophenyl)-2(2,2,2-trichloroethyl)oxirane), ethanol have been shown to inhibit GST activity (Abel et al., 2004, 2007; Magdalou et al., 1987; Yeates et al., 1989).

NAD(P)H quinone oxidoreductase (NQOR) is a major enzyme of xenobiotic metabolism that carries out obligatory two-electron reductions and thereby protects cells against mutagenicity and carcinogenity resulting from free radicals and toxic oxygen metabolites generated by the one electron reduction (Iqbal et al., 2003). NQOR activity has been related to bioactivation and cytotoxicity of antitumor quinone. Quinoids compounds, naphthoquinone series, quinoneimines, and 2,6-dichlorophenol-indophenol using as substrates for NQOR. Estradiol, farnesol, diallyl sulfide, phenolic antioxidants, 1,2-dithiole-3-thiones, isothiocyanates, Sudan III and 3-methylcholanthrene induced the expression of NQOR genes (Horn et al., 2005; Munday et al., 1999; Primiano et al., 1997; Sanchez et al., 2003). Diethyl clofibrate, sulfhydryl reagents (*p*-chloromeribenzoate, *O*-iodosobenzoate); thyronine (thyroxine, 3,3',5-triiodothyronine, and tetraiodothyronoacetate.) caused significant reduction of NQOR activities (Mesia-Vela et al., 2004).

Many reports of herb-drug interactions are focused only on the induction of the drug metabolizing enzymes. However, knowledge about the recovery of the induced drug metabolizing enzymes is also important for the design of appropriate drug therapy. Although the field *in vitro* assessment of drug metabolism and drug interactions has progressed sufficiently to allow preparation, additional work will be required to allow a comprehensive characterization of drug metabolism *in vitro* (including induction and inhibition). If *C. comosa* causes inductive and/or inhibitory effects on CYPs and/or phase II drug metabolizing enzymes that play a key role in drug metabolism, this plant is characterized as one of the most important etiology of herb-drug interaction. In addition, if *C. comosa* causes inductive and/or inhibitory effects on CYPs and/or phase II drug metabolizing enzymes, this plant is potentially causes an increase or decrease risks of toxicity/mutagenesis/carcinogenesis from many environmental chemicals that are detoxified or activated by these enzymes. To date, effects of *C. comosa* hexane and ethanolic extracts on rat hepatic phase II drug metabolizing enzymes have never been investigated. Thus, the objective of this study

was to determine if *C. comosa* altered the activity of the major phase II drug metabolizing enzymes (UDPGT, SULT, GST and NQOR).

Hypothesis

C. comosa hexane and ethanolic extracts demonstrated an inductive/or inhibitory effects on hepatic phase II drug metabolizing enzymes in rat liver.

Study design and process

1. Animal treatment with *C. comosa* hexane and ethanolic extracts.

2. Preparation of liver microsomes and cytosols.

3. Verification the methods using to determine phase II drug metabolizing enzymes (UDPGT, SULT, GST and NQOR) activities.

4. Detemination of phase II drug metabolizing enzymes (UDPGT, SULT, GST and NQOR) activities.

5. Data analysis.

Anticipated benefits from the study

1. A preliminary data of *C. comosa* hexane and ethanolic extracts whether it possessed an inductive and/or inhibitory effects on major phase II drug metabolizing enzymes (UDPGT, SULT, GST and NQOR). This would be useful to estimate the possibility of *C. comosa* extracts to increase and/or decrease risks of chemical-induced toxicities, mutagenesis and/or carcinogenesis.

2. The possibility of drug-drug interaction if the extracts were taken simultaneously with other medicines metabolized by phase II drug metabolizing enzymes (UDPGT, SULT, GST and NQOR).

CHAPTER II

LITERATURE REVIEW

Curcuma comosa Roxb., a herb belonging to Zingiberaceae family, has been locally known in Thai as Waan chak mod look. Its rhizome has been used for centuries in Asia for the treatment of female abnormalities such as hot flashes during menopause, easing menstrual pain and cramping. *C. comosa* can reduce premenstrual syndrome, suppressed abdominal pain and chronic pelvic disorders, increases the effect of ovarian/testicular hormones. The herbs appears to have estrogenic effect. It is used traditionally as general tonic after childbirth, dysmenorrhea, amenorrhea, menorrhagia and menopausal symptoms. Thai traditional practitioner has used its rhizome in woman for alleviation of postpartum uterine pain, enhancement of uterine involution and for anti-inflammation of the uterus after delivery, also as an aromatic stomachics, cholalogues and choleretics. In male, it has been employed to relief pain in scrotal sac herniation (สมาคมแพทย์แผนไทย, 2520). A number of different active principles of *C. comosa*, include: 1. Curcuminoids (curcumin, desmethyl curcumin, bismethoxycurcumin); 2. Diarylheptanoids (*trans*1,7-diphenyl-5-hydroxy-1-heptene, *trans*-1,7-diphenyl-6-heptene-3-one-5-ol, *trans*1,7-diphenly-3-acetoxy-6-heptene, *trans*-1,7-diphenyl-6-heptene-3-one, *trans*,*trans*1,7-diphenyl-1,3-heptadiene-5-ol, *trans*,*trans*1,7-diphenyl-4,6-heptadien-3-one, 1,7-diphenyl-(*1E*,*3E*,*5E*)heptatriene, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene, 7-(3,4-dihydroxyphenyl-5-hydroxy-1-phenyl-(1*E*)-1-heptene; 3. Acetophenones: phloracetophenone, 4,6-dihydroxy-2-*O*-(*β*-D-glucopyranosly)acetophenone (Suksamran et al., 1994, 1997). Pharmacological effects and potential toxicities of *C. comosa* have been reported as following:

1. Choleretic effects

A phloracetophenone glucoside, 4,6-dihydroxy-2-*O*-(*β*-D-glucopyranosyl) acetophenone, isolated from the *C. comosa* ethyl acetate and butanol extracts, exhibited choleretic effect (Suksamrarn et al., 1997). THA at dose of 100 mg/kg induced an increase of bile flow rate and bile acid output. A considerable decreased secretion of cholesterol and phospholipids, and lowered bile lithogenic index

attributed to great choleretic activity with enhancement of biliary bile acid secretion. THA increased the excretion of both bile acids and cholesterol into the intestinal lumen for elimination. THA also increased hepatic cholesterol 7α-hydroxylase activity which increased hepatic conversion of cholesterol to bile acid. These results suggested that THA exerted its action by increasing hepatic conversion of cholesterol to bile acid and enhancing the excretion of bile acids (Piyachaturawat et al., 2002b). THA increased CYP7A1 activity in human HepG2 cells by stimulating mRNA transcription (Charoenteeraboon et al., 2005).

2. Estrogenic-like effects

C. comosa hexane extract was the most effective in increasing of uterine weight, glycogen content, the cornification of vaginal epithelium. Also, it promoted growth and induced keratinization of vaginal mucosa. (Piyachaturawat et al., 1995a, 1995b). In 1998, Piyachaturawat and collaborates demonstrated that intragastric administration of hexane extract at dose of 500 mg/kg/d for 7 consecutive days in immature male rats significantly suppressed weights for testes, epididymis, ventral prostate, seminal vesicle and levator ani muscle. Histological examination revealed regression of the spermatogonium in the seminiferous tubules and necrosis of epithelial cells in the epididymis. Concurrent treatment with either hCG (5 IU/day) and testosterone (1 mg/kg) did not fully abolish the suppressing effects of the extracts (Piyachaturawat et al., 1998).

3. Anti-inflammatory effect

C. Comosa hexane extract possed a strong anti-inflammatory activity in activated microglia (Jantaratnotai et al., 2005). *C. comosa* extracts (hexane or ethanol) at 10 μg/ml or diarylheptanoids 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene of *C. comosa* reduced tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) secretion through attenuating IKKβ expression and NF-κB activation (Sodsai et al., 2006).

Toxicological effects

Acute toxicity of THA was depend on species and route of administration. For subacute toxicity study, THA at high doses (150 and 300 mg/kg/day) significantly increased plasma levels of alanine and aspartate aminotransferase, bilirubin, blood urea nitrogen and hepatic triglyceride content (Piyachaturawat et al., 2002). Subchronic exposure of THA was shown to cause an increase of platelet number and gastritis found in few rats (Chivapat et al., 2003).

Phytoestrogens

Phytoestrogens are plant-derived chemicals that have estrogenic activity (Rice et al., 2006). Based on their chemical structure, phytoestrogens can be classified into four main groups: **1) Isoflavonoids** are natural phenolic compounds constitute the largest group of natural isoflavonoids. In this subclass, the most thoroughly investigated and interesting compounds are genistein, daidzein, formonetin, biochanin A and prunetin. Isoflavonoids can also be found in Leguminosae (soybeans in particular). **2) Flavonoids** are usually subdivided into flavonols, flavanols (catechins, epicatechins, gallocatechins, epigallocatechins), anthocyanidin, chalcones, flavanones and flavones. Flavonoids are constituents of fruits, vegetables, nuts, tea and wine (Galati et al., 2004). **3) Lignans** are dimeric compounds. They are usually subdivided into pinoresinol, isolariciresinol, 7-hydroxymatairesinol, lariciresinol, secoisolariciresinol and matairesinol. Lignans are found in high fiber foods such as cereal, beans and flaxseeds. **4) Coumestans** have been isolated from a few fodder and pasture plant (split peas, pinto beans, lima beans, alfafa and clover sprouts). Most recently, there has been increasing interest in stilbene and resveratrol. The major dietary sources are grapes, wine, hops, beer and peanuts (Basly et al., 2005; Rice et al., 2006). These compounds have several beneficial properties for these following areas:

1. cancer

Numerous animal and cell studies suggested that isoflavones (daidzein, genistein and lignans) may protect against cancer, particularly breast and prostate cancers (Pfeiffer et al., 2005). *In vitro* studies indicated that genistein inhibited growth of both estrogen receptor-negative and estrogen receptor-positive cell lines, and induced the activity of phase II detoxification enzymes, NQOR and GST. Regulation of detoxification enzymes genes may partially explain these protective effects.

Regulation can occur at the electrophilic response element through ERβ transactivation (Bianco et al., 2005). Enterolactones, genistein, biochanin A and coumestrol are capable of NQOR induction in colo205 cells by promoting NQOR mRNA expression (Atherton et al., 2006; Wang et al., 1998). Many mechanisms of cancer prevention actions have been identified for flavonoids (including flavonols, flavones, catechins, flavonones, anthocyanidins and isoflavones) such as antiestrogenic activity, antiproliferation, induction of cell-cycle arrest and apoptosis, prevention of oxidation, induction of detoxification enzymes, regulation of host immune system and changes in cellular signaling (Birt et al., 2001).

2. Menopause

Phytoestrogens have shown to alleviate the frequency and severity hot flushes, night sweat, vaginal dryness and climacteric complaints (Brezinski et al., 2002; Albertazzi et al., 1998; Krebs et al., 2004).

3. Cardiovascular diseases

Isoflavones have been shown to modulate risk factors for cardiovascular disease *via* estrogenic activity, which have been identified as nonsteroidal estrogens (Bayer et al., 2001; Manach et al., 2004; Lampe et al., 2007).

4. Diabetes

Dietary supplement with soy phytoestrogens demonstrated significantly lower mean values of fasting insulin, insulin resistant and HbA(1C). Isoflavones have antidiabetic properties such as inhibition of intestinal brush borders glucose uptake, α-glucosidase inhibitors actions and tyrosine kinase inhibitory properties (Jayapogal et al., 2002; Lee et al., 2001). Genisteine binds to a transactivater PPARγ (Anderson et al., 1995; Friedenwald et al., 1990).

5. Osteoporosis

Genistein-, daidazein- or equol-supplement diet at 10 mg/kg BW/day for 3 months indicated a preventive effect on bone mineral density decline (Coxam, 2003; Dang et al., 2005; Phrakonkham et al., 2007). Phytoestrogens appear to have potential promise for maintenance of bone mass. Furthermore, genistein itself has been shown

to elicit osteoprotective effect in postmenopausal women (Dalais et al., 1998; Alekel et al., 2000, Gredel et al., 2003).

6. Immune system

Isoflavones such as genistein and daidzein dose-dependently affect T-lymphocyte functions including proliferation, cytokine production and cytotoxicity against tumor cell lines. Lignans affect T-lymphocyte cell lines by inducing the apoptosis and inhibiting proliferation (Birt et al., 2001; Lampe, 2007). The isoflavones, genistein, daidzein, *O*-desmethylangolensin, equol and the lignans secoisolariciresinol, matairesinol, enterodiol and enterolactone decreased the production of all assayed cytokines (IFN-γ, IL-2, IL-4, TNF-α) with genistein and equol showing the strongest suppressive effects with regard to IL-2 and IL-4 production (Gredel et al., 2003).

7. Serum lipid

Secoisolariciresinol diglucoside was shown to reduce total serum cholesterol, LDL cholesterol without an influence on HDL or total triglycerides. Soy protein rich diet decreases plasma low density lipoprotein (LDL) concentration. Several studies demonstrated that peptides of soybean globulin up-regulated LDL receptors and exhibited an effect on cholesterol metabolism. Of special interest was the expression of transcriptional factors such as SREBPs, PPARs and LXRs genes from HepG2 cells (Hohlfeld et al., 2002). Otherwise, the results from some researchers indicated that the hypocholesterolemic effects of soy protein may function by influencing lipid metabolism through altering lipid-mediated gene expression (Kirk et al., 1998; Sirtori et al., 2001).

8. Antioxidant

Quercetin, luteolin and genistein have been shown to inhibit oxidative DNA damage by the scavenging superoxide anion, singlet oxygen and lipid peroxyl-radicals. Secoisolariciresinol, matairesinol, hydroxyl-matairesinol and lariciresinol had high potency to inhibit lipid peroxidation. In addition, they could scavenge supeoxides and peroxyl radicals. Flavonoids, stilbenes, lignans and lignins are dietary plants with high content of total antioxidants. These phytoestrogens can protect cells

from free radicals and other toxic electrophiles by induce detoxification enzymes such as catalase, several types of GST, Glutathione peroxidase, Superoxide dismutase, γ-Glutamyl cysteine synthetase and NQOR (Blomhoff et al., 2006; Wang et al., 1998).

Effect of phytoestrogen on xenobiotic metabolizing enzymes

Some flavonoids such as chrysin, apigenin, luteolin, kampherol, quercetin, myricetin and naringenin have high potencies and selectivities for inhibition of CYP1A isoforms (Birt et al., 2001). 1% Arctiin induces CYP1A2 and 2B1 in rat liver (Hirose et al., 2000). Resveratrol modulates constitutive CYP1A1 and CYP1B1. Daidzein and genistein have no significant effect on CYP1A1, 1A2, 1B1 and 2E1 (Atherton et al., 2006). Lignans have been shown to induce CYP3A4 (Jacobs et al., 2005). Potent inhibition of CYP19 occurred with flavanones such as naringenin, hesperetin, erodictyol and naringin (Basly et al., 2005). Daidzein, formononetin, equol and 3'-hydroxy-daidzein can stimulate UDPGT1A1 but did not induce UDPGT1A6, 1A9 and 2B7 (Walle et al., 2002; Pfeiffer et al., 2005). Genistein stimulated UDPGT1A10 mRNA levels (Starlard-Davenport et al., 2007). The isoflavonoid biochanin A has been shown to induce UDPGT2B15, a testosterone-glucuronidating isoform, in prostate cancer cell (Galijatovic et al., 2000). UDPGT1A1 was induced in response to flavonoids and xenobiotics through the transactivation of 290-bp reporter gene in the UGT1A1-5'-flanking region, that was a multi-component enhancer containing constitutive androsterone receptor (CAR), pregnan X receptor (PXR) and AhR motifs (Sugatani et al., 2004).

Estrogen sulfotransferase (SULT1E1) was inhibited competitively by quercetin and resveratrol with quercetin being the most potent phytoestrogens displaying low hPXR activation (Nishiyama et al., 2002; Jacobs et al., 2005; Rice et al., 2006; Manach et al., 2004). Genistein, daidzein, quercetin and luteolin inhibited SULT1A1 activity (Basly et al., 2005). Some flavonoids, such as chrysin, apigenin, luteolin, kampherol, quercetin, myricetin and naringenin induced GST activity. *In vitro* studies using cultured human breast cancer cells indicate that genistein induced the activity of GST. The synthetic flavonoids (4'-bromoflavone) was the most potent *in vivo* inducer of GSH synthesis enzymes. This result indicated that metabolic conjugation pathways involving GSH may play an important role in protection rat liver against carcinogenesis (Galati et al., 2004).

Quercetin induces NQOR activity (Birt et al., 2001). Daidzein up-regulated NQOR mRNA and GST pi gene (Lin et al., 2007). The order of effectiveness of flavonoids found for inducing NQOR was galangin and kaempferol> quercetin>myricetin and apigenin, with epicatechin, catechin and taxiffolin being inactive (Galati et al., 2004; Rowlands et al., 1988). Genistein, biochanin A and resveratrol induce the expression of NQOR genes. Binding of these phytoestrogens to ERβ induces transcription of NQOR genes. This regulation can occur at the EpRE (Electrophile response element) of the NQOR promoter through ERβ transactivation. The binding of ERβ to EpRE may be involve ER co-activators, such as SRC-1 (steroid receptor coactivator-1) or GRIP1 (glucocorticoid-interacting receptor protein 1) that regulate transcription (Bianco et al., 2005). A summary of flavonoid-induced enzyme induction or inhibition is given in table 1

Table 1 Summary of flavonoid-induced enzyme induction or inhibition (↑ induction, ↓ inhibition, * in vivo study)

Phase I	Flavonoids	Effects
CYP1A1	Quercetin	↑mRNA expression and activity
	Kaempferol	↓TCDD or B[a]P-induced transcription
	Galangin	↓Activity
		↑mRNA expression
		↓TCDD or DMBA-induced transcription
	Green tea extracts	↑Activity*
	Theaflavins	↓Activity in the intestine*
	Xanthohumol	↓Activity
	Baicalein	↓DMBA-induced transcription
		↓Activity
	Dimethoxyflavone	↓Activity
		↓B[a]P-induced mRNA and protein expression, and Activi
	Chrysin	↑Activity
	Diosmin	↑mRNA expression
	Diosmetin	↑mRNA expression
		↓DMBA-induced transcription
		↓Activity
	Acacetin	↓Activity
	Flavone	↓Activity
	Hesperetin	↓Activity
	Biochanin A	↓DMBA-induced transcription and activity
	Genistein	↓DMBA-induced transcription and activity
	2′-Hydroxychalcone	↓mRNA expression and activity
CYP1A2	Quercetin	↓Activity
	Biapigenin	↓Activity
	Galangin	↓Activity
	Green tea extracts	↓TPA-induced transcription
		↑Activity*
	8-Prenylnaringenin	↓Activity
	Isoxanthohumol	↓Activity
	Diosmetin	↓Activity
	Acacetin	↓Activity
	Flavone	↓Activity
	Tangeretin	↓Activity
	Naringin	↓Activity*
	Genistein	↓Activity*
		↓Activity*
	Equol	↓Activity*
	Diadzein	↓Activity*
CYP1B1	Xanthohumol	↓Activity
	Baicalein	↓DMBA-induced transcription
	Diosmetin	↓Activity
	Acacetin	↓Activity
	Hesperetin	↓Activity
	Biochanin A	↓DMBA-induced transcription and activity
	Genistein	↓DMBA-induced transcription and activity
	Diadzein	↓Activity
	Formononetin	↓Activity
	2′-hydroxychalcone	↓mRNA expression and activity
CYP2E1	Genistein	↓Activity*
	Equol	↓Activity*
	Theaflavins	↓Protein level*
	Silybin (=Silibinin, Silybinin)	↓Activity
CYP3A4	Naringenin	↓Activity*
	Hyperforin	↓Activity
		↑mRNA expression
		↑Activity*
	Biapigenin	↓Activity
	Quercetin	↓Activity
	Silymarin	↓Activity
	Silybin	↓Activity (at high conc.)

Table 1 (con't) Summary of flavonoid-induced enzyme induction or inhibition
(↑induction, ↓inhibition, * in vivo study)

Phase I	Flavonoids	Effects
		↑Activity (at low conc.)
	Genistein	↓Activity
	Diadzein	↓Activity
	Glabridin	↓Activity
	Myricetin	↓Activity
CYP19	Biochanin A	↓Activity
		↓Activity
		↓Activity
	Formononetin	↓Activity
	Equol	↓Activity
	Chrysin	↓Activity
		↓Activity
		↓Activity
		↓Activity*
	Naringenin	↓Activity
		↓Activity
		↓Activity
	Flavone	↓Activity
	Apigenin	↓Activity
	Bicalein	↓Activity
	Galangin	↓Activity
Phase II		
UGT1A1	Chrysin	↑mRNA expression
		↑activity
	Galangin	↑mRNA expression
	Isorhamnetin	↑mRNA expression
	Tangeretin	↓Activity
	Naringenin	↓Activity
	Flavone	↓Activity
	Quercetin	↓Activity
UGT	Flavone	↑Activity*
		↑Activity*
		↑Activity*
	Quercetin	↑Activity*
		↑Activity
	Green tea	↑Activity*
		↑Activity*
	Biochanin A	↑Activity
	Formononetin	↑Activity
	Genistein	↑Activity
	Diadzein	↑Activity
	Naringenin	↑Activity
	Galangin	↑Activity
	Kaempferol	↑Activity
GST	Genistein	↑or ↓mRNA expression (cell type specific)
		↑Activity*
	Diadzein	↑Activity*
	Genistein + Diadzein	Prevent TPA-downregulated Activity*
	Flavone	↑Activity*
	Green tea extract	↑Activity*
	Morin	↑Activity*
		↑Activity*
	Silymarin	↑Activity*
GSTA2	Flavone	↑mRNA expression
	2'-amino-3' methoxyflavone	↑mRNA expression
GSTP1-1	Quercetin	↓Activity
QR	Tephropurpurin	↑Activity
	Xanthohumol	↑Activity

Table 1 (con't) Summary of flavonoid-induced enzyme induction or inhibition (↑ induction, ↓ inhibition, * in vivo study)

Phase II	Flavonoids	Effects
	Morin	↑Activity*
		↑Activity*
	Pinostrobin	↑Activity
	Kaempferol	↑Activity
	Galangin	↑Activity
	Quercetin	↑Activity
	Silymarin	↑Activity*
	Genistein	↑Activity
		↑Activity and n
		↑Activity*
	Diadzein	↑Activity
		↑Activity*
	Flavone	↑Activity*
SULT1A1 (P-PST or TS-PST)	Fisetin	↓Activity
	Galangin	↓Activity
	Quercetin	↓Activity
		↓Activity
		↓Activity
	Catechin	↓Activity
	Equol	↓Activity
	Flavone	↓Activity
	Myricetin	↓Activity
	Kaempferol	↓Activity
	Chrysin	↓Activity
	Apigenin	↓Activity
	Genistein	↓Activity
		↓Activity
		↓Activity
	Diadzein	↓Activity
		↓Activity
SULT1A3 SULT1E1	Baicalein	↓Activity
	Equol	↓Activity
	Quercetin	↓Activity
		↓Activity
	Genistein	↓Activity
	Diadzein	↓Activity
		↓Activity

Effect of estrogen on xenobiotic metabolizing enzymes

Estradiol elevated CYP1A1 expression through the hepatic estrogen receptor (ER) where the ER-E_2 complex can interfere with the CYP1A1 gene directly or alternatively may interact with AhR from cross-talk, and indirectly regulate CYP1A1 gene expression through XRE (Carrera et al., 2007; Mortensen et al., 2007; Kabayashi et al., 1999; Leung et al., 2007). *In vitro* and *in vivo* studies have shown that estrogenics components are inducers of CYP2C9 and CYP3A4 (Giersbergen et al., 2004). Chronic treatment with 1 or 3 mg of 17β-estradiol (E_2) did not alter NADPH-

dependent (CYP450) oxidation. Modulation by E_2 has been reported for CYP2C7 and CYP3A9 (Sanchez et al., 2003).

Estradiol regulation of the glucuronidation enzyme and mRNA levels of UDPGT2B15 of ERα breast cancer cell lines (MCF-7, BT-474, T47D and ZR75) in a time- and dose-dependent manner (Harrington et al., 2006). Treatment of the MCF-7 (ERα human breast cancer cell line) with estradiol (0.1-1 nM) procedures a dose-dependent up-regulation of UDPGT1A10 mRNA levels, but estradiol did not stimulate mRNA expression of ERβ breast cancer cell line. ERα can also affect UDPGT1A10 gene expression by binding to silencer elements in the promoter regions of estrogen target gene (Starlard-Davenport et al., 2007)

Sulfotransferase, SULT1A1 was not significantly affected in the livers of August-Copenhagen Irish rat following chronic treatment of estradiol (Sanchez et al., 2003). The estrogen sulfotransferase, SULT1E1 displayed much higher activity towards estrone and 17β-estradiol. The increasing effect of SULT1E1 activity in human MCF-7 breast cancer cell line is associated with decreased expression of ERα (Charles et al., 2007).

Exposure to natural estrogen (E_2) did not affect GST-A mRNA expression, although GST catalytic activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was elevated (Ansell et al., 2004). NQOR and GST activities were increased in liver cytosol of August-Copenhagen Irish (ACI) rats treated with E_2 for 6 weeks. The induction of NQOR and GST activities suggest that under physiological conditions, E_2 may protect against oxidative stress *via* elevation of these antioxidant enzymes (Sanchez et al., 2003). In tissue culture, treatment with estradiol for 8-9 months results in an increased expression of a μ-class glutathione *S*-transferase (hGSTYBX) (Hudson et al., 1998).

Xenobiotic biotransformation

Biotransformation is the process whereby a substance is changed from one chemical to another by a chemical reaction within the body. Metabolism or metabolic transformations are terms frequently used for biotransformation process. Metabolism of drugs and other foreign compounds (xenobiotics) can also occurs to some extent in all body tissues, but mostly in liver, lung, intestinal tract, kidney and skin (Goshman et al., 1999). The major organ for drug biotransformation is the liver. The metabolic

products are often less active than the parent drug or inactive. However, some biotransformation products (metabolites) may have enhanced activity or toxic effects. Formation of reactive metabolic intermediates is one of the causes for drug toxicity. The routes by which drugs may be metabolized include oxidation, reduction, hydrolysis, hydration, conjugation and condensation reactions. Drug metabolism is normally divided into two phases: phase I (or functionalisation reactions) acts as addition of a functional polar group. This typically results in a relatively small increase in hydrophililicity and may cause metabolic activation. Phase II (or conjugative reactions) are the true “detoxification” pathways, conjugation with a small hydrophilic endogenous substance-often, but not always, to a functional group provided by a phase I reaction-thereby significantly increasing hydrophilicity and facilitating excretion. A summary of metabolic reactions is given in table 2.

Table 2 The summary reactions in the biotransformation of xenobiotics (Gibson et al., 2001; Loannides., 1996)

Reactions	Enzymes	Localization	Substrates
Phase I Reactions: Transformations			
Oxidation	Mixed-function oxidase	Microsomes	Alkanes, alkenes, arenas, amines, thiones, thioethers
	Monoamine oxidases	Mitochondria	Amines
	Alcohol dehydrogenases	Cytosol	Alcohols
	Aldehyde dehydrogenases	Cytosol	Aldehydes
Reduction	Mixed-function oxidases	Microsomes	Azo and nitro group , *N*-oxides, arene oxides, alkyl halogenides
	Alcohol dehydrogenases	Cytosol	Aldehydes, ketones
Hydrolysis	Esterases	Cytosol Mitochondria Microsomes	Esters

Table 2 (con't) The summary reactions in the biotransformation of xenobiotics (Gibson et al., 2001; Loannides., 1996)

Reactions	Enzymes	Localization	Substrates
Phase II Reactions: Conjugations with:			
H_2O	Epoxide hydrolase	Microsomes Cytosol	Epoxides
Glutathione (GSH)[a]	Glutathione transferase	Microsomes Cytosol	Electrophiles
Glucuronic acid (UDPGA) [a]	UDP-glucuronosyltrans-ferase	Microsomes	Phenols, thiols, amines carboxylic acids
Sulfuric acid (SAM) [a]	Sulfotransferase	Cytosol	Phenols, thiols, amines
Methyl group	*N*- and *O*-methyl transferase	Microsomes Cytosol	Phenols, amines
Acetic acid (Acetyl-CoA)	*N*-acetyltransferase	Cytosol	Amines
Oxidation (NADH, NADPH)	NQOR	Cytosol	Quinoid compounds

[a] Abbreviations in brackets are co-substrates. UDPGA = uridine-3',5'-diphospho glucuronic acid; SAM = *S*-adenoslymethionine; CoA= coenzyme A

Phase I metabolisms

Phase I metabolisms includes oxidation, reduction, hydrolysis, hydration and isomerisations (Gibson et al., 2001). One of the major enzyme systems that determines the organism's capability of dealing with drugs and chemicals is represented by the cytochrome P450 monooxygenase (CYP450). The intracellular localization of phase I CYP450 is within smooth endoplasmic reticulum (SER). The CYP450 enzymes are now referred to as heme-thiolate proteins. In humans, about 40

different CYPs are present and these play critical roles by catalyzing reactions in: (a) the metabolism of drugs, environmental pollutants and other xenobiotics; (b) the biosynthesis of steroid hormones; (c) the oxidation of unsaturated fatty acids to intracellular messengers; and (d) the stereo- and region-specific metabolism of fat-soluble vitamins (Hasler et al., 1999).

Phase II metabolisms

The major phase II reactions are: glucuronide conjugation, sulfate conjugation, acetylation, amino acid conjugation, glutathione conjugation and methylation.

Glucuronidation

Glucuronidation represents the major route of sugar conjugation, UDP-glucuronosyltransferase enzymes (UDPGT) catalyze the linkage of glucuronic acid (derived from the co-enzyme UDPGA) with endobiotic (e.g. steroids and bilirubin) and drug substrates containing hydroxyl, amine, sulfhydryl and carboxylic acid functional groups. UDPGT can directly conjugate drugs without the prerequisite of phase I metabolism (Patten., 2006). The reaction that proceeds to a glucuronide conjugation is:

$$\text{R-OH} + \text{UDPGA} \longrightarrow \text{R-OGA} + \text{UDP}$$

Where R-OH is a xenobiotic or endobiotic and UDPGA is UDP-glucuronic acid. This reaction is catalysed by a family of enzymes, UDPGTs, which have evolved discrete specificities in response to the need to eliminate potentially toxic endobiotic and environmentally xenobiotic compounds. These ubiquitous microsomal enzymes are present principally in the liver, but also occur in a variety of extrahepatic tissues. Their location in the endoplasmic reticulum has important physiological effects in the neutralization of reactive intermediates generated by the CYP450 system and incontrolling the levels of reactive metabolites present in these tissues. There are more than 50 known microsomal membrane-bound isoenzymes in humans found in liver, lung, skin, intestinal, brain and olfactory epithelium. However the major site of glucuronidation is the liver. *O*-Glucuronides are formed from phenols, alcohols and carboxylic acids, carboxylic acids forming ‘ester’ glucuronides and the other ‘ether’ glucuronides e.g. glucuronidation of morphine, chloramphenicol and salicylic acid.

The *O*-glucuronides are often excreted in bile and thus released into the gut where they can be broken down to the parent compound by β-glucuronidase and possibly reabsorbed. An interesting example of alcohols is given by codeine, which following demethylation to morphine can undergo glucuronidation either at the phenolic, or at the secondary alcohol group, with concomitant formation of two distinct metabolites (morphine-3-glucuronide and morphine-6-glucuronide) with different pharmacological activities. A very recent study concludes that both compounds display opiate agonistic behaviour. Another major pathway of *O*-glucuronidation is represented by the formation of acylglucuronides, the ideal substrates for this alternative pathway being aliphatic and aromatic acids (acidic drugs, such as NSAIDs). Common substrates undergoing glucuronide conjugation are hydroxylamines and hydroxylamides. A recent example in the former category refers to the identification of and *O*-glucuronidation metabolite of an aryl piperazine and oral hypoglycaemic agent. A relatively small number of aromatic amines are first *N*-hydroxylated and then undergo *O*-glucuronidation. The reactively of the resulting *N*-*O*-glucuronides and their potential for hydrolytic cleavage with subsequent formation of nitrenium ions. *N*-glucuronidations are considered to be of secondary importance of which substrates undergoing this type of reaction are amines (mainly aromatic), amides, carboxyamides, sulfonamides, as well as different types of amines. It has also been suggested that tertiary amines can form glucuronide giving quarternary nitrogen conjugates. *N*-glucuronidation of aliphatic and aromatic amines as well as of some compounds with pyrimidynic structure has also been mentioned. Special significance was attributed to *N*-glucuronidation of lipophilic, basic tertiary amines, containing one or two methyl group in their structure. *N*-glucuronides may form spontaneously, i.e. without the presence of the enzymes (Coleman et al., 2005). *S*-Glucuronidation, thiol groups can react with the UDPGA in the presence of UDPGT to yield *S*-glucuronides, an example is the glucuronidation of the antabuse. Direct attachment of glucuronic acid to the carbon skeleton of drugs e.g. phenylbutazone has also been reported (*C*-glucuronidation) (Gibson et al., 2001; Nishiyama et al., 2006). This superfamily is divided into two families named UDPGT1 and UDPGT2 which is divided again into two subfamilies, UDPGT2A and UDPGT2B. UDPGT1 isozymes are implicated in the glucuronidation of bilirubin and small planar phenol compounds such as 4-nitrophenol. UDPGT2 isozymes are responsible for the glucuronidation of steroids,

bile acids, retinoids and numerous xenobiotics (Gueraud et al., 1998). To date, at least 16 human UDPGT enzymes have cloned and sequenced, and their cDNAs heterologously expressed in a cell system to demonstrate substrate specificity. UDPGT isoforms have been identified in a wide variety of human tissues, for example liver, small and large intestine, kidney, stomach and esophagus. As expected, the majority of the UDPGT isoforms are expressed in the liver at high levels. The known UDPGT isoforms is UDPGTs 1A1, 1A2, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2A1, 2B4, 2B7, 2B10, 2B15, 2B17 and 2B28. UDPGT multiple forms exhibit a quite pronounced tissue-specific pattern in man. For example UDPGTs 1A1, 1A3, 1A4, 1A6, 2B4, 2B7, 2B10, 2B11 and 2B15 are all expressed in human liver, thus providing the liver with a substantial capacity to glucuronidate a wide range of endogenous and xenobiotic compounds. UDPGT1A10 is expressed in many extrahepatic organs, an important observation in view of the fact that this form exhibits one of the widest ranges of substrate specificities of known UDPGTs, ranging from small phenolic compounds to large steroids. In addition, UDPGT1A10 is expressed in all regions of gastrointestinal tract, thus providing a first line of defence for the elimination of orally administered xenobiotics which have the capability to be directly glucuronidated. Bilirubin, morphine, estradiol (3-OH) and all-*trans*-retinoic acid are specific substrates for UDPGT1A1. Ketoprofen, Ibuprofen, fenoprofen, naproxen, ciprofibrate, clofibrate, valproic acid and morphine are substrates for UDPGT1A3. Androgens, naproxen, morphine, ciprofibrate, clofibrate, valproate, furosemide, difunisal and all-*trans*-retinoic acid are substrates for UDPGT1A6. Fenoprofen, furosemide, ibuprofen, ketoprofen, mefenamic acid, 4-catechol estroges, estradiol and estriol are substrates for UDPGT1A9. NSAIDs (ibuprofen, ketoprofen, naproxen, fenoprofen zomepirac, diflunisal), clofibric acid, valproic acid and estriol are substrates for UDPGT2B7. Dihydrotestosterone is substrate for UDPGT2B15 and C19 steroids (androsterone, dihydrotestosterone, testosterone) are substrates for UDPGT2B17 (Ritter., 2000)

In colon carcinoma Caco-2 cells UDPGT1A6 is found to be TCDD-inducible and thyroid hormone is shown to increase effect on UDPGT1A6 mRNA expression. Substrates, inducers and inhibitors of UDPGT are shown in table 3. Specific substrates are shown in table 4. In some cases, however, it is possible that these enzymes produce compounds with more potent activity than parent analog, such as morphine-6-glucuronide. Conjugates may also be immunoreactive, teratogenic or

carcinogenic; for example ketoprofen acylglucuronide, all-*trans*-retinoly-*β*-D-glucuronide and glucuronides of *N*-hydroxy arylamines, respectively (Smith et al., 1999; Collier et al., 2000)

Table 3 Substrates, inducers and inhibitors of UDPGT (Thomassin, 1985; Iwamura et al., 2005)

Enzyme	Substrates (for all UDPGT)	Inhibitors (for all UDPGT)	Inducers (for all UDPGT)
UDPGT	bilirubin, estradiol (3-OH), all-*trans*-retinoic acid, morphine (3-OH) , naproxen, NSAIDs (ibuprofen, ketoprofen, naproxen, pirprofen diflunisal), valproic acid, clofibric acid, bezafibrate, ciprofibrate, 17β-estradiol, 4-hydroxycoumarin, 7-hydroxycoumarin, eugenol, 4-methylumbelliferone, 2-naphthol, 3-methoxy phenol, carveol, pentazocine	triiodothyronine, 4-nitrophenol, 2-aminophenol, androsterone, *p*-nitrophenol, phenolphthalein, *p*-nitrophenol, benzo(*a*)pyrene-7,8-dihydrodiol-9,10-oxide, lanasol, lobenzarit	polychlorinated, biphenyls, polychlorinated dioxins, hypolipidemic drugs (peroxisome proliferators), phenolic antioxidants (BHA), barbiturates, thiocarbamates, 1,2-dithiol-3-thiones, isothiocyanates, *β*-naphthoflavone, phenobarbital, 3-methylcholanthrene, androgens, TCDD, bilirubin, peroxisome proliferators, perfluorodecanoic, clofibric acid, oltipraz, dexamethasone, DAS (dially sulfide), pyraxol

Table 4 Specific substrates of UDPGT (Coleman et al., 2005; Kiang et al., 2005)

Enzyme	Specific substrates
UDPGT1A1	acetaminophen, buprenorphine, carvedilol (glucuronidation 1), carvedilol (glucuronidation 2), ethinylestradiol, entacapone (3-*O*-glucuronidation), entacapone (glucuronidation 1), 7-ethyl-10-hydroxycamptothecin, etoposide, ezetimibe, *cis*-hydroxy-tamoxifen, trans-hydroxy-tamoxifen, morphine (3-*O*-glucuronidation), mycophenolic acid, nalorphine, naltrexone, retigabine, thiocoraline, troglitazone, bilirubin, estrogenic steroids only (not androgens or any other steroid molecules) such as 17α-ethinylestradiol, 2-hydroxyestradiol, benzopyrenes
UDPGT1A2	bilirubin
UDPGT1A3	amitriptyline, buprenorphine, ciprofibrate, clonixin, clofibrate, clozapine, cyproheptadine, diclofenac, ezetimibe, fenoprofen, flurbiprofen, ibuprofen, imipramine, ketoprofen, (*R*)-ketotifen, (*S*)-ketotifen, morphine, morphine (3-*O*-glucuronidation), naproxen, norbuprenorphine, pitavastatin, valproic acid, tertiary amines, flavonoids, phenolic compounds
UDPGT1A4	amitriptyline, chlorpromazine, clozapine, cotinine, *c*-posaconazole, cyproheptadine, diphenhydramine, doxepin, 7-ethyl-10-hydroxycamptothecin *cis*-hydroxy-tamoxifen, *trans*-hydroxy-tamoxifen, imipramine, (*R*)-ketotifen, trifluroperazene, aromatic amines, tricyclic antidepressants such as imipramine, (*S*)-ketotifen, nicotine, olanzapine, retigabine
UDPGT1A6	acetaminophen, 7-ethyl-10-hydroxycamptothecin, morphine (3-*O*- glucuronidation), 1-naphthol
UDPGT1A7	7-ethyl-10-hydroxycamptothecin, mycophenolic acid, endogenous lignds
UDPGT1A8	morphine (3-*O*-glucuronidation), mycophenolic acid, naloxone, naltrexone, niflumic acid, propofal, raloxifene (4-glucuronidation), raloxifene (6-glucuronidation), small and bulky phenolic chemicals
UDPGT1A9	acetaminophen, almokalant, cotinine, diclofenac, 5,6-dimethylxanthenone-4-acetic acid, entacapone, 7-ethyl-10-hydroxycamptothecin, flavopiridol, flurbiprofen, gemfibrazole, *cis*-hydroxy-tamoxifen, *trans*-hydroxy-tamoxifen, ibuprofen, ketoprofen, morphine (3-*O*-glucuronidation), mycophenolic acid, nicotine, niflumic acid, (*R*)-oxazepam, propofol, raloxifene (4-glucuronidation), raloxifene (6-glucuronidation), valproic acid, coumarins, flavones, amines, propofol

Table 4 (con't) Specific substrates of UDPGT (Kiang et al., 2005)

Enzyme	Specific substrates
UDPGT1A10	mycophenolic acid, morphine (3-*O*-glucuronidation), niflumic acid, raloxifene (4-glucuronidation), troglitazone, small and bulky aglycone
UDPGT2B4	carvedilol (glucuronidation 1), carvedilol (glucuronidation 2)
UDPGT2B7	almokalant, buprenorphine, benoxprofen, carvedilol (glucuronidation 1), carvedilol (glucuronidation 2), carbamazepine, clofibric acid, clonixin, codeine, cyclosporine A, diclofenac, diflunisal, 5,6-dimethylxanthenone-4-acetic acid, epirubicin, ezetimibe, fenoprofen, flurbiprofen, gemfibrozil, 3-hydroxyrofecoxib, ibuprofen, indomethacin, ketoprofen, maxipost, morphine (3-*O*-glucuronidation), morphine (6-*O*-glucuronidation), naltrexone, nalorphine, naproxen, (*S*)-oxazepam, (*R*)-oxazepam, pitavastatin, tacrolimus, tiaprofenic acid, valproic acid, zaltoprofen, zomepirac, zidovudine, catechol estrogens, 4-hydroxyestrone
UDPGT2B15	androstanediol, entacapone, 7-ethyl-10-hydroxycamptothecin, 3-hydroxyrofecoxib, *cis*-hydroxy-tamoxifen, *trans*-hydroxy-tamoxifen, (*S*)-oxazepam, tolcapone, phenols, dihydrotestosterone, 5α-androsterone, 3α-androsterone, 17β-diol, 8-hydroxyquinoline
UDPGT2B17	ibuprofen, C-19 steroids (androsterone, dihydrotestosterone, testosterone)
UDPGT2B28	17β-estradiol, testosterone

Sulfation

Sulfation is a major conjugation pathway for phenols, but also contributes to the biotransformation of alcohols, amines and to a lesser extent, thiols. It is also relevant in the metabolism of endogenous compounds such as catecholamine, neurotransmitter, steroid hormones, thyroxine and bile acids. Dopamine, epinephrine, progesterone and DHEA are some of the endogenous mediators that may be sulfated (Mcfadden, 1996). Moreover, the tyrosinyl group of peptides and proteins may represent sites of sulfation resulting in possible changes in their properties. The resulting compounds are generally less active and more polar, thus more readily excreted in the urine. Sulfate conjugation is a multiplestep process, comprising activation of inorganic sulfate first, by converting it *via* ATP to adenosine-5'-phosphosulfate (APS), and further to the activated form, known as PAPS, 3'-phosphoadenosine-5'-phosphosulfate, as shown in equations. Each step involves a specific enzyme, present in cytosol.

$$ATP + SO_4^{2-} \xrightarrow{\text{ATP-sulfurase}} APS + PPi$$

$$APS + ATP \xrightarrow{\text{ATP-phosphokinase}} PAPS + ADP$$

The reaction by which sulfotransferase catalyses the transfer of a sulfuryl group from PAPS to an acceptor molecule is shown in the following reaction:

$$R\text{-}OH + PAPS \xrightarrow{\text{Sulfotransferase}} R\text{-}OSO_3H + PAP$$

The availability of PAPS and its precursor inorganic sulfate strongly determine the rate of reaction. In humans, sulfotransferase are found in the liver, small intestine, brain, kidneys and platelets. Two forms of sulfotransferase are known to exist, namely a “thermolabile” (TL) form, responsible for the sulfation of dopamine (and other catecholamines) and a “thermostable” (TS) form, which catalyses the sulfation of a variety of phenolic compounds. The total pool of sulfate in the body is normally limited and can be easily exhausted. Thus, with increasing doses of a drug, sulfate conjugation will become a less significant pathway. For a competing substrate, at high doses glucuronidation usually predominates over sulfation, which instead prevails at low substrate doses due to the kinetics of the two reaction and the limited supply of PAPS in the cell compared to UDPGA (Gibson et al., 2001). Sulfate conjugation is most common for phenols and to a lesser extent for alcohols, yielding highly ionic and polar sulfates, metabolites that are readily excreted in the urine. Different drug sulfate conjugates are excreted mostly in urine, but in the case of steroids, biliary elimination is more prevalent. However, in the small intestine, through mediation of certain sulfatases, the parent drug or its metabolites may be reabsorbed into the portal circulation. After oral administration of a drug, the intestine represents an important site of sulfation. For drug whose primary metabolic pathway is sulfation, the result is a pre-systemic first pass effect, which decreases the bioavailability. Some drugs in this category are acetaminophen, steroid hormones, α-methyldopa, isoproterenol and albuterol. Various forms of the enzyme have been described named after their preferred substrates although classification by molecular and gene structure is becoming more common. The phenol, alcohol and arylamine

sulfotransferase are fairly non-specific and will metabolise a wide range of drugs and xenobiotics but the steroid sulfotransferases are specific for a single steroid or a number of steroids of a particular type. For example, estrone sulfotransferase will sulfate estrone and to a lesser extent, other estrogens while testosterone is sulfated by another sulfotransferase. The sulfotransferase exist in multiple enzyme forms and at least 45 SULTs have been identified in mammals with at least 11 genes in man, the latter being dispersed on five separate chromosomes. The mammalian SULTs are classified into gene families (SULTs 1-5), with families being differentiated from each other by having less than 45% amino acid sequence similarity to the other families. The families are further sub-divided into sub-families (45-60% similar) and individual genes being uniquely identified by and Arabic number (e.g. SULT1A1). Mammalian SULTs fall into the SULT1 and SULT2 families, a SULT3 family has recently been identified in DNA sequence data bases, although the enzymes have not been characterized to date. A single member of the SULT6 family has been found in chickens. SULT1 has five sub-families (A-E) and SULT2 (A, B) (Gibson et al., 2001). SULT family is the major group of human sulfotransferase, which contains four subfamilies (1A, 1B, 1C and 1E) and considerable number of isoforms, including 1As (not 1A3), 1A3, 1B1, 1C1, 1C2, 1D1 and the estrogen sulfotransferase 1E1. SULT 1A3 (not found in the liver) as well as 1E1 are potent sulfators of xenobiotic estrogens and are inhibited by estrone and quercetin. However, the most important isoform from the xenobiotic metabolism point of view is the phenol and aryl amine sulfating sulfotransferase SULT1A1. SULT2 family, these enzymes are hydroxysteroid sulfotransferase and there are only two subfamilies, SULT2A and SULT2B, with SULT2A1 and SULT2B1 being the best understood. There are a large number of individual enzymes which sulfonate a variety of sex hormones and hydroxysteroids. SULT1B1 metabolized sex steroids and predictably is found in the placenta, uterus and prostate and is thought to be heavily involved in the regulation of androgen levels (Chen, 2004; Coleman et al., 2005; Falany et al., 2006). Each SULT has distinct substrate preferences, but may also exhibit broad and overlapping substrate specificity to span the diversity of chemicals requiring sulfonation. For example, SULT1A3 catalyses dopamine sulfonation but also accepts *p*-nitrophenol with lower affinity. SULT1A1, with shares 93% identity with SULT1A3, also sulfonates dopamine and *p*-nitrophenol but the reverse preference, and in addition can sulfonate a wide range of hydrophobic molecules including xenobiotic and

endogenous substrates 3,3'-di-iodothyronin and 17β-estradiol (Gamage et al., 2003). Substrates, inducers and inhibitors of SULT are shown in table 5. Specific substrates are shown in table 6.

In contrast, *N*-sulfates, analogous to *N*-glucuronides, are able to promote cytotoxicity by facilitation the formation of reactive electrophilic intermediates. Sulfation of *N*-oxygenated aromatic amines is an activation process for some arylamines that can eliminate the sulfate to an electrophilic species capable of reacting with proteins or DNA. The metabolic activation of the carcinogenic arylmethanols, such as 7-hydroxymethylchrysene was achieved with the major liver hydroxysteroid sulfotransferase (Ogura et al., 1994). 1-Hydroxymethylpyrene is activated to a potent mutagen by hydroxysteroid sulfotransferase in the presence of hepatic cytosol (Glatt et al., 1994). The industrial solvent 2-nitropropane (2-NP) is genotoxic hepatocarcinogen in rats. The genotoxicity has been attributed to sulfotransferase-mediated formation of DNA-reactive nitrenium ions from the anionic ion form of 2-NP, propane 2-nitronate (Kreis et al., 2000).

Table 5 Substrates, inducers and inhibitors of SULT (Allera et al., 2004; Wang et al., 2006)

Enzyme	Substrates	Inhibitors (for all SULT)	Inducers (for all SULT)
SULT	3,3'-di-iodothyronin, 17β-estradiol, 2-naphthol, 4-phenylphenol, 4-ethylresorcinol, 2,3-dihydrohydroxybenzoic acid, 4-isopropylphenol, 4-methylphenol, 4-isopropylphenol, catechol, minoxidil, paracetamol, hydroxyarylamines, iodothyronines, tyramine, 5-hydroxytryptamine, salbutamol, isoprenaline,	polychlorobiphenylols, 4-chlorobiphenyl-3'-ol dichloronitrophenol, triphenyltin, fenarimol, prochloraz, methyltestosterone, clomiphene, diuron, salicylic acid, quercetin, galangin, kaempferol, fisetin, myricetin, chrysin, apigenin, genistein, curcumin, ellagic acid,	progesterone, medrogestone, nomegestrol acetate, promegestone or tibolone, 4-OH-tamoxifen, retinoic acid, endogenous and exogenous estrone triamcinolone acetonide, farnesol, pentachlorophenol

Table 5 (con't) Substrates, inducers and inhibitors of SULT (Allera et al., 2004; Wang et al., 2006)

Enzyme	Substrates	Inhibitors (for all SULT)	Inducers (for all SULT)
	dobutamine, iodothyronines, *N*-hydroxy -2-acetylaminofluorene, estrone, estradiol, estriol, 17α-ethinyl estradiol, equilenin, diethylstilbestrol, thyroxine, DHEA, pregnenolone, cholesterol, cortisol, benzyl alcohols of PAHs, testosterone, bile aminophenols, tyrosine methyl ester, epinephrine, peptides that contain *N*-terminal tyrosine residues (e.g., enkephalins, cholecystokinin heptapeptide), hydroxysteroids (e.g., testosterone, cortisone, hydrocortisone, aldosterone), ethanol, 1-butanol, isoamyl alcohol	thiol reagents, 2,6-dichloro-4-nitrophenol, pentachlorophenol	

Table 6 Specific substrates of SULT (Duanmu et al., 2000; Ohkimoto et al., 2004)

Enzyme	Specific substrate
SULT1A1	estrone, 17β-estradiol, 2-naphthol, 7-azido-4-methylcoumarin, acetaminophen, *p*-nitrophenol, *N*-hydroxy-2-acetylaminofluorine, minoxidil, thyroid hormones, 4-chlorophenol, 4-methoxyphenol, epinephrine, tyrosine methyl ester, tyramine, 2-cyanoethyl-*N*-hydroxythioacetimidate, peptides that contain *N*-terminal tyrosine residues such as enkephalins, cholecystokinin heptapeptides

Table 6 (con't) Specific substrates of SULT (Duanmu et al., 2000; Ohkimoto et al., 2004)

Enzyme	Specific substrate
SULT1A2	2-naphthol, aminophenol, 4-chlorophenol, 4-methoxyphenol, epinephrine, tyrosine methyl ester, tyramine, 2-cyanoethyl-*N*-hydroxythioacetimidate
SULT1A3	dopamine, 2-naphthol, aminophenol, 4-chlorophenol, 4-methoxyphenol epinephrine, tyrosine methyl ester, tyramine, 2-cyanoethyl-*N*-hydroxythioacetimidate
SULT1B1	dopamine, norepinephrine, thyroid hormones such as 3,3',5-triiodothyronine (T_3), 3,3',5'-triiodothyronine (rT_3), 3,3'-diiodothyronine (T_2), thyroxine, 2-naphthol, aminophenol, 4-chlorophenol, 4-methoxyphenol, epinephrine, tyrosine methyl ester, tyramine, 2-cyanoethyl-*N*-hydroxythioacetimidate
SULT1C1	2-naphthol, aminophenol, 4-chlorophenol, 4-methoxyphenol, epinephrine, tyrosine methyl ester, tyramine, 2-cyanoethyl-*N*-hydroxythioacetimidate
SULT1C2	*p*-nitrophenol, 2-naphthol, aminophenol, 4-chlorophenol, 4-methoxyphenol, epinephrine, tyrosine methyl ester, tyramine, 2-cyanoethyl-*N*-hydroxythioacetimidate
SULT1D1	2-naphthol, aminophenol, 4-chlorophenol, 4-methoxyphenol, epinephrine, tyrosine methyl ester, tyramine, 2-cyanoethyl-*N*-hydroxythioacetimidate
SULT1E1	2-naphthol, aminophenol, 4-chlorophenol, 4-methoxyphenol, epinephrine, tyrosine methyl ester, tyramine, 2-cyanoethyl-*N*-hydroxythioacetimidate, estrone, 17β-estradiol, 3,3',5-triiodothyronine (T_3), thyroxine, L-dopa, dehydroepiandrosterone, dopamine, quercetin, genistein, daidzein
SULT2A1	dehydroepiandrosterone, 1-hydroxymethylpyrene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, 5-hydroxymethylchrysene, testosterone, β-estradiol, cortisol, ethanol, 1-butanol, isoamyl alcohol, 1-amyl alcohol
SULT2B1	dehydroepiandrosterone, 1-hydroxymethylpyrene, 7-hydroxymethyl-12-methylbenz[*a*]anthracene, 5-hydroxymethylchrysene, testosterone, 17β-estradiol, cortisol, ethanol, 1-butanol, isoamyl alcohol, 1-amyl alcohol

Glutathione conjugation

Glutathione [N-(N-*L*-γ-glutatmyl-L-cysteinyl)glycine], an atypical tripeptide, is an endogenous compound, recognized as playing a protective role within the body in removal of potentially toxic elctrophilic compounds. Glutathione (GSH) is present

at highest concentration in the liver, with higher values in the cytosol and also in mitochondria and nucleus. GSH conjugation involves the formation of a thioether link between the GSH and electrophilic compounds. The reaction considered as the result of nucleophilic attack by GSH on electrophilic carbon atoms, with leaving functional groups such as halogen, sulfate and nitro, ring opening (in the case of small ring ethers-epoxides, β-lactones) and the addition to in the activated β-carbon of an α, β-unsaturated carbonyl compound. Thus conjugation with glutathione usually results in detoxication of the electrophilic compounds by preventing their reaction with nucleophilic centers in macromolecules such as proteins and nucleic acids. The electrophilic substrates for glutathione are commonly generated by prior metabolism of xenobiotics or by displacement of suitable electron withdrawing groups in nitro or halo-alkanes, benzenes and sulfonic acid esters by the sulfur atoms of glutathione and it is usually eliminated as mercapturic acid after further metabolism of the *S*-substituted glutathione. The glutathione conjugates may be excreted directly in urine (or more usually in bile) but more commonly undergo further metabolism. Many GSH conjugates undergo futher enzymatic modification by hydrolysis of the glutathione *S*-conjugate at the γ-glutamyl bond. This specific reaction is catalysed by the enzyme γ-glutamyl transferase. The tripeptide glutathione (Gly-Cys-Glu), once attached to the acceptor molecule, can be attacked by this specific enzyme, which removes the glutamate yielding a dipeptide; the latter may be further attacked by a peptidase which removes the glycine, thus forming the cysteine conjugate of the xenobiotic. In the final step, mediated by spefic *N*-acetylases, the cysteine conjugate previously formed may undergo *N*-acetylation (*via* the normal acetylation pathway), yielding the *N*-acetylcysteine conjugation of mercapturic acid. The first two enzymes are most commonly found in the liver and kidney cytosol, while highest *N*-acetyltransferase (NAT) activity is found in the proximal tubules. Depending on the nature of the substrate and the species investigated, each of the three conjugated metabolites (glycylcysteine, cysteine and mercapturic acid conjugates) may appear as excretion products (Figure 1).

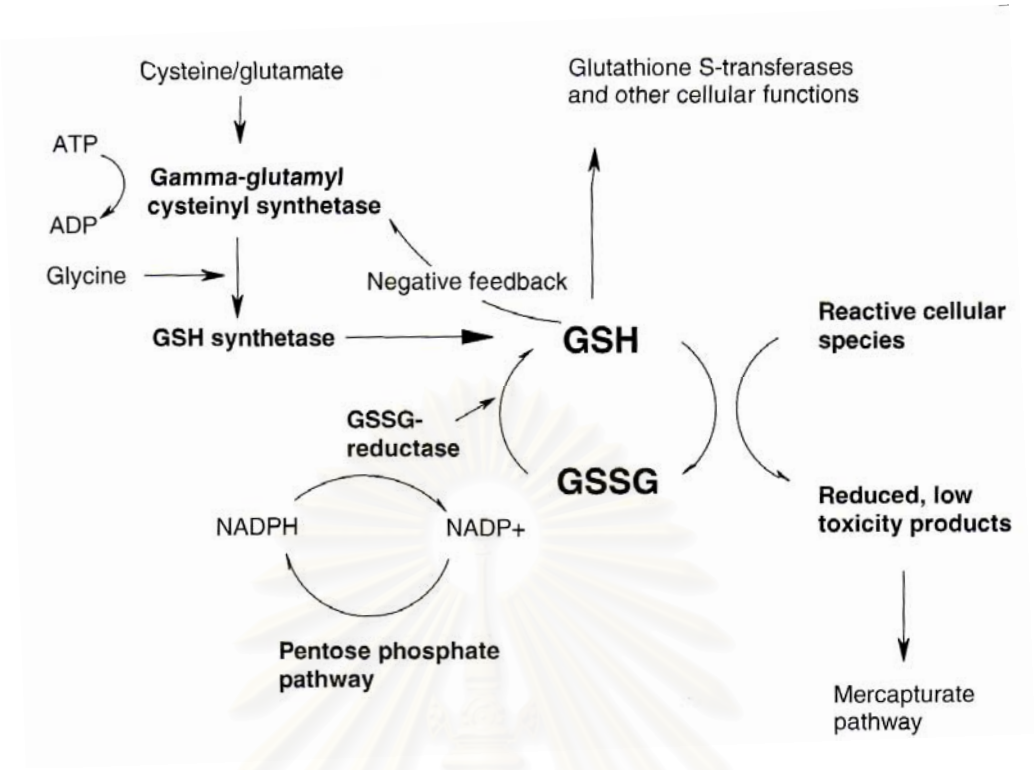


Figure 1. The GSH maintenance system in man

The glutathione *S*-transferases (GST) provide the essential active promotion of GSH reaction with electrophilic agents. This results in the formation of thioethers, which often are rearranged through further metabolism (mercapturate pathway) to form mercapturates, which are stable and non-toxic. The active site of GST enzyme (found near the *N*-terminous of the enzyme) is obviously required to bind both a range of possible substrates as well as GSH. Thiols also by their nature bind reactive and are not very stable to compensate for this. GSTs use hydrogen bonding at the GSH binding site to stabilize the thiol and prevent it from oxidizing before the substrate can be bound. Almost all the GSTs are found in the cytosol, although some are associated with the endoplasmic reticulum. The hydrophobic GSTs in endoplasmic reticulum are structurally different from the cytosolic GSTs and they metabolized leukotrienes and prostaglandins (Coleman et al., 2005). GSTs are thought to play a physiological role in initiating the detoxication of potential alkylating agents, including pharmacologically active compounds. These enzymes catalyze the reaction of compounds with –SH group of glutathione, thereby neutralizing their elctrophilic sites and rendering the products more water-soluble. Over 20 isoforms of GSTs are known to expressed in man, The nomenclature system has been confusing, a unifying

nomenclature for the soluble human transferases has been suggested and classified into gene family: Alpha (A), Mu (M), Pi (P), Sigma (S), Theta (T), Zeta, Omega and Kappa and reflect their subunit composits. Thus GSTA1-1 is a glutathione *S*-transferase (GST) of the Alpha class, consisting of homodimer of two identical '1-type sub-units', which found in only a few tissues of the body, including kidneys, intestine, lung, liver and testis. The substrate-binding site of the Alpha GSTs is most efficient at processing small hydrophobic molecules. There are three other human GSTA isoforms, including GSTA2, 3 and 4. The catalytic action of GSTA4 is inhibited by ethacrynic acid, lipid hydroperoxidase and 4-hydroxylalkenes (products of lipid breakdown). The representative Mu class, GSTM1-1 has a larger more open active site than the alpha GSTs and it contains a deeper binding cleft than the GSTP variants. GSTM1 are found in the liver, brain, testis, kidney and lung and oxidize may bulkier electrophilic agents, such as 1-chloro-2,4-dinitrobenzene (CDNB); aflatoxin B1-epoxide; *trans*-4-phenyl-3-buten-2-one and benzpyrene diols. GST Pi class GSTP1-1 is widespread, except for the liver, especially common in tumor cells. It processes a variety of toxicologically dangerous agents as well as endogenous species. These include CDNB, acrolein, adenine, propenal, benzyl isothiocyanate and 4-vinylpyridine. Aside from their functions in xenobiotic metabolism, GST Pi and Mu class GSTs appear to regulate a mitogen-activated protein (MAP) kinase pathway that is part of the apoptosis control system. The role of GSTP1 is especially troublesome in the induction of resistance to alkylating agents in cancer chemotherapy. Part of GSTP1 and other isoforms in the series defend the tumor cells by direct detoxification as well as by blocking apoptosis through their effect on MAP kinase. GST Theta class enzymes differ from the other GSTs as they do not use the tyrosine residue to catalyse the reaction between the substrate and GSH. Serine is accomplishes this activity in the GST-T isoform and it is likely that the site is capable of some structural rearrangement that assists in the catalytic process. This GST is associated with the metabolism of environmental and industrial carcinogens, including planar polycyclic aromatic hydrocarbons, halomethanes, dihalomethanes and ethylene oxide. Interestingly, GST-T in erythrocytes is identical to the hepatic version. So methyl bromide or ethylene oxide turnover by the enzyme in sampled erythrocytes is used to determine if an individual express this isoform. GST Omega class enzyme process CDNB, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole, *p*-nitrophenol acetate particularly effectively and are found in most tissues. These isoforms are thought to be responsible

for protein repair, where thiol adducts are trimmed off cytosolic structures as the enzyme acts as a thiol transferase. It has very large and open hydrophobic binding site, which allows it to bind polypeptide chains. This isoform is also involved in preventing cellular apoptosis by blocking calcium ion mobilization from intracellular stores (Coleman et al., 2005). Substrates, inducers and inhibitors of GST are shown in table 7. Specific substrates of GST are shown in table 8.

Table 7 Substrates, inducers and inhibitors of GST
(Baijail et al., 1997; Wallin et al., 1988; Turella et al., 2005)

Enzyme	Substrates	Inhibitors (for all GST)	Inducers (for all GST)
GST	bilirubin, estradiol, cortisol, testosterone, tetracycline, penicillin, ethacrynic acid, vitamin K_3, 17β-estradiol, paracetamol, sulfobromophthalein, parathion, urethane, *p*-nitrobenzylchloride (NBC), 1,2-epoxy-3-(*p*-nitrophenoxy)propane, hexachloro-1,3-butadiene nitrobenzene, 1-chloro-2,4-dinitrobenzene, 4-nitropyridine-*N*-oxide, *p*-nitrophenethyl bromide, *p*-nitrobenzyl chloride, 1,2-naphthalene oxide, allly alcohol, iodomethane 1-menaphthyl sulfate, *trans*-4-phenyl-3-buten-2-one,	equine estrogens, sulfobromophthalein, tridiphane(2-(3,5-dichlorophenyl)-2(2,2,2-trichloroethly)oxirane), ethanol, clofibate, ethacrynic acid, GSH analogs, gossypol, indomethacin, misonidazole, piriprost, sulfasalazine, terrapin199, selected 7-nitro-2,1,3-benzoxadiazole, SKF derivatives, lanosol, lobenzarit	17β-estradiol, 4-nonylphenol, butyrated hydroxyanisole, ethoxyequin, oltipraz, cumarin, indole-3-carbinol, TCDD, 3-methylcholanthrene, polycyclic aromatic hydrocarbons, folic acid, *trans*-stilbene oxide, nitrogen mustard alkylating agent, (melphalan, bromophenol, benzylidenealkanones, benzylidenecycloalkanones, butyrated hydroxyanisole, butyrated hydroxytoluene), chlorambucil, cyclophosphamide, ifosfamide, mechlorethamine, aziridines, epoxides, thiotepa, mitomycin C,

Table 7 (con't) Substrates, inducers and inhibitors of GST
(Baijail et al., 1997; Wallin et al., 1988; Turella et al., 2005)

Enzyme	Substrates	Inhibitors (for all GST)	Inducers (for all GST)
	sex steroids, androgens, transferring, testosterone, estradiol, atrazine, Δ^5-androsterone-3,17-dione, maleylacetoacetate, nitromethane, nitroethane, 1-nitropropane, 2-nitropropane, 2-nitroethanol, 3-nitro-2-butanol,		diazoquone, alkyl sulfonates, busulfan, nitrosoureas, carmustine, lomustine, nimustine, procarbazine, dacarbazine, polychlorinated biphenyls, polychlorinated dioxins, hypolipidemic drugs (peroxisome proliferators), antioxidants, safroles, musk xylene, barbiturates, *trans*-stilbene oxide, phenobarbital, carnesol, carnosic acid, dichloromethane, styrene, sulfobromophthalein

Table 8 Specific substrates of GST (Coleman et al., 2005)

Enzyme	Specific substrates
GST Alpha	-
GST Mu	*trans*-4-phenyl-3-buten-2-one, ethacrynic acid, Δ^3-androstene-3,17-dione
GST Pi	1-chloro-2,4-dinitrobenzene (CDNB), acrolein, adenine, propenal, benzyl isothiocyanate, 4-vinylpyridine, atrazine, 7-nitro-2,1,3-benzoxadiazole, trichloroethylene
GST Theta	polycyclic aromatic hydrocarbons, halomethanes, dihalomethanes, ethylene oxide, 1,1-dichloropropene
GST Omega	1-chloro-2,4-dinitrobenzene (CDNB), 7-chloro-4-nitrobenzo-oxa-1,3-diazole, *p*-nitrophenol acetate, *S*-(4-nitrophenacyl)glutathione

Table 8 (con't) Specific substrates of GST (Coleman et al., 2005)

Enzyme	Specific substrates
GST Theta	-

In general glutathione conjugation is regarded as a detoxicataion reaction. However depending on the properties of substrate, bioactivation is also possible. Four types of activation reaction have been recognized: direct-acting compounds, conjugates that are activated through cysteine conjugate beta-lyase, conjugates that are activated through redox cycling and lastly conjugates that release the original reactive parent compound (Bladeren et al., 2000). Toxicity was found for a cysteine conjugate was aplastic anemia due to a cysteine adduct of trichloroethylene. Hepatic glutathione conjugation to dihaloalkenyl or 1,1-difluoroalkyl glutathione *S*-conjugates, *via* an addition-elimination or simple addition reaction. For a number of halogenated alkenes such as hexachlorobutadiene, the microsomal GST are much more important than the cytosolic enzymes. However, for trichloroethylene it was shown that hGSTM1-1 and hGSTP1-1 are responsible for the formation of the glutathione conjugates. Glutathione dependent reactions have been implicated in the nephrotoxicity of a number of cyclic aromatic compounds such as bromobenzene and *p*-aminophenol. For bromobenzene, the nephrotoxicity is caused by glutathione conjugates derived from bromohydroquinone. The glutathione moiety is responsible for the targeting to the kidney, where the hydroquinone is oxidized to a quinone and causes cell damage by covalent binding as well as *via* reactive oxygen species as result of redox cycling. *p*-Aminophenol is oxidized enzymatically to the benzoquinone imine, which the undergoes the same reaction sequence as bromohydroquinone. Ethylene dibromide, tetrachloro 1,4-benzoquinone, hydroquinone, hexachlorobutadiene, chloro-2,4-dinitrobenzene, benzylisothio-cyanate, furazolldone metabolite, and 1,1-dichloropropene are bioactivated through glutathione conjugation (Bladeren et al., 2000). Polychlorinated alkenes are bioactivated in a complex *via* glutathione-dependent pathway (Dekant et al., 2003; Granville et al., 2004). GST catalyze the conjugation of the tripeptide GSH with alkyl halides and related compounds. If a second leaving group is present, the substrate at least a potential bis-electrophile and the initial conjugate may be susceptible to further

attack by the sulfur atom. This process can yield potent electrophiles that modify DNA and are genotoxic. Much of chemistry is understood in the context of the halide order and size of rings generated in reactive sulfonium ions. Similar chemistry has been demonstrated with the active site cysteine residue in the DNA repair protein (Guengerich., 2005). Substrates are bioactivated by GST are shown in the table 9.

Table 9 Substrates are bioactivated by GST (Bladeren et al., 2000; Dekant et al., 2003)

Substrates are bioactivated by GST	
dihaloalkyl	bromohydroquinone
hexachlorobutadiene	ethylene dibromide
trichloroethylene	tetrachloro-1,4-benzoquinone
cyclic aromatic compound	hydroquinone
bromobenzene	benzyl isothiocyanate
p-aminophenol	furazolldone metabolite
benzoquinone imine	1,1 dichloropropene
polychlorinated alkene	

NAD(P)H quinoneoxidoreductase (NQOR)

NAD(P)H quinoneoxidoreductase (NQOR), DT diaphorase is a major enzyme of xenobiotic metabolism that carries out obligatory two-electron reductions and thereby protects cells against mutagenicity and carcinogenity resulting from free radicals and toxic oxygen metabolites generated by the one electron reduction (Iqbal et al., 2003). Ernster and Navazio reported the occurrence of a highly active diaphorase in the soluble fraction of rat liver homogenates which catalyses the oxidation of NADH and NADPH. Its role as a protective device against the cytotoxicity and mutagenicity of quinone-derived oxygen radicals, and its involvement in vitamin K-dependent protein carboxylation. NQOR catalyzes the two-electron reduction of quinoid compounds to hydroquinones:

$$Q + NAD(P)H + H^{+} \longrightarrow QH_2 + NAD(P)^{+}$$

Where Q is a quinone, It also reacts with a number of other electron acceptors, for example, various dyes and nitro compounds. NQOR is widely distributed in the animal kingdom, although activity varies greatly from one species to another. Among animal tissues, liver is one of the richest sources of the enzyme, but other tissues, including brain, heart, lung, kidney, small intestine, skeletal muscle and mammary gland, exhibit varying NQOR activities. On fractionation of liver homogenates, the bulk (>90%), NQOR is recovered in the cytosolic fraction, whereas minor portions of the enzyme associated with mitochondria and microsomes. Appreciable NQOR activities are found in different tumors and in preneoplastic liver nodules as well as in various cell cultures (Ernster, 1967, 1990). NQOR activity is encoded by four genetic loci known as NQO1, NQO2, NQO3 and NQO4. The majority of NQOR is coded for by the NQO1 gene but roles of the other three are poorly understood. NQO1 (NAD(P)H: quinone oxidoreductase) is a flavoprotein that catalyzes the two-electron reduction of quinones and quinoid compounds to hydroquinones, using either NADH or NADPH as the electron donor. NQO2 (dihydronicotinamide riboside: quinone oxidoreductase) has nucleotides sequence identity to DT-diaphorase and is considered to be an isozyme of DT diaphorase (Chen et al., 2000). NQO1 and NQO2 are homologous for 54% of cDNA and 49% of protein. NQO2 is expressed in fewer tissues (including heart, lung, brain and skeletal muscle) than NQO1. NQO1 and NQO2 differ in their cofactor requirement with NQO2 using dihydronicotinamide riboside (NRH) rather than NADH or NADPH as the electron donor. NQO2 is resistant to typical inhibitors of NQOR such as dicumarol (Danson et al., 2004). Although NQO1 has been historically associated with generation of hydroquinones from reactive quinones, additional substrates including nitrosoamine, nitro and azo chemical moieties have been identified. NQO1 is also capable of scavenging superoxide anions generated during oxidative stress and regenerating reduced forms of protective endogenous antioxidant compounds. There is very low expression of NQO1 mRNA and protein in normal human livers, with slightly greater mRNA levels observed in biliary epithelial cells. NQO2 mRNA is greater in hepatocytes compared to NQO1 mRNA and is turn thought to play a more critical role in maintaining low levels of quinones in hepatocytes. Consequently, it has been suggested that human NQO1 does not play a major role in hepatic xenobiotic metabolism under normal conditions. Instead, NQO1 may be more important during periods of hepatic oxidative stress and damage. NQO1 mRNA protein and activity are markedly increased in

mouse liver following bile duct ligation, a model of obstructive cholestasis. Similar elevation in rodent NQO1 mRNA also occur after exposure to centrilobular hepatotoxicants such as acetaminophen, carbon tetrachloride and bromobenzene. Up regulation of NQO1 may represent a common response to liver injury with an oxidative stress. The isoforms of NQO1 showed broad substrate specificities towards four different quinones: menadione, vitamin K_1, benzo(*a*)pyrene-3,6-quinone, cyclized-dopamine ortho-quinone, The NQO2 reacts with biotinylated lectins which are specific for *N*-acetylgalactosamine, mannose, fucose and galactosly(*β*-1,3)-*N*-acetylgalactosamine while NQO4 reacts only biotinylated lectins specific for mannose and *N*-acetylgalactosamine (Segura-Aguilar et al., 1991). Several data were obtained using DCPIP as an electron acceptor. Substrates of NQOR are shown in table 10.

Table 10 Substrates of NQOR (Ernster et al., 1962, 1990)

Substrates of NQOR	
1,4,dihydroxynaphthalene, 1,4,9,10-tetrahydroxyanthracene, 1,4-dihydroxy-9,10 anthraquinone, *p*-benzoquinone, tetramethyl-*p*-benzoquinone, menadione, 1,4-naphthoquinone, 9,10-anthraquinone, sodium anthraquinone, *β*-sulfonate, 9,10-phenanthrenequinone, 2,3,5,6-tetramethyl-1,4-benzoquinone, thiazines, viologens, disulfide, oxygen, mono- and dehydroascorbate, tetrazolium dye, MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide, benzene, catechol estrogen-3,4-quinones, *α*-tocopherolquinone, *p*-benzoquinone, 2-methyl-2,3-epoxy-*p*-benzoquinone, 2,6-dimethyl-2,3-epoxy-*p*-benzoquinone, benzo(*a*)pyrene-3,6-quinone, cyclized dopamine *O*-quinone, 9,10-phenanthrenequinone, 2,6- dichlorophenol-indophenol, methyleneblue, 2-acetamido-*N,N*-dimethylindoaniline,	1,4-naphthoquinone, 1,2-naphthoquinone, 2-methyl-1,4-naphthoquinone, 2,3-dimethyl-1,4-naphthoquinone, vitamin K_1, 2,3-dimethoxy-1,4-naphthoquinone, 2-hydroxyl-1,4- naphthoquinone, 5-hydroxyl-1,4- naphthoquinone, 2-methyl-5-hydroxyl-1,4-naphthoquinone, 5,8-dihydroxy-1,4-naphthoquinone, 3-glutathionyl-1,4-naphthoquinone, 2,3-glutathionyl-1,4-naphthoquinone, 2-methyl-3-glutathionyl-1,4-naphthoquinone, 3-glutathionyl-5-hydroxy-1,4-naphthoquinone, 2-methyl-3-glutathionyl-5-hydroxy-1,2,4-naphthoquinone, 3-glutathionyl-5,8-dihydroxy-1,4-naphthoquinone, 2,3-epoxy-1,4-naphthoquinone, 2,3-epoxy-2-methyl-1,4-naphthoquinone, 2,3-epoxy-2,3-dimethyl-1,4-naphthoquinone, *N*-acetyl-*p*-benzoquinoneimine, 2-amino-1,4-naphthoquinoneimine, *N,N*-dimethylindoaniline

On the other hand, diethyl clofibrate caused significant reductions activities of NQO1 (Mesia-Vela et al., 2004). The inhibition is independent of the concentration of the electron acceptor. Other inhibitors of NQOR are of three categories: certain sulfhydryl reagents (*p*-chloromeribenzoate, *O*-iodosobenzoate); thyronine (thyroxine, 3,3',5-triiodothyronine and tetraiodothyronoacetate.); and flavins antagonists (FAD, FMN, atabrine, chlorpromazine). Dicumarol, which is the most potent inhibitor of NQOR found so far, inhibits the enzyme to 50% at a concentration 10^{-8} M without albumin. The dicumarol inhibition is independent of the nature of the electron acceptor, the same degree of inhibition being obtained when DCPIP is replaced by *p*-benzoquinone, vitamin K_3 or ferricyanide. The extent of dicumarol inhibition of was independent of the amount of NQOR. The inhibition proved to be competitive with respect to reduced pyridine nucleotides. Both NADH and NAD(P)H could counteract the dicumarol inhibition in reactions with DCPIP as well as in the coupled reaction system with vitamin K_3 and cytochrome *c* as electron acceptors. The affinity of the enzyme towards dicumarol was extremely high, shown by the dissociation constants for the enzyme-inhibitor complex (Ernster et al., 1962). The Dicumarol inhibition was not competitive with regard to the electron acceptor (Ernster et al., 1960). Specific substrates, inducers and inhibitors of NQOR are shown in table 11.

Table 11 Specific substrates, inducers and inhibitors of NQOR (Ernster et al., 1960; Benson et al., 1980)

Enzyme	Specific substrates	Inhibitors (for all NQOR)	Inducers (for all NQOR)
NQOR	menadione, vitamin K_1, benzo(*a*)pyrene-3,6-quinone, cyclized-dopamine ortho-quinone, biotinylated lectins, *N*-acetylgalactosamine, mannose, galactosly(*β*-1,3)-*N*-acetylgalactosamine,	clofibrate, sulfhydryl reagents (*p*-chloromeribenzoate, *O*-iodosobenzoate), thyronine (thyroxine, 3,3',5-triiodothyronine, tetraiodothyronoacetate), flavin antagonists (FAD, FMN, atabrine, chlorpromazine),	estradiol, farnesol, diallyl sulfide, phenolic antioxidants, 1,2-dithiole-3-thiones, isothiocyanates, flavone, coumarins, Sudan III [1-[[4 phenylazo) phenyl[azo]-2-naphthalenol], 3-methylcholanthrene, serum albumin, neutral phospholipids, detergents,

Table 11 (con't) Specific substrates, inducers and inhibitors of NQOR (Ernster et al., 1960; Benson et al., 1980)

Enzyme	Specific substrates	Inhibitors (for all NQOR)	Inducers (for all NQOR)
NQOR	DCPIP, methylene blue, ferricyanide, *p*-benzoquinone, 2-methyl- and 2,6-dimethyl-benzoquine, 1,2- and 1,4-naphthoquinone, vitamin K_3	dicumarol	hydrophobic quinones, benzoquinones, naphthoquinones, ubiquinones, vitamin K derivative, 2(3)-*tert*-butyl-4-hydroxyanisole, phenolic antioxidants (e.g., *tert*-butyl-hydroquinone), azo dyes-1-phenylazo-2-naphthol (Sudan I), polycyclic aromatic hydrocarbons, lactones, flavonoids, sulfur compounds (dithiocarbamates, 1,2-dithiol-3-thiones), aromatic isothiocyanates, BHA (1,4-dialkylphenols), 1,4-diphenols (*tert*- butyl-hydroquinone), 1,2-and 1,4-diphenols, acrylate, cortonate, cinnamate analogues, tetrachlorodibenzo-*p*-dioxin (TCDD)

NQOR activity has been related to bioactivation and cytotoxicity of antitumor quinone. A pair of human colon adenocarcinoma cell lines, HT29 and BE, were used to examine the role of NQOR in antitumor quinone induced apoptosis. HT29 have elevated levels of NQOR due to a point mutation which results in a complete lack of NQOR activity. MWDZQ, a quinone that is efficiently bioactivated by NQOR, induced apoptosis both in HT29 and BE cells, as assessed by morphological criteria

and formation of oligonucleosomal fragments of DNA. Two other quinone compounds which are substrates for NQOR, i.e., streptomycin and mitomycin C, also preferentially induced apoptosis in HT29 cells. In this study suggest that bioreductive activation of antitumor quinones by NQOR results in induction of apoptosis in human colon carcinoma cells (Sun et al., 1996). It is supposed that the main cytotoxicity mechanism of antitumor aziridinyl-substituted benzoquinones is their two-electron reduction to alkylating products by NQOR. The aziridyl-benzoquinones, di(*N*-ethylamino) analogue and 1,4,5,8-tetrahydroxynaphthalene were active as NQOR substrates. These findings indicate that in addition to the activation by NQOR, the oxidative stress presumably initiated by single-electron transferring enzymes may be an important factor in the cytotoxicity (Nemeikaite-Ceniene et al., 2003).

Factors affecting drug metabolism

Drugs can be metabolised by many different pathways and many factors can determine which pathway is used by which drug and to what extent a particular drug is biotransformed by a particular pathway. These factor range from the species of organism studied to the environment in which that organism liver. The factors affecting drug metabolism will be split into internal factors (species, genetic, age, sex, hormones, diseases) and external factors (diets and environments)

Species differences

In practice, genetic diversity is manifested in differences in the single DNA nucleotides and/or whole genes that code for particular protein that is different in structure and function to the majority. These differences may manifest as a substitution of a single amino acid with another, or a whole amino acid sequence may be different. These are termed ‘polymorphisms’ and their study is termed ‘pharmacogenetics’ or ‘pharmacogenomics’. The most common polymorphisms seem to be due to change in one nucleotide, which means that gene now specific to the wrong amino acid in a critical position, so reducing catalytic activity or it means a full stop occurs too early on the gene and incomplete mRNA is made which causes translation of and incomplete protein that is also ineffective and/or unstable. Polymorphisms arise due to mutations, but persist in human populations, but persist in human populations due to factors that may involve some advantage of the milder forms of the polymorphism (heterozygotes). Similarity, if some xenobiotic agent can

only be cleared, or detoxified, by a particular enzyme that is subject to a polymorphism, then the extremes may occur of either drug failure or drug toxicity. Most polymorphisms are a problem clinically due to an inability of the poor metabolizers to remove the drug from the system. Drug failure can occur if the agent is administered as pro-drug and requires some metabolic conversion to an active metabolite. Drug accumulation can lead to unpleasant side effects and loss of patient tolerance for the agent (Coleman et al., 2005). It is believed that polymorphisms in UDPGTs are found in the promoters, or their contolling systems, of these isoforms. One of the main UDPGTs, UDPGT1A1 is the subjected to polymorphic. The most severe, Crigler Najjar syndrome-1 is fatal, but CN-2 is survival and Gilbert's syndrome is also relatively mild and is the commonest UDPGT polymorphism. It is apparent that susceptibility to some cancers linked with exposure to some environmental toxins such as PAHs as well as hormone dependent cancers, may be influenced by UDPGT status. If a drug relies on the clearance of a reactive toxic metabolite to a glucuronide, then the various UDPGT polymorphisms in the use of anticolorectal cancer drug irinotecan. Glucuronidation normally protects the gut as the conjugate is not toxic and UDPGT1A1 polymorphisms abolish this protective pathway.

The role of SULTs in the activation of carcinogens is becoming more apparent. One of the major influences on SULT activity is their polymorphic nature; in the case of one of the most important toxicologically relevant SULTs, SULT1A, this isoform exists as two variants, SULT1A1*1 and SULT1A1*2. The *2 variant differs only in the exchange of one amino acid for another. This single amino acid change has profound effects on the stability and catalytic activity of the isoform. The *2 variants is also less subject to substrate inhibition, which may have consequences regarding the general feedback metabolic control of the *2 variant. Although susceptibility to carcinogenesis depends on myriad factors, SULT activity regarding some specific carcinogens such as acetylfluorene.

GSTs are polymorphic and much research has been directed at linking increased predisposition to cytotoxicity and carcinogenicity with defective GST phenotypes. This is caused by a gene deletion that also occurs in GST-Theta isoforms. It is difficult to link a lack of expression of GST isoforms and increased DNA damage partly because of other detoxifying enzymes such as epoxide hydrolase (EH) might be operating and masking the effects on the test cell system. EH itself is subject to a

polymorphism, with a fast and slow variant of the enzyme. Carcinogenesis may be due to a complex mix of factors, where different enzyme expression and activities may combine with particular reactive species from specific parent xenobiotics which lead to DNA damage (Lampe et al., 2007).

Age

It has long been recognized that the young, particularly the newborn and the elderly of many animals are more susceptible to drug action. Studies on the development of drug metabolising capacity have indicated that the increased sensitivity of neonates may be related to their very low or, at times, unmeasurable drug metabolizing capacity which subsequent develops in a species-, strain-, substrate- and sex-dependent manner until adult levels of enzyme activity are achieved. The decrease in drug-metabolising capacity in old age also appeared to be dependent of these factors although other specific factors may be involved.

Sex

Qualitative and quantitative differences in both phases of drug metabolism are related to sex as well. Female rats required only half the dose of a barbiturate compared to male rats to induce sleep. This was due to the reduced capacity of the female to metabolise the barbiturates. Sex differences have been intensively studied, sometimes related to species or ages, are now being observed for a wide range of substrates, including commonly prescribed drugs or even endogenous compounds, including steroid sex compounds. An example, the *in vitro* investigation of sex and species differences in the metabolism of BOF-4272, a drug intended for the treatment of hyperuricaemia. On the other hand, results of other investigations examine the influence of sex and age on different enzyme activities.

Diseases

Pathological status, in cirrhosis seems absolutely reasonable that drug metabolism should be impaired. A comparative study was performed on normal mice to investigate the effects of drug-induced liver injury using prednisolone vs Angelica Sinensis Polysaccharides (ASP). ASP was shown to increase content and catalytic activity of several enzymes: different demethylase, hydroxylases and GSH-related enzymes. In contrast, prednisolone significantly decreased the liver mitochondrial glutathione content, whereas all enzymes activities were increased.

Hormones

Hormones, known to play a major role in the general metabolism, have similarly been proven to control the biotransformation of drugs, in direct connection with other factors such as age, sex, or in particular physiological stages such as pregnancy. The apparent connection between sex and age with the control of the growth hormone (GH) was the focus of interesting cDNA cloning investigations. Thyroid status contributes to differences for several drug administered in equi-active doses on several forms of UDPGT. As experimental animals, rats having different thyroid hormonal were employed, namely normal (control group), hypothyroid and hyperthyroid. The drugs tested were ciprofibrate, bezafibrate, fenofibrate, and clofibrate. The responses were markedly modulated by the thyroid status, with an average increase of about 5% in hyperthyroid animals. The results confirmed the role of hormonal control upon the enzyme induction displayed by certain drugs (or other xenobiotics).

There are however, other factors, from outside the body that can also have a profound influence on drug metabolism. The body can be exposed to these factors by design (e.g. substances taken as food, alcohol and tobacco smoke) or by accident (air, water and food contaminants or pollutants). The effect of protein deficiency on phase II drug metabolism is more complex, with some activities decreasing, e.g. paracetamol glucuronidation, while others increase, e.g. 4-nitrophenol glucuronidation. For GST an alteration in subunit composition is ween when a protein-depletion diet is used. In man, excess vitamin C ingestion decreases the ability of the glucuronidation conjugate estrogens with sulfate. This is thought to be due to a competition of ascorbic acid and the estrogens for the limited supply of the cofactor required for sulfation (PAPS). Zinc deficiency lead to reduced UDPGT and GST. Selenium deficiency has also been shown to markedly inhibit expression of the estrogen sulfotransferase gene in the rat. The most common effect of tobacco smoking is an increase in biotransformation of drugs, an effect similar to that seen for ingestion of charcoal-broiled meat. Indeed, there is a common factor: the polycyclic hydrocarbon benzo(*a*)pyrene. Smoking as found to increase drug clearance phase II metabolism have been shown to be affected by tobacco smoking. Heavy metal: lead, mercury, cadmium, gadolinium have been shown to decreased CYP450 activity. Industrial pollutants: TCDD, solvent, polychlorinated biphenyls is an increase

4-nitrophenol glucuronidation was also seen (Gibson et al., 2001; Zamek-Gliszczynski et al., 2006).

CHAPTER III

MATERIALS AND METHODS

Materials

1. Experimental animals

Adult male Wistar rats of 180 to 230 g body weight were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. They were housed in air-conditioned animal facility at the Faculty of Medicine, Srinakharinwirot University, Bangkok. They were kept in a room maintained at 25±2 °C and 55±5 % humidity. All animals were maintained on a 12-hour light/dark cycle and provided freely with standard food pellets (C.P. company, Thailand) and tap water. The rats were acclimatized to the animal facility for 7 days before being entered into an experiment. Body weights of rats as well as food and water consumption were recorded at weekly intervals (Appendix A).

2. Instruments

The experimental instruments included :

1. Autopipetts 20, 100, 200, 1000 and 5000 µl (Gibson, France)
2. Metabolic shaker bath (Heto, Denmark)
3. pH meter (Beckman Instruments, USA)
4. Potter-Elvehjem homogenizer with pestle and glass homogenizing vessels (Hedolph, Germany)
5. Refrigerated centrifuge (Allegra X-12 R, USA)
6. Refrigerated superspeed centrifuge (Beckman Instruments, USA)
7. Refrigerated ultracentrifuge (Beckman Instruments, USA)
8. Sonicator (Elma, Germany)
9. Spectrophotometer (Jasco, Japan)
10. Surgical equipments
11. Thermometer
12. Timer
13. Ultra-low temperature freezer (Forma Scientific Inc., USA)
14. Vortex mixer (Clay Adams,USA; CT Laboratory)

3. Chemicals

These following chemicals were purchased from Sigma Chemical Co. Ltd., USA: Adenosine 3'-phosphate-5'-phosphosulfate (PAPS) , bovine serum albumin (BSA), cupric sulfate, 1-chloro-2,4-dinitrobenzene (CDNB), 2,6-dichlorophenol-indophenol (DCPIP), dicumarol, ethylenediaminetetraacetic acid (EDTA), Folin & Ciocalteu's phenol reagent, glutathione reduced form (GSH), magnesium chloride ($MgCl_2$) , nicotinamide adenine dinucleotide reduced form (NADH), *p*-nitrophenol sulfate, sodium carbonate (Na_2CO_3), sodium citrate, sodium hydroxide (NaOH), sodium phosphate dibasic anhydrous (Na_2HPO_4), triton X-100, trizma base, UDP-glucuronic acid

Ethanol, hydrochloric acid (HCl), *p*-nitrophenol, potassium chloride (KCl), potassium phosphate monobasic anhydrous (KH_2PO_4) and trichloroacetic acid (TCA) were purchased from Merck, Germany.

2-Naphthol were purchased from Aldrich, USA.

Potassium phosphate dibasic anhydrous (K_2HPO_4) and sodium chloride (NaCl) were purchased from Ajax Finechem, Australia.

Ultrapure water was prepared by ELGASTAT MAXIMA UF® (ELGA Ltd, England).

Methods

1. Preparation of *C. comosa* extract

C. comosa hexane extract and *C. comosa* ethanolic extract were supplied by Professor Apichart Suksamrarn, Department of Chemistry, Faculty of Sciences, Ramkhamhaeng University. The extracts of *C. comosa* were prepared as following:

1.1 Preparation of *C. comosa* hexane extract

C. comosa rhizomes were collected from Kampaengsan District, Nakorn pathom Province, Thailand. Rhizomes were dried, sliced into small pieces and ground to a coarsely powder. Thirty kilograms of pulverized dried rhizomes was extracted with *n*-hexane in Soxhlet extractor, subsequently the suspension was filtered and evaporated to dryness under rotary evaporator and sucked residual solvent with high vacuum pump to give a pale brownish viscous oil (1.01 kg). *C. comosa* hexane extract was stored in a light protected, tightly closed container and kept at room temperature.

1.2 Preparation of *C. comosa* ethanolic extract

The hexane-extracted marc was subsequently extracted with 95% ethanol, filtered and evaporated to dryness under rotary evaporator and dried again with high vacuum pump. The ethanolic extract was obtained as a dark reddish brown viscous oil (1.3 kg).

2. Characteristic of *C. comosa* extracts

Hexane and ethanolic extracts of *C. comosa* were characterized by Suksamrarn et al. (unpublished data) and the TLC chromatogram were shown in the appendix E. The major constituent of hexane extract was 1,7-diphenyl-5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene (1). The major constituents of ethanolic extract were 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene (6) and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene (7).

2. Animal treatment

2.1 Animal treatment for verification of the methods

Twenty four rats were randomly divided into 6 groups of 4 animals/group.

UDPGT: Each group was injected intraperitoneally with corn oil or β-naphtho-flavone, once daily for three consecutive days as following:

1. Control group: Rats were injected intraperitoneally with corn oil at 1 ml/kg/day.
2. Induced group: Rats were injected intraperitoneally with β-naphthoflavone at 80 mg/kg/day (Hu et al., 1994).

SULT and NQOR: Each group was injected intramuscularly with corn oil or estradiol valerate, once daily for seven consecutive days as following:

1. Control group: Rats were injected intramuscularly with corn oil at 1 ml/kg/day.
2. Induced group: Rats were injected intramuscularly with estradiol valerate at 500 μg/kg/day (Kabayashi et al., 1999; Sonia et al., 2004).

GST: Each group was injected intraperitoneally with normal saline or phenobarbital, once daily for three consecutive days as following:

1. Control group: Rats were injected intraperitoneally with normal saline at 1 ml/kg/day.
2. Induced group: Rats were injected intraperitoneally with phenobarbital at 80 mg/kg/day (Soucek and Gut et al., 1992; Tredger et al., 2002).

The day following the animal treatment, each animal was anesthesized with diethyl ether before collecting livers. Rat liver cytosolic and microsomal fractions were prepared according to the method described by Lake (1987) with some modifications.

2.2 Animal treatment for study the effects of *C. comosa* extracts on phase II drug metabolizing enzymes

Fifty rats were randomly divided into 5 groups of 10 animals/group. Each group was treated orally by gavage with the vehicle or *C. comosa* extracts, once daily for thirty consecutive days as following:

1. Group I : Rats were treated orally with corn oil at 1 ml/kg/day.
2. Group II: Rats were treated orally with *C. comosa* hexane extract at 250 mg/kg/day.
3. Group III: Rats were treated orally with *C. comosa* hexane extract at 500 mg/kg/day.
4. Group IV: Rats were treated orally with *C. comosa* ethanolic extract at 250 mg/kg/day.
5. Group V: Rats were treated orally with *C. comosa* ethanolic extract at 500 mg/kg/day.

The suspension of *C. comosa* hexane extract and *C. comosa* ethanolic extract for animal administration were prepared freshly by weighing 250 mg and 500 mg of both extracts, dissolving in 1 ml of corn oil to make suspensions of concentration of 250 mg/ml and 500 mg/ml.

C. comosa hexane extract at the doses of 250 mg/kg/day and 500 mg/kg/day in this study, were previously reported to possess estrogenic like action in male rats (Piyachaturawat et al., 1998, 1999).

C. comosa ethanolic extract at the doses of 250 mg/kg/day and 500 mg/kg/day in this study exhibited hypocholesterolemic effect (Piyachaturawat et al., 2002).

3. Preparation of liver cytosolic and microsomal fractions.

At the end of the experimental period, the overnight-fasted animals were anesthesized with diethyl ether before collecting livers. Liver weight of each rat was recorded as shown in appendix A. Rat liver cytosolic and microsomal fractions were prepared according to the method described by Lake (1987) with some modifications.

Reagents

1. 0.9% w/v of NaCl
2. 0.1 M Phosphate buffer pH 7.4
   One liter of 0.1 M phosphate buffer pH 7.4 containing 1.78 g of KH_2PO_4, 9.55 g of Na_2HPO_4 and 11.50 g of KCl, adjusted to pH 7.4 with NaOH or HCl.
3. 0.1 M Phosphate buffer pH 7.4 containing 20% v/v glycerol
   One liter of 0.1 M phosphate buffer pH 7.4 containing 200 ml of glycerol, adjusted to pH 7.4 with NaOH or HCl.

Procedures

1. Under anesthesized with diethyl ether, liver of rat was perfused through the portal vein with 0.9% w/v NaCl until the entire liver become pale.
2. Liver was excised, then rinsed with ice-cold 0.9% w/v NaCl and blotted dry with gauze.
3. The whole liver was weighed, minced with scissor and homogenized in 3 fold volume of ice-cold 0.1 M phosphate buffer pH 7.4.
4. The homogenate was centrifuged at 10,000 *g* for 30 minutes at 4°C, using refrigerated superspeed centrifuge to sediment intact cells, cell debris, nuclei and mitochondria.
5. The supernatant was transferred into a ultracentrifuge tube then recentrifuged at 100,000 *g* for 60 minutes at 4°C, using refrigerated ultracentrifuge.
6. The supernatant contained cytosolic fraction was aliquoted collected in microtubes and stored at -80°C.
7. The pellet (microsomal fraction) was suspended in 5 ml of 0.1 M phosphate buffer pH 7.4 containing 20% v/v glycerol. One milliter of microsomal fraction was aliquoted, kept in microtubes and stored at -80°C.

4. Determination of protein concentrations in liver cytosolic and microsomal fractions

The protein concentrations in liver cytosolic and microsomal fractions were measured according to the method modified from the method of Lowry et al. (1951).

Reagents

1. 2% w/v Na_2CO_3

   Anhydrous Na_2CO_3 30 g was dissolved in ultrapure water 1.5 L.
2. 0.5 M NaOH

   NaOH 5 g was made up to 250 ml in ultrapure water.
3. 2% w/v Sodium citrate

   Sodium citrate 0.4 g was dissolved in ultrapure water 20 ml.
4. 1% w/v Cupric sulfate

   Cupric sulfate 0.2 g was dissolved in ultrapure water 20 ml.
5. 1 mg/ml BSA in 0.5 M NaOH

   BSA 0.02 g was dissolved in 0.5 M NaOH 20 ml.
6. Folin & Ciocalteu's phenol reagent
7. Working protein reagent

The solution consisted of 2% w/v Na_2CO_3, 0.5 M NaOH, 2% w/v sodium citrate and 1% w/v cupric sulfate solutions in a 100:10:1:1 ratio by volume, respectively. This agent was freshly prepared and must be clear during in the assay.

Procedures

1. Tubes were labeled in duplicate for 7 standards (0, 50, 100, 150, 200, 250 and 300 µg) and for each unknown sample.
2. 0.5 M NaOH and 1 mg/ml BSA were added into each standard tubes as following:

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

Each tube was vortex-mixed throughly, after addition of these reagents.

3. Each unknown sample tube consisted of 490 µl of 0.5 M NaOH and 10 µl of cytosol or microsome.
4. After adding 6.5 ml of working protein reagent to each tube, the tubes were placed at room-temperature for 10 minutes.
5. Two hundred microliter of Folin & Ciocalteu's phenol reagent was added to each tube, the tube was vortex-mixed throughly for at least 30 seconds. The tubes were allowed to stand at room temperature for at least 30 minutes.
6. The absorbance of the solution in each tube was measured at 500 nm by spectrophotometer.

Calculation of protein concentration

1. A standard curve was plotted between the absorbance and the corresponding amount of BSA protein. The linear regression line was constructed and the linear regression equation was calculated.
2. Amount of protein in each sample could be determined by using its absorbance and the linear regression equation. The protein concentration was calculated by divided the amount of protein by the volume of cytosolic or microsomal sample used in the reaction and the unit was expressed as mg/ml or µg/µl.

5. Enzyme assays

All enzyme assays were performed in duplicate. Every method was verified for linearity, precision and capacity of the methods before using for determination of the enzyme activity in the cytosolic or microsomal samples.

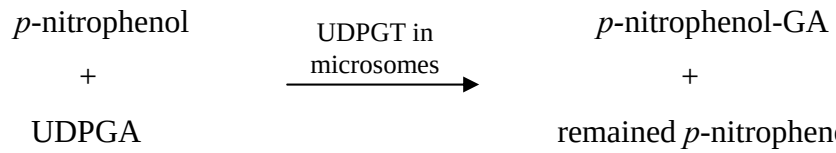
5.1 Assay of UDP-glucuronosyltransferase (UDPGT) activity

UDPGT activity was measured using *p*-nitrophenol as a substrate and UDPGA as a co-enzyme according to the method of Bock et al (1973) with some modifications.

Principle of the assay

The principle of the reaction was shown as following:

Reaction:



The remained *p*-nitrophenol was measured spectrophotometrically at 405 nm.

Reagents

1. 1 M Tris-HCl buffer pH 7.4 (MW of Trizma base = 121.1)
   Trizma base 121.1 g was dissolved and made up to 1 L with ultrapure water.
   The solution was adjusted to pH 7.4 with NaOH or HCl.
2. 50 mM $MgCl_2$·hexahydrate (MW = 203.31)
   $MgCl_2$·hexahydrate 1.017 g was dissolved and made up to 100 ml with ultrapure water.
3. 5% w/v Trichloroacetic acid (MW = 163.39)
   Trichloroacetic acid 50 g was dissolved in 1 L of ultrapure water.
4. 2 M NaOH (MW = 40)
   NaOH 20 g was dissolved and made up to 250 ml with ultrapure water.
5. 0.25% w/v Triton X-100
   Triton X-100 0.05 g was dissolved in 20 ml of ultrapure water
6. 5 mM *p*-nitrophenol (MW = 139.11)
   p-Nitrophenol 0.0174 g was dissolved and made up to 25 ml with ultrapure water.
   This agent was freshly prepared and kept in a light protected container.
7. 30 mM UDPGA (MW = 646.2)
   UDPGA 0.01939 g was dissolved in 1 ml of ultrapure water.

Procedures

1. A total volume of 0.5 ml reaction mixture contained 600 µg of microsomal protein, 180 µl of 1 M Tris-HCl buffer pH 7.4, 20 µl of 0.25% w/v Triton X-100, 50 µl of 50 mM $MgCl_2$ and 50 µl of 5 mM *p*-nitrophenol.
2. The solution in each tube was vortex-mixed and preincubated for 2 minutes at 37°C in a metabolic shaker bath.
3. The enzyme reaction was started by an addition of 50 µl of 30 mM UDPGA whereas 50 µl of 1 M Tris-HCl buffer pH 7.4 was added in the corresponding blank tube. All tubes were vortex-mixed and incubated for 10 minutes at 37°C.
4. The reaction was stopped with 1 ml of ice-cold 5% trichloroacetic acid, each tube was vortex-mixed and standed on ice.
5. The reaction mixture was centrifuged to remove protein pellet at 3,200 rpm for 15 minutes at 4°C.
6. One milliliter of the supernatant was transferred into a clean glass test tube.

7. Two hundred fifty microliter of 2 M NaOH and 2 ml of ultrapure water were added into the tube and vortex-mixed throughly. The tubes were protected from light.
8. The absorbance of *p*-nitrophenol in the solution was measured spectrophotometrically at 405 nm.
9. Amount of *p*-nitrophenol was determined by comparing its absorbance against a standard curve of *p*-nitrophenol.
11. A standard curve of *p*-nitrophenol was generated by 5 concentrations of 5 mM *p*-nitrophenol solutions. One hundred and twenty microliter of 1 M Tris-HCl buffer pH 7.4 and 666 µl of 5% trichloroacetic acid were added into each standard tube containing various concentrations of *p*-nitrophenol which were prepared as following:

p-nitrophenol concentration (nmol/ml)	90	110	130	150	170
5 mM *p*-nitrophenol (µl)	18	22	26	30	34
ultrapure water (µl)	196	192	188	184	180

Absorbance of the solution in each tube was measured spectrophotometrically at 405 nm.

Calculation of UDPGT activity

Amount of *p*-nitrophenol remaining in the reaction tube was determined by comparing its absorbance against a *p*-nitrophenol standard curve. The disappearance of *p*-nitrophenol was determined by subtracting the measured remaining *p*-nitrophenol in the sample tube from that in the blank tube. Microsomal UDPGT activity was calculated from amount of the disappered *p*-nitrophenol divided by the time of incubation (10 minutes) and amount of microsomal protein used in the reaction (0.6 mg).

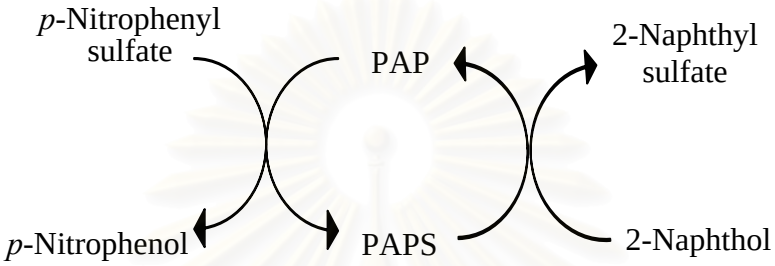
5.2 Assay of sulfotransferase (SULT) activity

SULT activity was determined using 2-naphthol as a substrate as well as adenosine 3'-phosphate-5'-phosphosulfate (PAPS) and *p*-nitrophenylsulfate as co-enzymes according to the method of Frame et al. (2000) with some modifications.

Principle of the assay

The principle of the reaction was shown as following:

Reaction:



The product (*p*-nitrophenol) was measured spectrophotometrically at 405 nm.

Reagents (Stock solutions)

1. 0.0668 M Potassium phosphate buffer pH 6.5 (KH_2PO_4 MW = 136.09, K_2HPO_4 MW = 174.18)

   Twenty two point eighty eight milliliter of 0.1336 M KH_2PO_4 was mixed with 10.52 ml of 0.1336 M K_2HPO_4. The solution was diluted to a total volume of 1 L with ultrapure water, adjusted to pH 6.5 with NaOH or HCl (Deangelis, 2007).

2. 2% w/v $MgCl_2$ (MW = 203.31)

   $MgCl_2$·hexahydrate 2 g was dissolved in 100 ml of ultrapure water.

3 0.025% w/v PAPS (MW = 507.26)

   PAPS 0.005 g was dissolved in 20 ml of ultrapure water.

4. 2.5% w/v *p*-Nitrophenylsulfate (MW = 257.27)

   p-Nitrophenylsulfate 0.125 g was dissolved in 5 ml of ultrapure water. This agent was freshly prepared.

5. 0.05% w/v 2-Naphthol (MW = 144.17)

   2-Naphthol 0.01 g was dissolved in 0.8 ml of ethanol and then adjusted to 20 ml with ultrapure water. This agent was freshly prepared.

6. 0.25 M Tris-HCl buffer pH 8.7 (MW of Trizma base = 121.1)

   Trizma base 30.275 g was made up to 1 L with ultrapure water and the solution was adjusted to pH 8.7 with NaOH or HCl.

Procedures

1. A total volume of 1.5 ml reaction mixture contained 750 µg of cytosomal protein, 0.668 M potassium phosphate buffer pH 6.5, 76 µl of 2% w/v $MgCl_2$, 62 µl of 0.025% w/v PAPS and 76 µl of 2.5% w/v *p*-nitrophenylsulfate. The tubes were protected from light.
2. The reaction mixture was preincubed for 2 minutes at 37 °C in a metabolic shaker bath.
3. The enzyme reaction was started by an addition of either 44 µl of 0.05% w/v 2-naphthol or 44 µl of solvent as a blank. The tubes were vortex-mixed and incubated for 15 minutes at the same conditions.
4. The reaction was stopped by adding 1.5 ml of ice-cold 0.25 M Tris-HCl pH 8.7 and the tube was standed on ice.
5. The reaction mixture was centrifuged at 3,200 rpm for 10 minutes at 4°C.
6. The supernatant was transferred into a clean glass test tube which was protected from light.
7. The formation of *p*-nitrophenol was measured by spectrophotometer at 405 nm within 30 minutes.

Calculation of SULT activity

Concentration of *p*-nitrophenol formed was determined from the extinction coefficient of 18.4 $mM^{-1}cm^{-1}$, which directly correlated to the concentration of 2-naphthylsulfate formed (Mulder et al.,1977). Results were expressed as nmol/ min per mg protein by subtracting the blank absorbance from that of the sample, dividing the amount of *p*-nitrophenol by the time of 15 minutes incubation and amount of cytosolic protein (0.75 mg) used in reaction. Using Beer's law and assuming a cuvette path length of 1 cm. The equation was given by:

$$\text{SULT activity} = \Delta A \times 91.7 \quad \text{(nmol/min per mg protein)}$$

5.3. Assay of glutathione *S*-transferase (GST)

GST activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate and glutathione reduced form (GSH) as a co-enzyme according to the method of Habig et al. (1980) with some modifications.

Principle of the assay

The principle of the reaction was shown as following:

Reaction:

GST + CDNB $\xrightarrow{\text{GST in cytosol}}$ conjugation of CDNB with glutathione + H_2O

The product (conjugation of CDNB with glutathione) was measured spectrophotometrically at 340 nm.

Reagents

1. 0.1 M Sodium phosphate buffer pH 6.5 containing 1 mM EDTA sodium (Na_2HPO_4 MW = 142, EDTA sodium MW = 372.2)
   Sodium phosphate 7.1 g and EDTA sodium 0.1861 g were dissolved and made up to 500 ml with ultrapure water. The solution was adjusted to pH 6.5 with NaOH or HCl.
2. 20 mM CDNB in 95% ethanol (MW = 202.6)
   CDNB 0.0406 g was dissolved in 10 ml of 95% ethanol. This solution was freshly prepared and kept in light-protected container.
3. 20 mM GSH (MW = 307.33)
   GSH 0.0614 g was dissolved in 10 ml of ultrapure water. This agent was freshly prepared and protected from light.

Procedures

1. The reaction mixture was prepared in a pair of cuvette. A total volume of 1 ml reaction mixture containing 890 µl of 0.1 M sodium phosphate buffer pH 6.5 containing 1 mM EDTA sodium, 50 µl of 20 mM CDNB and 50 µl of 20 mM GSH were added to both the reference and sample cuvettes. The cuvettes were covered with paraffin and mixed well.
2. Both cuvettes were put in a spectrophotometer and the absorbance was adjusted to zero at 340 nm.

3. The enzyme reaction was started by an addition of 10 µl of 0.1 M sodium phosphate buffer pH 6.5 containing 1 mM EDTA sodium or 10 µl of cytosol, into either reference or sample cuvettes, respectively.
4. Both cuvettes were put back immediately into the spectrophotometer and then started measuring the absorbance.
5. An increase of the absorbance was monitored for 10 seconds.

Calculation of GST activity

The concentration of 2,4-dinitrophenylglutathione was determined from the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ (Jakoby, 1985). GST activity was calculated by dividing the concentration of 2,4-dinitrophenylglutathione by the time and amount of cytosolic protein (10 µl x protein concentration (µg/µl) used in the reaction. GST activity was expressed as nmol/min per mg protein. Using Beer's law and assuming a cuvette path length of 1 cm. The equation was given by:

$$\text{GST activity} = \frac{\Delta A \times 3125000}{\text{protein concentration (mg/ml)}} \quad \text{(nmol/min per mg protein)}$$

5.4 Assay of NAD(P)H quinoneoxidoreductase (NQOR) activity

NQOR activity was determined using 2,6-dichlorophenol-indophenol (DCPIP) as a substrate and NADH as a co-enzyme according to the method of Ernster (1990) with some modifications.

Principle of the assay

The principle of the reaction was shown as following:

Reaction:

DCPIP (2,6-dichlorophenol-indophenol) $\xrightarrow{\text{NQOR in cytosol}}$ Reduced DCPIP

The product (reduced DCPIP) was measured spectrophotometrically at 600 nm.

Reagents (Stock solutions)

1. 0.68% w/v Tris-HCl buffer pH 7.5 (MW of Trizma base = 121.1)

   Trizma base 6.8 g was dissolved in 1 L of ultrapure water. The solution was adjusted to pH 7.5 with NaOH or HCl.

2. 5% w/v Triton X-100

   Triton X-100 2.5 g was dissolved in 50 ml of ultrapure water.

3. 2% w/v NADH (MW = 709.4)

   NADH 0.03 g was dissolved in 1.5 ml of ultrapure water. This agent was freshly prepared.

4. 0.1% w/v DCPIP (MW = 290.08)

   DCPIP 0.05 g was dissolved in 50 ml of ultrapure water and the solution was kept in light protected container.

5. 0.0125% w/v Dicumarol (MW = 336.3)

   Dicumarol 0.0125 g was dissolved in 25 µl of 5 M NaOH and made up to 100 ml with ultrapure water.

6. 5 M NaOH (MW = 40)

   NaOH 4 g was dissolved and made up to 20 ml with ultrapure water.

Procedures

1. The reaction mixture was prepared in a cuvette. A total volume of 3 ml reaction mixture containing 0.68% w/v Tris-HCl buffer pH 7.5, 48 µl of 5% w/v Triton X-100, 54 µl of 2% w/v NADH and 45 µl of 0.1% w/v DCPIP, were added into the sample cuvette. The cuvette was covered with paraffin and mixed well. Three milliliter of ultrapure water was added into the reference cuvette.
2. The reaction was started by an addition of cytosol (containing 500 µg of the cytosolic protein) into the sample cuvette.
3. The sample cuvette was immediately placed back to the spectrophotometer and the absorbance was recorded.
4. The decrease of absorbance was monitored for 10 seconds.
5. In this study, cytosolic samples were nonpurified preparations and may contain other diaphorase enzymes therefore, dicumarol which was a selective inhibitor of the NQOR activity was added into the reaction. The parallel reaction was performed in the presence of 81 µl of 0.0125% w/v dicumarol instead of ultrapure water.

Calculation of NQOR activity

NQOR activity was determined from the extinction coefficient of 21 mM^{-1} cm^{-1} at 600 nm (Ernster, 1967, 1990). Absorbance difference between uninhibited sample and dicumarol-inhibited sample was divided by the time and amount of cytosolic protein (500 µg) used in the reaction. Unit of NQOR activity was expressed as nmol/min per mg protein. Using Beer's law and assuming a cuvette path length of 1 cm. The equation was given by:

$$\text{NQOR activity} = \Delta A \times 1904.7 \quad \text{(nmol/min per mg protein)}$$

6. Verification of the methods used for enzyme activity assay

Before using the methods for assay activities of UDPGT, SULT, GST and NQOR in the samples, the methods were verified for the precision as well as linearity with respect to amount of protein used in the reaction. In addition, the capacity of the methods to determine an inducing effect of an inducer was also verified regarding the methods for determination of UDPGT, SULT, GST and NQOR activities.

1. Precision assay

1.1 Intraday precision assay

One microsomal sample (for UDPGT) and one cytosolic sample (for SULT, GST and NQOR) were used to verify for intraday precision.

For UDPGT activity assay, a microsomal sample was used to determine UDPGT activity for 5 times in the same day. The results were calculated for mean ($\overline{X}$) and standard deviation (SD) and the % coefficient of variation (%CV) was calculated as following:

$$\%CV = \frac{SD}{\overline{X}} \times 100$$

For SULT, GST and NQOR activities assay, a cytosolic sample was used to determine SULT, GST and NQOR activities for 5 times in the same day. The results were calculated for $\overline{X}$, SD and thus %CV of the intraday precision as mentioned above.

1.2 Interday precision assay

The same microsomal and cytosolic samples in 1.1 were used to determine UDPGT, SULT, GST and NQOR activities daily for 5 days. Each day, each assay was performed in duplicate. The results were calculated for $\overline{X}$, SD and thus %CV of interday precision as mentioned aboved.

2. Linearity assay

Linearity assays for UDPGT, SULT, GST and NQOR were performed by analyzing activity of the enzymes using microsomal or cytosolic sample using various amounts of protein content in the sample. Coefficient of determination (r^2) between amounts of microsomal/cytosolic protein and the corresponding absorbance of the final reaction product was then calculated. Linearity assays of each enzyme were described in detail as following:

2.1 Linearity assay of the method for determination of UDPGT activity: Liver microsome was prepared from β-naphthoflavone-induced rat (as mentioned above in 2.1 page 49). Using various amounts of microsomal protein (200, 400, 600 and 800 µg) per 0.5 ml of the reaction mixture, the reactions were performed as mentioned above for UDPGT activity assay. Coefficient of determination between amounts of microsomal protein and the corresponding spectrophotometrically absorbance of the final product solutions was calculated.

2.2 Linearity assay of the method for determination of SULT activity: Liver cytosol was prepared from normal rat (as mentioned above in 2.1 page 49). Using various amounts of cytosolic protein(188, 375, 750, 1125 and 1275 µg) per 1.5 ml of the reaction mixture, the reactions were performed as mentioned above for SULT activity assay. Coefficient of determination between amounts of cytosolic protein and the corresponding spectrophotometrically absorbance of the final product solutions was calculated.

2.3 Linearity assay of the method for determination of GST activity: Liver cytosol was prepared from phenobarbital-induced rat (as mentioned above in 2.1 page 49). Using various amounts of cytosolic protein (368, 553, 737, 921 and 1106 µg) per 1 ml of the reaction mixture, the reactions were performed as mentioned above for GST activity assay. Coefficient of determination between amounts of cytosolic protein and the corresponding spectrophotometrically absorbance of the final product solutions was calculated.

2.4 Linearity assay of the method for determination of NQOR activity: Liver cytosol was prepared from estradiol-induced rat (as mentioned above in 2.1 page 49). Using various amounts of cytosolic protein (100, 200, 300, 400, 500 and 600 µg) per 3 ml of the reaction mixture, the reaction were performed as mentioned above for NQOR activity assay. Coefficient of determination between amounts of cytosolic protein and the corresponding spectrophotometrically absorbance of the final product solutions was calculated.

3. Capacity of the methods to determine an inducing effect of an inducer:

3.1 Capacity of the UDPGT activity assay to determine an inducing effect of a known UDPGT inducer: Liver microsomes were prepared from control rats and β-naphthoflavone-induced rats as mentioned above in 2.1 page 49. The reactions were performed as mentioned above for UDPGT activity assay using *p*-nitrophenol as a substrate.

3.2 Capacity of the SULT activity assay to determine an inducing effect of a known SULT inducer: Liver cytosols were prepared from control rats and estradiol valerate-induced rats as mentioned above in 2.1 page 49. The reactions were performed as mentioned above for SULT activity assay using 2-naphthol as a substrate.

3.3 Capacity of the GST activity assay to determine an inducing effect of a known GST inducer: Liver cytosols were prepared from control rats and phenobarbital-induced rats as mentioned above in 2.1 page 49. The reactions were performed as mentioned above for GST activity assay using CDNB as a substrate.

3.4 Capacity of the NQOR activity assay to determine an inducing effect of a known NQOR inducer: Liver cytosols were prepared from control rats and estradiol valerate-induced rats as mentioned above. The reactions were performed as mentioned above for NQOR activity assay using DCPIP as a substrate.

7. Statistical analysis

The data were reported as means ($\overline{X}$) ± standard error of the means (SEM). Mean differences between *C. comosa* treated groups and control group were compared using one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls test and $p < 0.05$ was considered to be significant.

Mean difference between induced group and control group was compared using Independent T-Test and $p < 0.05$ was considered to be significant.

CHAPTER IV

RESULTS

1. Verification of the methods used for enzyme activity assay

1.1 Precision assay

Precision of the assay for determination of UDPGT, SULT, GST and NQOR activities as shown by %CV for the intra-day and inter-day precisions were shown in table 12

1.2 Linearity assay

Linearity of each enzyme assay was determined and the correlation was shown by r^2. For UDPGT, SULT, GST and NQOR activity assay, r^2 of the assays were 0.9976, 0.9954, 0.9547 and 0.9953, respectively (Figure 2-5)

1.3 Capacity of the methods to determine the inducing effect of an inducer

The methods used in this study such as UDPGT, SULT, GST and NQOR activity assays could detect the inductive effect caused by the known inducer. β-naphthoflavone was shown to significantly induce UDPGT activity (Figure 6). Estradiol valerate significantly induced SULT activity (Figure 7) whereas phenobarbital significantly induced GST activity (Figure 8) and estradiol valerate significantly caused an increase of NQOR activity (Figure 9).

Table 12 Precision of the assay for determination of UDPGT, SULT, GST and NQOR activities.

Enzyme activity assay	**%CV**	
	Intraday precision	**Interday precision**
UDPGT	6.83	7.39
SULT	0.75	2.42
GST	5.45	5.69
NQOR	7.58	8.75

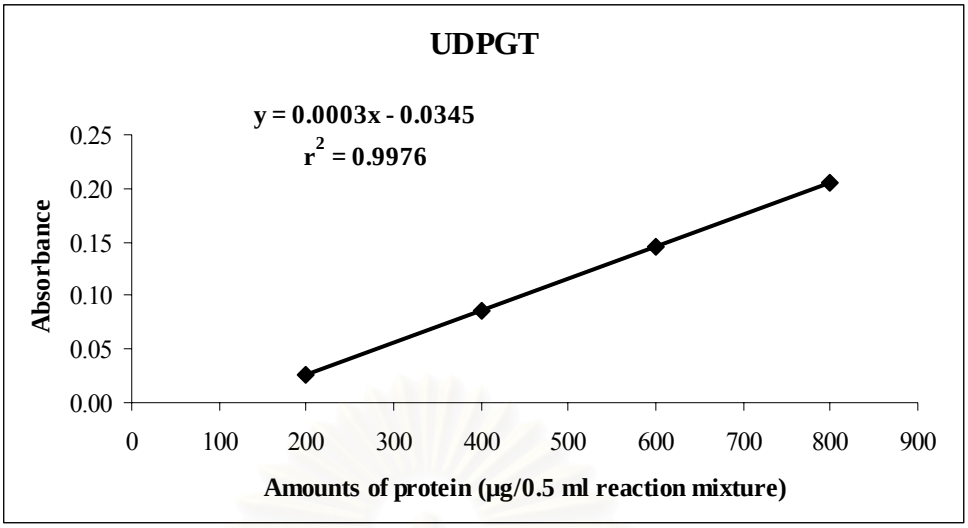


Figure 2 Linearity of the method for determination of UDPGT activity. The line was acquired by plotting the amounts of microsomal protein (in the ranges of 200-800 µg/0.5 ml reaction mixture) against the corresponding spectrophotometrically absorbance. The correlation coefficient was 0.9976. Each point represented the mean of duplicated reactions.

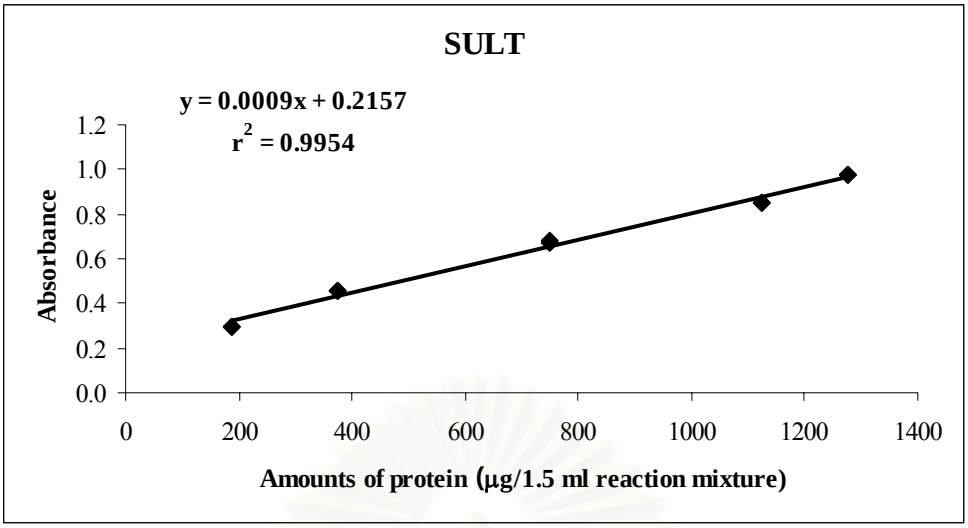


Figure 3 Linearity of the method for determination of SULT activity. The line was acquired by plotting the amounts of cytosolic protein in the ranges of 188-1275 µg/ 1.5 ml reaction mixture against the corresponding spectrophotometrically absorbance. The correlation coefficient was 0.9954. Each point represented the mean of duplicated reactions.

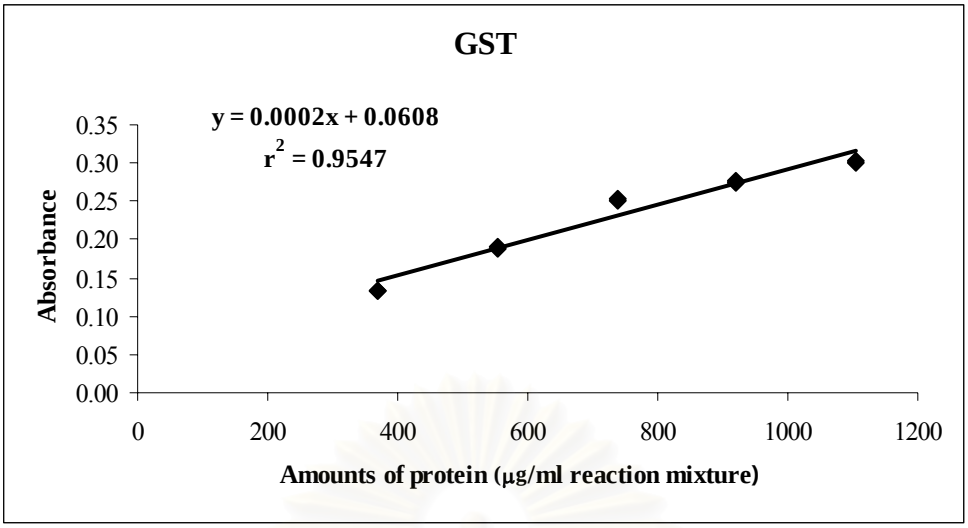


Figure 4 Linearity of the method for determination of GST activity. The line was acquired by plotting the amounts of cytosolic protein (in the ranges of 368-1106 μg/ml reaction mixture) against the corresponding spectrophotometrically absorbance. The correlation coefficient was 0.9547. Each point represented the mean of duplicated reactions.

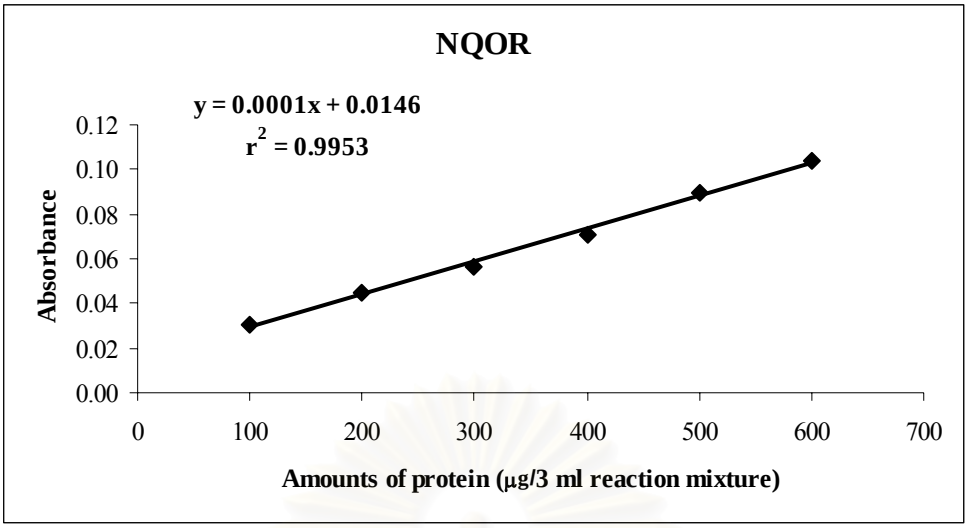


Figure 5 Linearity of the method for determination of NQOR activity. The line was acquired by plotting the amounts of cytosolic protein (in the ranges of 100-600 μg/ 3 ml reaction mixture) against the corresponding spectrophotometrically absorbance. The correlation coefficient was 0.9953. Each point represented the mean of duplicated reactions.

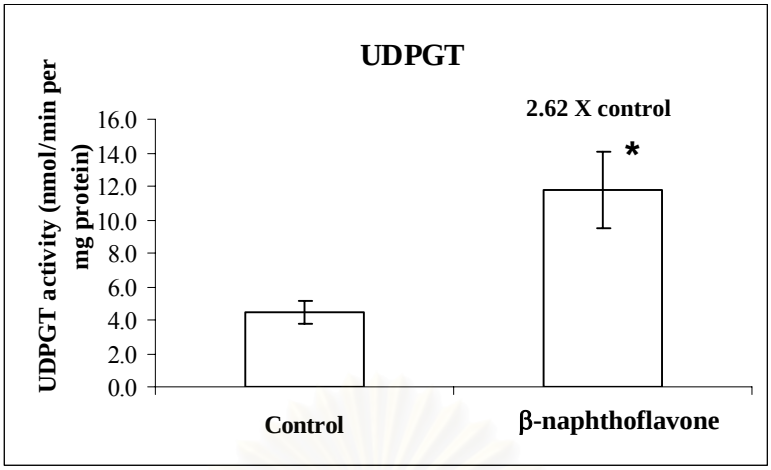


Figure 6 Effect of an inducer, β-naphthoflavone on UDPGT activity in rats. Rats were injected intraperitoneally with corn oil (1ml/kg/day) in the control group or β-naphthoflavone (80 mg/kg/day) in the induced group, daily for 3 consecutive days. Data were presented as mean ± SEM of 4 rats/group. For statistical analysis, Independent T-Test was carried out, $p < 0.05$ was considered statistically significant.
*$p < 0.05$; β-naphthoflavone-treated group vs control group

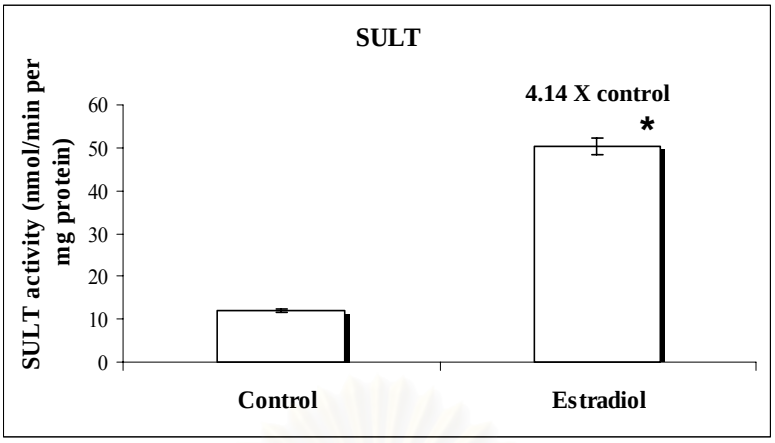


Figure 7 Effect of an inducer, estradiol valerate on SULT activity in rats. Rats were injected intramuscularly with corn oil (1 ml/kg/day) in the control group and estradiol valerate (500 µg/kg/day) in the induced group, daily for 7 consecutive days. Data were presented as mean ± SEM of 4 rats/group. For statistical analysis, Independent T-Test was carried out, $p < 0.05$ was considered statistically significant.

* $p < 0.05$; estradiol-treated group vs control group

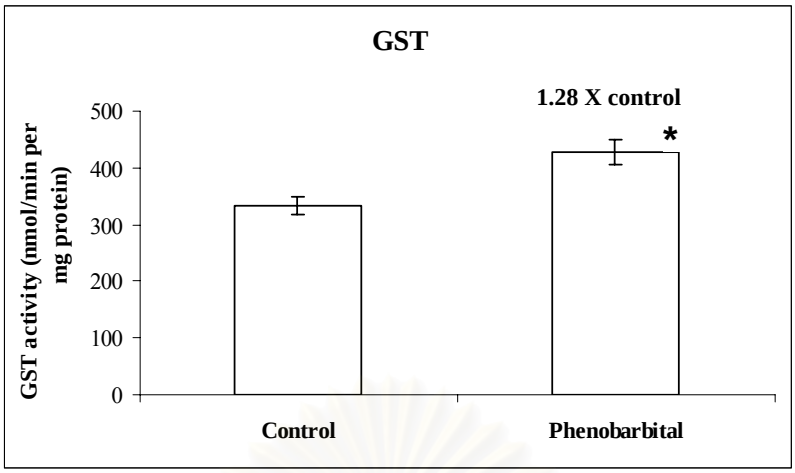


Figure 8 Effect of an inducer, phenobarbital on GST activity in rats. Rats were injected intraperitoneally with normal saline (1 ml/kg/day) in the control group or phenobarbital (80 mg/kg/day) in the induced group, daily for 3 consecutive days. Data were presented as mean ± SEM of 4 rats/group. For statistical analysis, Independent T-Test was carried out, $p < 0.05$ was considered statistically significant.

*$p < 0.05$; phenobarbital-treated group vs control group

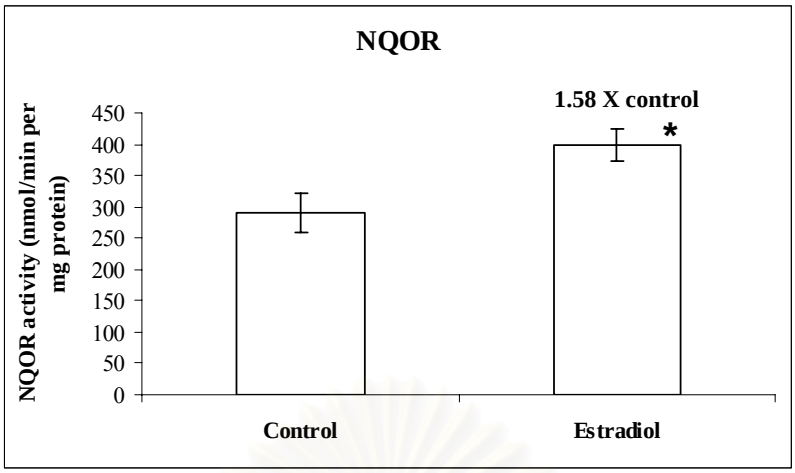


Figure 9 Effect of an inducer, estradiol valerate on NQOR activity in rats. Rats were injected intramuscularly with corn oil (1 ml/kg/day) in the control group and estradiol valerate (500 μg/kg/day) in the induced group, daily for 7 consecutive days. Data were presented as mean ± SEM of 4 rats/group. For statistical analysis, Independent T-Test was carried out, $p < 0.05$ was considered statistically significant.

* $p < 0.05$; estradiol-treated group vs control group

2. Effect of *C. comosa* hexane extract and ethanolic extract on phase II drug metabolizing enzymes

Oral administration of *C. comosa* extracts to rats daily for 30 consecutive days demonstrated different effects on phase II drug metabolizing enzymes. For UDPGT, *C. comosa* hexane extract caused an increase of UDPGT activity at both doses given to rats whereas *C. comosa* ethanolic extract did not affect this enzyme activity (Figure 10). Both *C. comosa* hexane extract and ethanolic extract did not modulate the activities of SULT (Figure 11) and GST (Figure 12). For NQOR, only *C. comosa* hexane extract at the dosage of 500 mg/kg/day caused an increase of NQOR activity whereas the lower doses (250 mg/kg/day) of *C. comosa* hexane extract and both doses of *C. Comosa* ethanolic extracts did not affect the activity of this enzyme (Figure 13).

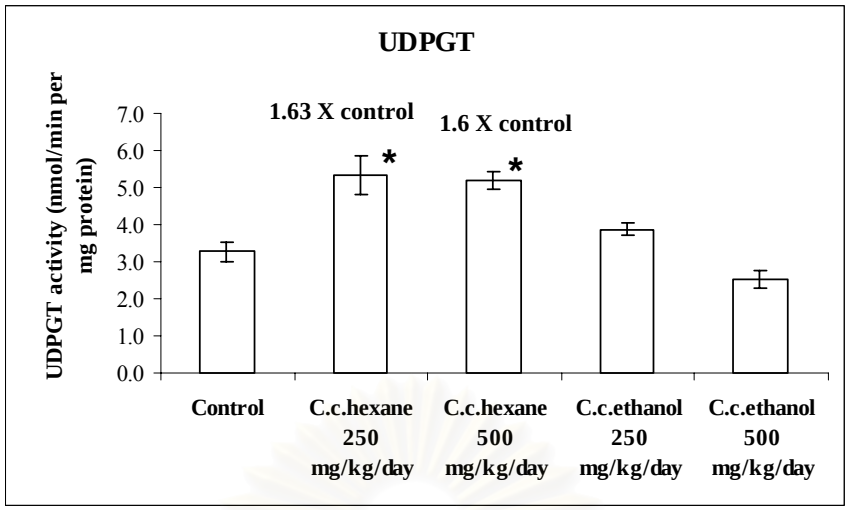


Figure 10 Effects of *C. comosa* hexane extract and ethanolic extract on UDPGT activity in rats. Rats were treated orally with corn oil (1 ml/kg/day) in the control group, *C. comosa* hexane extracts (250 or 500 mg/kg/day) and *C. comosa* ethanolic extracts (250 or 500 mg/kg/day) for 30 consecutive days. Data were presented as mean ± SEM of 9-10 rats/group. For statistical analysis, one-way ANOVA and Student-Newman-Keuls test were carried out, $p < 0.05$ was considered statistically significant.

* $p < 0.05$, *C. comosa* treated group vs control group.

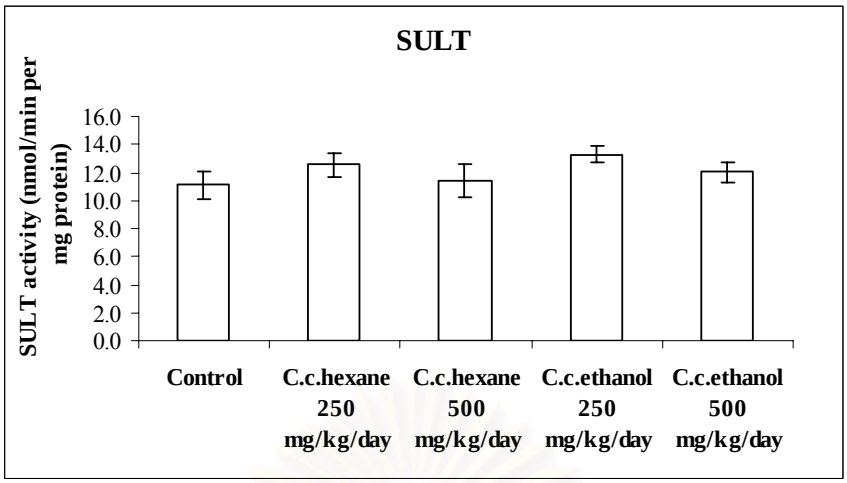


Figure 11 Effects of *C. comosa* hexane extract and ethanolic extract on SULT activity in rats. Rats were treated orally with corn oil (1 ml/kg/day) in the control group, *C. comosa* hexane extracts (250 or 500 mg/kg/day) and *C. comosa* ethanolic extracts (250 or 500 mg/kg/day) for 30 consecutive days. Data were presented as mean ± SEM of 9-10 rats/group. For statistical analysis, one-way ANOVA and Student-Newman-Keuls test were carried out, $p < 0.05$ was considered statistically significant.

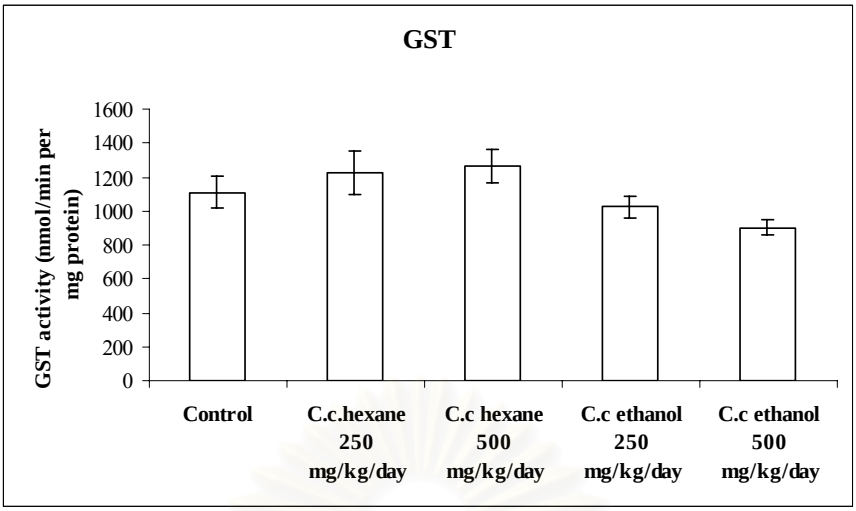


Figure 12 Effects of *C. comosa* hexane extract and ethanolic extract on GST activity in rats. Rats were treated orally with corn oil (1 ml/kg/day) in the control group, *C. comosa* hexane extracts (250 or 500 mg/kg/day) and *C. comosa* ethanolic extracts (250 or 500 mg/kg/day) for 30 consecutive days. Data were presented as mean ± SEM of 9-10 rats/group. For statistical analysis, one-way ANOVA and Student-Newman-Keuls test were carried out, $p < 0.05$ was considered statistically significant.

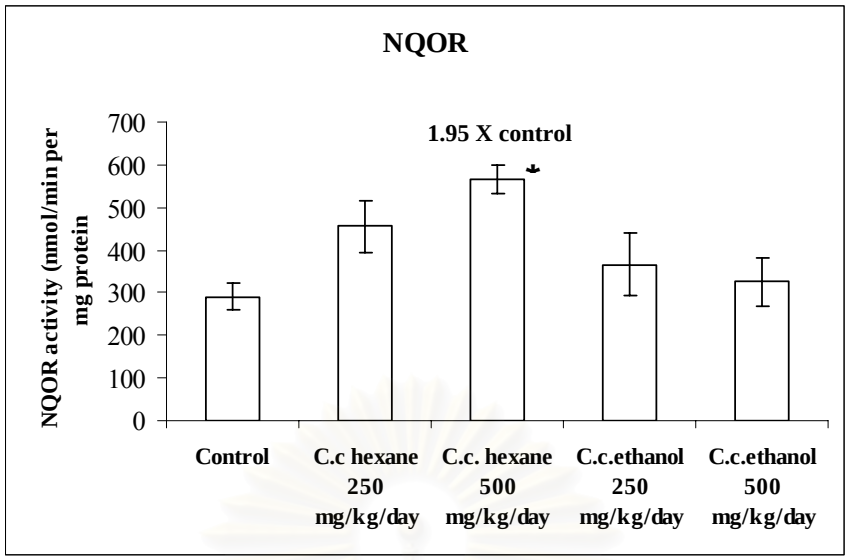


Figure 13 Effects of *C. comosa* hexane extract and ethanolic extract on NQOR activity in rats. Rats were treated orally with corn oil (1 ml/kg/day) in the control group, *C. comosa* hexane extracts (250 or 500 mg/kg/day) and *C. comosa* ethanolic extracts (250 or 500 mg/kg/day) for 30 consecutive days. Data were presented as mean ± SEM of 9-10 rats/group. For statistical analysis, one-way ANOVA and Student-Newman-Keuls test were carried out, $p < 0.05$ was considered statistically significant.

* $p < 0.05$, *C. comosa* treated group vs control group.

CHAPTER V

DISCUSSION AND CONCLUSION

This study primarily investigated effect of *C. comosa* on phase II drug metabolizing enzymes, UDPGT, SULT, GST and NQOR. This would partly give preliminary information of *C. comosa* in term of drug-drug interaction potential and a potential either to increase risk of xenobiotic induced mutagenesis/carcinogenesis or, in the opposite way to afford antimutagenic/anticarcinogenic effects against xenobiotic induced carcinogenesis. In this study, *C. comosa* hexane and ethanolic extracts were administered to rats at dosages of 250 and 500 mg/kg/day, the dosages which were shown to possess choleretic effect (Piyachaturawat et al., 1999, 2000), as well as estrogenic effect (Piyachaturawat et al., 1995, 1999). The major constituent of hexane extract was 1,7-diphenyl-5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene. The major constituents of ethanolic extract were 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene. Inducing of CYP1A1 activity by both *C. comosa* hexane extract (given at the doses of 250 and 500 mg/kg/day) and ethanolic extract given at the dose of 250 mg/kg/day has been reported. An increase of CYP1A1 activity by *C. comosa* is of interest regarding the concern of *C. comosa* administration that may cause an increased risk of human to several environmental-induced mutagenesis/ carcinogenesis (Kittichanun, 2006). CYP1A1 is an important enzyme that play a key role in the metabolic activation of a large group of polycyclic aromatic hydrocarbons (PAHs) to biologically reactive metabolites that interact with DNA, resulting in carcinogenesis (Gibson et al., 2001). CYP2B1/2B2 activities were significantly increased in rats treated with both *C. comosa* hexane extract and ethanolic extract (Kittichanun et al., 2006). Actually CYP2B1/2B2 are not expressed in human liver. Amino acid sequence homology of human CYP2B6 is closet to rat CYP2B1/2B2. CYP2B6 plays a major role in metabolism of cyclosporine, cyclofosfamide and ifosfamide. CYP2B6 also associates with the metabolic activation of procarcinogen such as 6-aminochrysene and styrene resulting in the chemical-induced carcinogenesis (Omiecinski et al., 1998). To investigate effects of *C. comosa* on phase II drug metabolizing enzymes responsible for drug metabolism as well as carcinogen detoxification, and *ex vivo* study was performed using *C. comosa* treated rat livers for

the preparations of cytosols and microsomes. Enzyme induction and some forms of enzyme inhibition can be simultaneously investigated by this experimental model. The results showed that *C. comosa* hexane extract at dosages of 250 and 500 mg/kg/day increased the activity of UDPGT and 500 mg/kg/day of *C. comosa* hexane extract increased NQOR activity. Mostly, UDPGT plays a major role in the detoxification of drugs and xenobiotics either the parent compounds or their metabolites from phase I metabolism. Such drugs/xenobiotics or their metabolites that are detoxified by UDPGT include morphine, 4-methylumbelliferone, eugenol, 2-naphthol, chloramphenicol, androsterone (Iwamura et al., 2005). NQOR is also a detoxification enzyme in the flavin monooxygenase reactions. Drugs/xenobiotics that are detoxified by NQOR include *p*-benzoquinone, 1,4-naphthoquinone, 2,6-dichlorophenol-indophenol, methyleneblue (Ernster, 1990). Thus, an increase of UDPGT and NQOR by *C. comosa* hexane extract indicated an advantage effect of this extract in term of a decrease of toxicity, antimutagenic and/or anticarcinogenic effect against many drugs and/or xenobiotics. However, an increase of UDPGT and NQOR by *C. comosa* might be concerned in term of drug-drug interaction resulting in a decrease efficacy of many drugs that are metabolized by these 2 enzymes. Such example included administration of some drugs that are metabolized by UDPGT (such as chloramphenicol, ketoprofen, ibuprofen) and NQOR (such as α-tocopherol, CoQ_{10}, quinoid compounds). No effects of *C. comosa* hexane and ethanolic extracts on both SULT and GST activities. Generally, SULT is a family of enzyme responsible for detoxification of many drugs/xenobiotics and/or their metabolites. Examples of drugs/xenobiotics that are detoxified by SULT include 2-naphthol, 4-phenylphenol, *p*-nitrophenol and estradiol (Coughtrie et al., 2001). An exception is also reported with the opposite role of SULT to bioactivate substances resulting in reactive metabolites which are toxic. The example for this issue is 2-AAF which is bioactivated by SULT resulting in sulfation of *N*-hydroxy-acetylaminofluorene which is non-stable and liver carcinogenic (Gibson et al., 2002). For GST, a family of enzymes that play a major role in detoxification of most electrophilic metabolites from phase I metabolism. Examples of reactive metabolites that are detoxified by glutathione conjugate, that is catalyzed by GST are aflatoxin B1-epoxide, *trans*-4-phenyl-3-buten-2-one, benzpyrene diols (David et al., 1983; Wallin et al., 1988). Therefore, no effect of *C. comosa* extracts on both SULT and GST activities indicated no advantage effects of these extracts regarding antimutagenic/anticarcinogenic/

protective effects against other xenobiotic toxicities. The findings of the effects of *C. comosa* on phase II enzymes found in this study provided a preliminary information in animals. Inter-animal variability of these enzymes (UDPGT, SULT, GST and NQOR) is normally existed (Coleman et al., 2005). Interpretation of these data from animals to human should be concerned. In addition, effects of *C. comosa* extracts were investigated only in some important phase II enzymes, effect of these extracts on other phase II enzymes should be further explored.

Before using the methods for determination the activities of UDPGT, SULT, GST and NQOR. Verifications of the methods were performed. Precision and linearity of the methods were assured. The results showed that both intraday and interday precision of all the methods as shown by %CV were all less than 10% which were generally accepted if %CV less than 15% (United States Pharmacopeia, 1995). Linearity of the assays were also strongly satisfied with very high correlation between the amount of microsomal protein/cytosolic protein and the absorbance of final product solution. In addition, capacity of the methods to detect inductive effects of a known inducer was also investigated. UDPGT assay method could detect an inductive effect of a positive inducer, β-naphthoflavone (Soucek and Gut, 1992; Tredger et al., 2002). SULT assay method could detect an inductive effect of a known SULT inducer, estradiol valerate (Seth et al., 2000; Pasqualini et al., 2005). GST assay method could detect an inductive effect of a known GST inducer, phenobarbital (Soucek and Gut, 1992; Tredger et al., 2002) whereas NQOR assay method could detect an inductive effect of a known NQOR inducer, estradiol valerate (Sonia et al., 2004; Kobayashi et al., 2005).

In conclusion, subacute effects of *C. comosa* on phase II drug metabolizing enzymes (UDPGT, GST, SULT, NQOR) were investigated. Two doses (250 and 500 mg/kg/day) of hexane and ethanolic extracts were given orally to male rats for 30 days compared to a control group given corn oil in the same manner. The results showed that *C. comosa* hexane extract at doses of 250 and 500 mg/kg/day increased UDPGT activity and only the 500 mg/kg/day of *C. comosa* hexane extract increased NQOR activity. The activities of GST and SULT were not significantly different between the treatment and the control group. Further studies on the effect of *C. comosa* in long term used as well as mechanism of induction of phase II drug metabolizing enzymes was suggests. Effects of these extracts on another phase II drug metabolizing enzymes should also be explored.

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APPENDICES

Appendix A
Body weight and liver weight

Table A1 Body weight of an individual rat in the control group

Rat No.	Day 0	Day 7	Day 14	Day 21	Day 28
1	301.80	302.20	331.00	354.20	359.30
2	297.60	312.00	330.20	351.20	366.00
3	305.60	316.50	346.40	355.00	378.50
4	285.40	315.50	334.50	348.00	372.00
5	305.00	328.70	348.10	365.00	384.50
6	336.20	356.50	374.60	385.90	402.00
7	339.60	359.20	382.60	397.00	407.50
8	335.10	343.60	354.50	367.00	369.00
9	387.00	412.10	421.40	456.00	469.20
10	333.50	355.40	369.20	379.50	389.30
Average	322.68	340.17	359.25	375.88	389.73
SEM	9.39	10.32	9.01	10.26	10.09

Unit express as g

Table A2 Body weight of an individual rat in the *C. comosa* hexane extract 250 mg/kg/day group

Rat No.	Day 0	Day 7	Day 14	Day 21	Day 28
1	280.00	299.50	307.20	331.30	334.10
2	300.70	306.10	302.60	329.50	332.70
3	283.40	313.30	326.30	338.00	345.00
4	279.20	307.50	309.70	319.00	322.00
5	281.50	288.30	284.10	278.70	289.00
6	289.70	307.60	306.50	312.60	315.50
7	295.80	300.20	304.00	313.70	315.50
8	318.00	323.90	324.20	317.50	307.50
9	355.50	366.60	381.60	386.60	307.50
10	304.00	310.90	316.30	312.00	392.50
Average	298.78	312.39	316.25	323.89	326.13
SEM	7.44	6.71	8.18	8.63	8.93

Unit express as g

Table A3 Body weight of an individual rat in the *C. comosa* hexane extract 500 mg/kg/day group

Rat No.	Day 0	Day 7	Day 14	Day 21	Day 28
1	288.20	303.20	299.50	305.80	300.40
2	309.80	293.80	301.00	305.00	316.10
3	305.50	310.00	316.20	329.00	311.90
4	285.80	320.50	333.80	342.40	352.90
5	343.60	345.00	353.40	366.10	367.00
6	314.00	322.20	330.90	324.50	329.00
7	360.70	366.30	381.30	387.00	387.50
8	332.60	332.20	337.00	343.50	321.00
9	353.00	362.20	361.10	360.50	-
Average	321.47	328.38	334.91	340.42	338.67
SEM	9.11	8.45	9.09	9.19	9.89

Unit express as g

(-) Missing value = rat was dead betweentimes of the study.

Table A4 Body weight of an individual rat in the *C. comosa* ethanolic extract 250 mg/kg/day group

Rat No.	Day 0	Day 7	Day 14	Day 21	Day 28
1	279.90	285.10	320.20	343.30	357.00
2	284.30	324.00	342.70	363.60	343.80
3	292.20	308.40	323.90	326.80	330.50
4	301.50	317.20	326.70	345.50	355.90
5	344.50	305.50	327.20	348.00	331.00
6	338.90	355.70	371.30	383.60	392.50
7	297.40	310.30	330.00	334.00	347.50
8	331.20	344.10	369.60	331.90	357.00
9	315.00	332.80	346.00	380.00	391.30
10	323.40	340.70	348.50	350.00	345.60
Average	310.83	322.38	340.61	350.67	355.21
SEM	7.29	6.73	5.83	6.15	6.81

Unit express as g

Table A5 Body weight of an individual rat in the *C. comosa* ethanolic extract 500 mg/kg/day group

Rat No.	Day 0	Day 7	Day 14	Day 21	Day 28
1	298.60	294.00	306.20	320.00	342.60
2	307.20	304.00	324.00	336.00	351.50
3	313.40	321.30	355.70	372.00	384.00
4	305.00	315.60	330.30	335.90	334.00
5	323.70	329.30	336.00	344.10	356.00
6	348.20	361.40	371.00	380.80	387.90
7	287.50	297.40	301.40	306.00	314.00
8	321.30	330.30	342.40	349.50	355.00
9	365.30	386.30	392.50	398.40	401.00
10	319.00	326.10	340.00	350.10	360.00
Average	318.91	326.62	339.94	349.19	358.44
SEM	8.16	10.12	9.84	9.88	9.30

Unit express as g

Table A6 Liver weight of individual rat

Rat No.	Control	*C. comosa* extract groups			
		C.c.hexane extract 250 mg/kg/day	C.c.hexane extract 500 mg/kg/day	C.c.ethanolic extract 250 mg/kg/day	C.c.ethanolic extract 500 mg/kg/day
1	12.32	13.48	12.26	13.99	11.39
2	13.18	15.31	14.85	11.25	11.94
3	10.61	12.64	11.53	10.42	12.18
4	9.95	12.10	12.79	11.09	10.97
5	10.25	8.82	13.56	9.82	12.55
6	10.00	10.51	12.78	11.33	10.32
7	11.69	10.92	16.00	10.90	9.55
8	8.74	12.47	13.86	10.00	13.98
9	15.49	19.46	-	13.00	13.94
10	13.70	13.07	17.55	10.61	13.68
Average	11.59	12.88	13.91	11.24	12.05
SEM	0.66	0.92	0.65	0.41	0.48

Unit express as g

(-) Missing value = rat was dead betweentimes of the study.

Appendix B

Verification of the methods for determination of UDPGT, SULT, GST and NQOR activities

Table B1 Intra-day and inter-day precisions of the assay for determination of UDPGT activity

Number of the assay	UDPGT activity (nmol/min per mg protein)	
	Intra-day precision	Inter-day precision
1	0.120	0.100
2	0.115	0.120
3	0.113	0.115
4	0.100	0.113
5	0.108	0.121
Mean	0.111	0.114
SD	0.007	0.008
%CV	6.830	7.389

Table B2 Intra-day and inter-day precisions of the assay for determination of SULT activity

Number of the assay	SULT activity (nmol/min per mg protein)	
	Intra-day precision	Inter-day precision
1	0.409	0.414
2	0.411	0.428
3	0.407	0.411
4	0.414	0.400
5	0.414	0.414
Mean	0.411	0.413
SD	0.003	0.010
%CV	0.750	2.416

Table B3 Intra-day and inter-day precisions of the assay for determination of GST activity

Number of the assay	GST activity (nmol/min per mg protein)	
	Intra-day precision	Inter-day precision
1	0.174	0.180
2	0.179	0.162
3	0.160	0.177
4	0.175	0.179
5	0.159	0.160
Mean	0.170	0.172
SD	0.009	0.010
%CV	5.452	5.689

Table B4 Intra-day and inter-day precisions of the assay for determination of NQOR activity

Number of the assay	NQOR activity (nmol/min per mg protein)	
	Intra-day precision	Inter-day precision
1	0.073	0.073
2	0.074	0.074
3	0.088	0.064
4	0.079	0.060
5	0.080	0.068
Mean	0.079	0.068
SD	0.006	0.006
%CV	7.582	8.751

Table B5 Linearity of the method for determination of UDPGT activity in rats

Microsome concentration (μg)	Δ absorbance
200	0.026
400	0.086
600	0.146
800	0.206

Table B6 Linearity of the method for determination of SULT activity in rats

Cytosol concentration (μg)	Δ absorbance
188	0.298
375	0.466
750	0.678
1125	0.858
1275	0.979

Table B7 Linearity of the method for determination of GST activity in rats

Cytosol concentration (μg)	Δ absorbance
368	0.133
553	0.190
737	0.253
921	0.276
1106	0.302

Table B8 Linearity of the method for determination of NQOR activity in rats

Cytosol concentration (μg)	Δ absorbance
100	0.031
200	0.045
300	0.057
400	0.071
500	0.090
600	0.104

Table B9 Effect of an inducer, β-naphthoflavone on UDPGT activity in rats

No.rat	UDPGT activity (nmol/min per mg protein)	
	Control	**β-naphthoflavone**
1	3.28	21.15
2	4.00	6.30
3	4.03	12.82
4	6.57	6.67
Mean ± SEM	4.47 ± 0.67	11.73* ± 2.28

Table B10 Effect of an inducer, estradiol valerate on SULT activity in rats

No.rat	SULT activity (nmol/min per mg protein)	
	Control	**Estradiol**
1	12.09	50.66
2	11.24	44.34
3	12.75	53.23
4	12.40	52.86
Mean ± SEM	12.12 ± 0.32	50.27* ± 2.06

Table B11 Effect of an inducer, phenobarbital on GST activity in rats

No.rat	GST activity (nmol/min per mg protein)	
	Control	**Phenobarbital**
1	318.93	409.66
2	350.22	379.49
3	370.37	444.50
4	298.79	418.79
Mean ± SEM	334.58 ± 15.94	428.11* ± 21.49

Table B12 Effect of an inducer, estradiol valerate on NQOR activity in rats

No.rat	NQOR activity (nmol/min per mg protein)	
	Control	**Estradiol**
1	251.43	417.14
2	280.00	400.00
3	285.71	462.86
4	291.43	468.57
Mean ± SEM	277.14 ± 7.70	437.14* ± 16.90

Appendix C
Enzyme activity study

Table C1 Microsomal protein concentration of individual rat

Rat No.	Control	*C. comosa* extract treated groups			
		C.c.hexane extract 250 mg/kg/day	C.c.hexane extract 500 mg/kg/day	C.c.ethanolic extract 250 mg/kg/day	C.c.ethanolic extract 500 mg/kg/day
1	30.56	45.96	46.26	38.56	40.96
2	16.26	37.46	41.66	30.26	37.26
3	42.46	42.76	54.96	43.26	43.86
4	42.36	35.96	52.76	63.23	47.36
5	28.86	35.06	50.56	46.36	46.16
6	26.23	44.56	37.96	39.46	53.26
7	62.86	60.16	67.66	50.56	68.06
8	50.46	53.06	72.16	53.96	47.66
9	51.69	53.76	-	63.26	68.76
10	49.76	45.96	74.26	58.46	71.31
Average	40.15	45.47	55.36	48.74	52.46
SEM	4.29	2.48	3.94	3.34	3.73

Unit expressed as mg/ml

(-) Missing value = rat was dead betweentimes of the study

Table C2 Cytosolic protein concentration of individual rat

Rat No.	Control	*C. comosa* extract treated groups			
		C.c.hexane extract 250 mg/kg/day	C.c.hexane extract 500 mg/kg/day	C.c.ethanolic extract 250 mg/kg/day	C.c.ethanolic extract 500 mg/kg/day
1	40.29	38.62	43.29	24.84	44.23
2	31.98	50.23	27.73	37.10	38.66
3	21.85	10.91	20.98	32.04	27.98
4	17.91	22.66	32.85	26.35	25.29
5	24.79	24.34	34.18	18.57	23.96
6	24.57	28.29	22.12	21.40	22.68
7	25.07	26.68	34.01	19.68	24.57
8	22.23	29.46	23.90	24.40	27.34
9	38.18	37.84	-	30.73	31.73
10	31.57	30.68	35.51	23.90	36.10
Average	27.84	29.97	30.51	25.90	30.25
SEM	2.32	3.35	2.45	1.85	2.28

Unit expressed as mg/ml

(-) Missing value = rat was dead betweentimes of the study

Table C3 Hepatic microsomal UDPGT activity of individual rat

Rat No.	Control	*C. comosa* extract treated groups			
		C.c.hexane extract 250 mg/kg/day	C.c.hexane extract 500 mg/kg/day	C.c.ethanolic extract 250 mg/kg/day	C.c.ethanolic extract 500 mg/kg/day
1	4.01	4.13	3.67	3.97	2.00
2	4.12	5.00	6.53	3.40	3.12
3	5.44	6.42	5.12	4.35	3.77
4	1.94	8.40	4.83	3.52	2.87
5	3.49	2.38	5.32	5.58	2.18
6	3.60	6.07	5.38	4.45	4.51
7	1.45	7.78	6.43	3.13	1.05
8	1.97	8.25	3.60	3.28	1.83
9	2.95	1.73	-	3.08	2.78
10	3.67	3.17	5.88	3.92	1.21
Average	3.27	5.33	5.20	3.87	2.53
SEM	0.27	0.54	0.24	0.17	0.24

Unit expressed as nmol/min per mg protein

(-) Missing value = rat was dead betweentimes of the study

Table C4 Hepatic cytosolic SULT activity of individual rat

Rat No.	Control	*C. comosa* extract treated groups			
		C.c.hexane extract 250 mg/kg/day	C.c.hexane extract 500 mg/kg/day	C.c.ethanolic extract 250 mg/kg/day	C.c.ethanolic extract 500 mg/kg/day
1	2.81	18.05	9.54	18.63	18.77
2	15.44	12.09	19.81	12.10	14.20
3	12.30	10.96	8.76	12.59	11.80
4	10.48	18.95	4.61	16.32	10.13
5	11.45	10.66	12.93	8.44	8.52
6	3.79	7.84	5.71	12.48	8.64
7	12.39	11.24	12.98	13.80	12.76
8	12.41	12.75	12.75	12.87	8.68
9	12.42	10.03	-	13.06	13.92
10	12.38	12.40	12.67	12.40	12.40
Average	11.09	12.54	11.37	13.27	12.00
SEM	0.97	0.84	1.20	0.59	0.78

Unit expressed as nmol/min per mg protein

(-) Missing value = rat was dead betweentimes of the study

Table C5 Hepatic cytosolic GST activity of individual rat

Rat No.	Control	*C. comosa* extract treated groups			
		C.c.hexane extract 250 mg/kg/day	C.c.hexane extract 500 mg/kg/day	C.c.ethanolic extract 250 mg/kg/day	C.c.ethanolic extract 500 mg/kg/day
1	484.77	971.00	732.70	1421.60	657.08
2	879.46	690.57	1763.66	728.61	778.83
3	1330.09	2033.68	1631.02	858.30	1027.52
4	1657.59	1510.09	1350.84	919.12	908.21
5	1374.04	1104.15	1211.42	1026.52	808.64
6	1125.61	1331.08	1222.03	1022.20	964.51
7	972.28	1058.09	1231.26	1206.81	1023.86
8	1054.32	1076.67	1183.32	1120.65	1080.15
9	1031.30	1321.35	-	864.38	965.17
10	1385.81	1018.58	1073.64	1072.18	798.39
Average	1111.07	1228.58	1266.65	1024.04	901.24
SEM	95.36	128.29	100.17	62.75	42.80

Unit expressed as nmol/min per mg protein

(-) Missing value = rat was dead betweentimes of the study

Table C6 Hepatic cytosolic NQOR activity of individual rat

Rat No.	Control	*C. comosa* extract treated groups			
		C.c.hexane extract 250 mg/kg/day	C.c.hexane extract 500 mg/kg/day	C.c.ethanolic extract 250 mg/kg/day	C.c.ethanolic extract 500 mg/kg/day
1	217.14	80.00	908.57	668.57	262.86
2	85.71	280.00	1417.14	822.86	342.86
3	382.86	457.14	411.43	234.29	314.29
4	462.86	628.57	548.57	228.57	600.00
5	251.43	451.43	640.00	388.57	788.57
6	280.00	685.71	611.43	474.29	325.71
7	337.14	297.14	582.86	314.29	314.29
8	222.86	582.86	508.57	297.14	308.57
9	285.71	457.14	-	74.29	354.29
10	377.14	640.00	662.86	154.29	377.14
Average	290.39	456.00	566.53	365.71	325.00
SEM	30.37	60.56	32.62	73.49	58.24

Unit expressed as nmol/min per mg protein

(-) Missing value = rat was dead betweentimes of the study

Appendix D

Study Protocol Approval by Ethic Committee of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

No. 99/2007

Study Protocol Approval

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

Study Title: EFFECTS OF *CURCUMA COMOSA* EXTRACTS ON RAT HEPATIC PHASE II DRUG METABOLIZING ENZYMES

Study Code: -

Centre: CHULALONGKORN UNIVERSITY

Principal Investigator : MISS NEERANART JIWAPORNKUPT

Protocol Date : December 7, 2006

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

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

Chairman of Ethics Committee: .....Withaya Janthasoot.....

(Withaya Janthasoot)

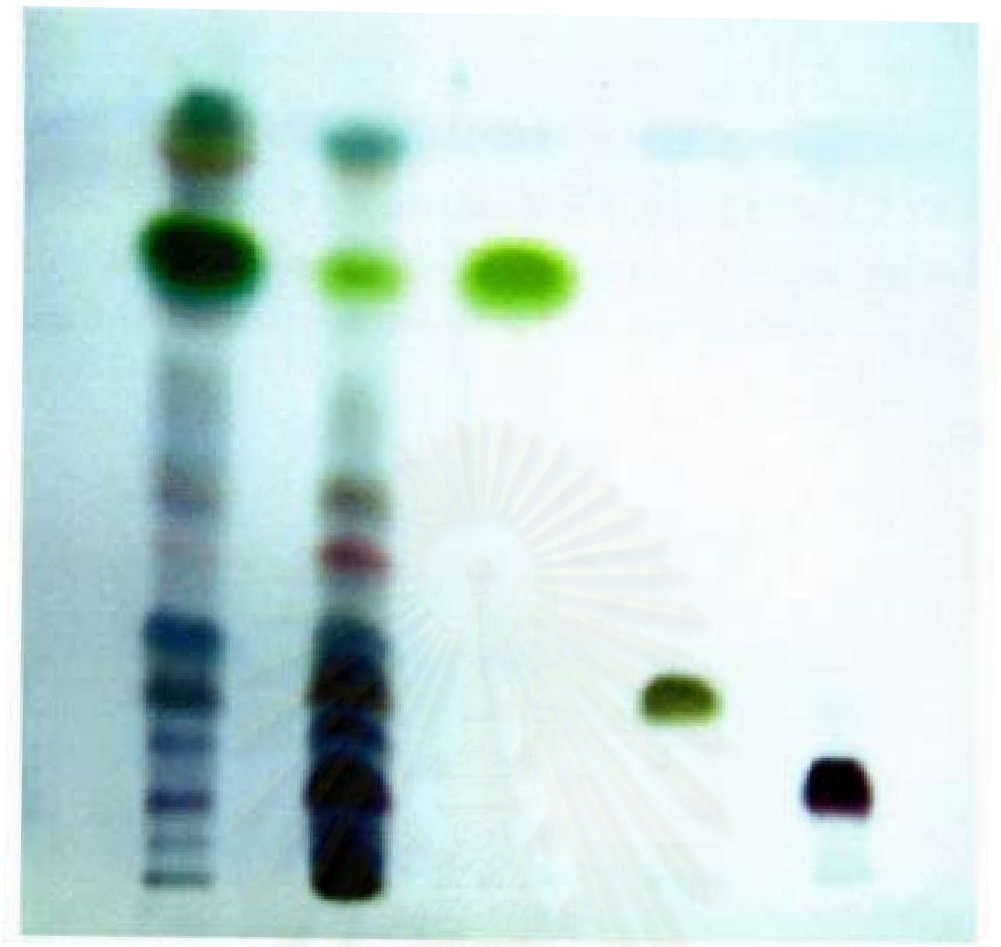
Secretary of Ethics Committee: .....Pasarapa Towiwat.....

(Pasarapa Towiwat, Ph.D.)

Date of Approval: January 29, 2007

Appendix E

Thin layer chromatography (TLC) of *C. comosa* extracts



Solvent system

CH_2Cl_2 : MeOH (40 : 1)

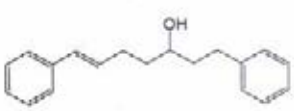
Spot 1 = *C. comosa* hexane extract 2.5 mg (250 μg/ml)

Spot 2 = *C. comosa* ethanolic extract 5.0 mg (500 μg/ml)

Spot 3 = trans-1,7-Diphenyl-5-hydroxy-1-heptene (1) 1.0 mg (100 μg/ml)

Spot 4 = 5-Hydroxy-7-(4-hydroxyphenyl)-1-phenyl-(1*E*)-1-heptene) (2) 1.0 mg (100 μg/ml)

Spot 5 = 7-(3,4-Dihydroxyphenyl)-5-hydroxy-1-phenyl-(1*E*)-1-heptene) (3) 1.0 mg (100 μg/ml)



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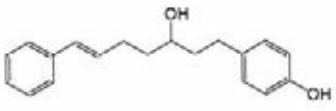

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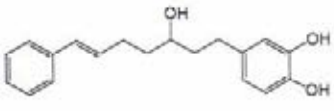

(3)

CURRICUM VITAE

Miss Neeranart Jiwapornkupt was born in June 3, 1981 in Suratthani, Thailand. She graduated with a Bachelor Degree of Pharmaceutical Sciences in 2003 from the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.