ผลของการได้รับกวาวเครือขาวแบบกึ่งเรื้อรังต่อเอนไซม์ไซโตโครมพี 450 ในตับและค่าชีวเคมีคลินิกในเลือดของหนูขาว

นางสาวกิตติยา เจริญกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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EFFECTS OF SUBCHRONIC EXPOSURE OF *PUERARIA MIRIFICA* ON HEPATIC CYTOCHROME P450 AND BLOOD CLINICAL BIOCHEMISTRY PARAMETERS IN RATS

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กวาวเครือขาว (*Pueraria mirifica* Airy Shaw and Suvatabandhu) เป็นสมุนไพรพื้นบ้านที่นิยมใช้เป็นยาอายุวัฒนะ การศึกษานี้มุ่งศึกษาผลของกวาวเครือขาวต่อเอนไซม์ไซโตโครม พี450 (CYP) ในตับ และค่าชีวเคมีคลินิกต่างๆ ในเลือดของหนูขาวพันธุ์เพศผู้พันธุ์วิสตาร์ โดยแบ่งหนูขาวแบบสุ่มเป็น 4 กลุ่ม กลุ่มละ 10 ตัว ดังต่อไปนี้ กลุ่มที่ได้รับอาหารปกติ, กลุ่มทีได้รับอาหารปกติและกวาวเครือขาว, กลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูง และกลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูงและกวาวเครือขาว หนูขาวได้รับกวาวเครือขาวขนาด 100 มิลลิกรัม/กิโลกรัม/วัน โดยวิธีป้อนทางปาก เป็นเวลา 90 วัน เมื่อครบระยะเวลา ทำให้หนูหมดความรู้สึก เก็บตัวอย่างเลือดจากหัวใจเพื่อตรวจค่าโลหิตวิทยาและแยก ซีรั่มตรวจค่าชีวเคมีคลินิก นำตับมาเตรียมไมโครโซม เพื่อวัดค่าสมรรถนะของเอนไซม์ ผลการทดลองพบว่ากวาวเครือขาวทำให้การเพิ่มของน้ำหนักหนูขาวต่ำกว่ากลุ่มควบคุม แต่ไม่มีผลต่อค่าโลหิตวิทยาและค่าชีวเคมีคลินิกดังต่อไปนี้ hemoglobin, hematocrit, WBC count, %differential WBC, platelet count, RBC morphology, glucose, BUN, SCr, total bilirubin และ direct bilirubin กวาวเครือขาวไม่มีผลต่อค่า AST, ALT และ ALP ในซีรั่มของหนูที่ได้รับอาหารปกติ อาหารคลอเรสเตอรอลสูงมีผลทำให้ AST, ALT และ ALP สูงแต่ค่าเหล่านี้ลดลงเมื่อให้กวาวเครือขาว กวาวเครือขาวทำให้ค่า total cholesterol และ LDL-C ในซีรั่มลดลงอย่างมีนัยสำคัญทั้งในกลุ่มที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง ในขณะที่ค่าไตรกลีเซอไรด์สูงขึ้นอย่างมีนัยสำคัญในกลุ่มที่ได้รับอาหารปกติ แต่มีค่าลดลงในกลุ่มที่ได้รับอาหารคลอเรสเตอรอลสูง กวาวเครือขาวทำให้ค่า HDL-C ในซีรั่มลดลงอย่างมีนัยสำคัญทั้งกลุ่มที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง ส่วนอัตราส่วนของ LDL-C ต่อ HDL-C มีค่าต่ำลงอย่างมีนัยสำคัญเฉพาะในกลุ่มที่ได้รับอาหารคลเอรสเตอรอลสูง สำหรับผลต่อสมรรถนะของเอนไซม์ CYP พบว่า กวาวเครือขาวมีผลยับยั้งสมรรถนะของ CYP2B1&2B2 ในหนูทั้งกลุ่มที่ได้รับอาหารปกติและอาหารคลอเรสเตอรอลสูง ส่วนสมรรถนะของ CYP1A2 และ CYP2E1 ลดลงเฉพาะในกลุ่มที่ได้รับกวาวเครือขาวร่วมกับอาหารปกติ กวาวเครือขาวไม่มีผลต่อสมรรถนะของ CYP1A1 เมื่อทำการทดสอบแบบ *in vitro* พบผลของกวาวเครือขาวในการยับยั้ง CYP2B1&2B2 และ CYP2E1 เช่นเดียวกัน ถึงแม้ว่ากวาวเครือขาวจะมีผลที่เป็นประโยชน์ต่อค่าไขมันในเลือดและไม่มีผลพิษใดๆ ต่อตับ ไต และระบบเลือด ผลที่ไม่พึงปรารถนาของกวาวเครือขาวที่พบคือมีผลเพิ่มไตรกลีเซอไรด์ในหนูที่ได้รับอาหารปกติ ผลของกวาวเครือขาวในการยับยั้ง CYP1A2, CYP2B1&2B2 และ CYP2E1 ชี้บ่งแนวโน้มในทางที่เป็นประโยชน์ของสารนี้ในเรื่องของการกระตุ้นฤทธิ์ของสารก่อมะเร็ง ควรทำการศึกษาต่อไปถึงผลของกวาวเครือขาวที่ขนาดต่างๆ ผลของการใช้สารนี้ในระยะเวลานาน รวมทั้งกลไกที่ใช้อธิบายผลที่เกิดขึ้น นอกจากนี้ควรทำการศึกษาผลของสารนี้ต่อCYP isoform อื่นๆ ที่ยังไม่ได้ทำการศึกษาด้วย

ภาควิชา เภสัชวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
ปีการศึกษา 2544	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

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KEYWORDS: *PUERARIA MIRIFICA*/ CYTOCHROME P450/ CHOLESTEROL LOWERING EFFECT/ BLOOD CLINICAL BIOCHEMISTRY PARAMETERS/ TOXICITY

KITTIYA CHAROENKUL: EFFECTS OF SUBCHRONIC EXPOSURE OF *PUERARIA MIRIFICA* ON HEPATIC CYTOCHROME P450 AND BLOOD CLINICAL BIOCHEMISTRY PARAMETERS IN RATS. THESIS ADVISOR: ASST. PROF. POL. LT. COL. DR. SOMSONG LAWANPRASERT, THESIS CO-ADVISOR: ASSOC. PROF. DR. SUPATRA SRICHAIRAT, 82 pp.

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Pueraria mirifica Airy Shaw and Suvatabandhu, known locally as Kwao Keur, is considered to be a rejuvenating folk medicine. In this study, the subchronic effects of *P.mirifica* on hepatic cytochrome P450 (CYP) and blood clinical biochemistry parameters were investigated in male Wistar rats. Rats were randomly divided into four treatment groups as following: normal diet-fed group; normal diet-fed supplemented with *P.mirifica* group; high cholesterol diet-fed group; high cholesterol diet-fed supplemented with *P.mirifica* group. Each group consisted of 10 rats. *P.mirifica* was administered orally at a dosage of 100 mg/kg/day for 90 consecutive days. At the end of the treatment, animals were anesthesized. Blood samples were collected by heart puncture and serum sample were determined for clinical biochemistry parameters. Microsomes were prepared from livers for enzyme assays. The results showed that body weight of rats given *P.mirifica* in either normal diet or high cholesterol diet conditions were significantly lower than their corresponding control groups. There was no significant difference of these following blood clinical biochemistry parameters: hemoglobin, hematocrit, WBC count, %differential WBC, platelet count, RBC morphology, glucose, BUN, SCr, total bilirubin, and direct bilirubin in all experimental groups. *P.mirifica* did not affect serum level of AST, ALT, and ALP in normal diet-fed condition. High cholesterol diet-fed condition caused a significant increase of AST, ALT, and ALP but *P.mirfica* helped attenuate these effects. *P.mirifica* significantly decreased serum total cholesterol and LDL-C in either normal diet-fed or high cholesterol diet-fed rats. Serum triglyceride was increased in normal diet-fed rats but decreased in high cholesterol diet-fed rats. *P.mirifica* caused a significant decrease of HDL-C in both normal and high cholesterol diet-fed rats whereas its improvement in the LDL-C/HDL-C ratio was shown only in high cholesterol diet-fed rats. Concerning the effects on CYPs, *P.mirifica* significantly inhibited CYP2B1&2B2 in either normal diet or high cholesterol diet-fed rats. Its inhibition effect of CYP1A2 and CYP2E1 was found only in normal diet-fed rats. No effect of *P.mirifica* was found on CYP1A1. Inhibition effects of *P.mirifica* on CYP2B1&2B2 and CYP2E1 were also found in the *in vitro* study. Although, *P.mirifica* demonstrated a benefit on lipid profile and did not show any toxic effects on liver, kidney, and blood system in this study, an increment of serum triglyceride in normal rat receiving *P.mirifica*, however, is not favorable. Inhibition effects of P.mirifica on CYP1A2, CYP2B1&2B2 and CYP2E1 indicated a beneficial potential of the compound on chemical-induced carcinogens via these enzymes. Effects of *P.mirifica* at various doses, long term used as well as mechanism of effects should be further investigated. Effects of this compound on other isoforms of CYP should also be explored.

Department of Pharmacology	Student's signature…….
Field of study Pharmacology	Advisor's signature……
Academic 2001	Co-advisor's signature…….

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List of Abbreviations

Ah receptor	= aliphatic hydrocarbon receptor
β	= beta
°C	= degree celcius
μl	= microlitre
β-NF	= beta-napthoflavone
α	= alpha
ALP	= alkaline phosphatase
ALT	= alanine aminotransferase
AST	= aspartate aminotransferase
BROD	= benzyloxyresorufin o-dealkylation
BSA	= bovine serum albumin
BUN	= blood urea nitrogen
CBC	= complete blood count
cm	= centimetre
CYP	= cytochrome P450
DMSO	= dimethylsulfoxide
EDTA	= ethylene diamine tetra acetic acid
EROD	= ethoxyresorufin o-dealkylation
et al.	= et alii (and other)
g	= gram
G6P	= glucose 6-phosphate
G6PD	= glucose 6-phosphate dehydrogenase
GST	= glutathione S-transferase
HDL-C	= high density lipoprotein cholesterol
i.p.	= intraperitonium
IC_{50}	= 50% inhibition concentration
kg	= kilogram
L	= litre
LD_{50}	= median lethal dose

LDL-C	= low density lipoprotein cholesterol
M	= molar
mg	= milligram
mg/kg	= milligram per kilogram body weight
mM	= millimolar
MROD	= methoxyresorufin o-dealkylation
NADP	= nicotinamide adenine dinucleotide phosphate (reduced form)
NADPH	= nicotinamide adenine dinucleotide phosphate
nm	= nanometer
nmol	= nanomole
pmol	= picromole
PROD	= pentoxyresorufin o-dealkylation
RBC	= red blood cell
SCr	= serum creatinine
SEM	= standard error of mean
TCA	= trichloroacetic acid
Tris	= tris (hydroxymethyl) aminomethane
UDPGT	= uridine-5'-diphospho-glucuronyltransferase
v/v	= volume by volume
w/v	= weight by volume
WBC	= white blood cell

CHAPTER I

INTRODUCTION

Pueraria mirifica Airy Shaw and Suvatabandhu, known locally as “Kwao Keur”, has been used as a rejuvenating medicine suggested for adult not recommended for young people. Several indications of this plant were suggested for a traditional purpose such as skin enrichment, thickening and blackening hair, a relief of weakness, an increase of an appetite, treatment of insomnia, and breast enlargement in women (หลวงอนุสารสุนทร, 2474). These uses of *P.mirifica* in traditional medicine and folklore may be attributed to its estrogenic properties of the constituents. Several previous studies demonstrated that this plant possessed various compounds including phytoestrogens, the compounds with estrogen-like biological activity. Phytoestrogens found in tuberous roots of *P.mirifica* include miroestrol (Caine, 1960), kwakhurin (Tahara et al., 1987), puerarin (Ingham et al., 1986a), coumestrol, daidzin, daidzein, mirificin (Ingham et al.,1986b), genistein, genistin (Ingham et al., 1989) and deoxymiroestrol (Chansakaow et al., 2000a). Besides phytoestrogens, this plant also comprises other nonestrogenic compounds such as isomiroestrol (Chansakaow et al, 2000b), puemiricarpene (Joshi and Kamart, 1973), and mirificoumestan (Ingham et al, 1988) etc..

Epidemiological studies showed that frequent consumption of phytoestrogen rich diet, as seen in traditional Asian food, is associated with lower risks of many diseases such as breast, prostate, and colon cancers as well as cardiovascular diseases (Murkies et al., 1998; Setchell et al., 1998; Knight and Eden, 1996). Several studies suggested that genistein and daidzein possess cancer chemopreventive effects (Adlercreutz, 1990; Barnes, 1995; Kennedy, 1995; Steele et al., 1995), of which the specific mechanisms have not been clearly identified. *In vitro* and *in vivo* studies found that genistein exhibited antiproliferative effects in human breast cancer cells (Zava and Duwe, 1997). It also inhibited tyrosine specific protein kinases (Akiyama et al., 1987), DNA topoisomerase II (Yamashita et al., 1990), epidermal growth factor-induced phophatidylinositol turnover

(Imoto et al., 1988) and angiogenesis (Fotsis et al., 1993). CYPs involve in metabolic bioactivations of mutagens/carcinogens such as CYP1A1, CYP1A2 and CYP2E1 (Rendic and Di Carlo, 1997). Inhibition of the enzymes involved in the activation of carcinogen and/or mutagen as well as stimulation of the enzyme in detoxification pathways (i.e. GST, UDPGT etc.) are among the hypotheses proposed by several groups of study to explain the anticarcinogenic effects of phytoestrogens with flavonoid structure (Roberts-Kirchhoff et al., 1999; Wiseman and Duffy, 2001). For example, genistein was shown to inhibit CYP1A1, CYP1A2, and CYP2E1. Isoflavone phytoestrogens, genistein and daidzein, which are found mostly in soy foods, also possess a benefit in reducing risk of cardiovascular diseases by decreasing of total cholesterol, LDL-C, and triglyceride but increasing of HDL-C in both normal and hypercholesterolemic conditions (Murkies et al, 1998; Cassidy et al, 1994). The cardioprotective effects of these compounds may be attributed to its estrogenic like-activity.

However, the cancer chemopreventive and/or cardioprotective potential of *P.mirifica* have never been investigated. So far, there have been no studies regarding the effects of *P.mirifica* on CYPs involving in activations of chemical carcinogens such as CYPs1A1, 1A2, 2B1, 2B2, 2E1, 3A4, etc.. Inhibition effects of *P.mirifica* if exist on CYPs that play a key role in carcinogenic and/or mutagenic activation of many environmental chemicals would partly be a potential information for this plant to reduce risk of chemical carcinogenenesis or vice versa if it possesses the induction effects. In addition, there are few studies regarding the subchronic toxicity as well as the cholesterol-lowering effect of *P.mirifica* (ทรงพล ชีวะพัฒน์ และคณะ, 2543). Therefore, the objectives of this study were primarily to investigate subchronic effects of *P.mirifica* on CYP such as CYPs 1A1, 1A2, 2B1, 2B2 and 2E1, which were involved in carcinogen and/or mutagen activation. Moreover, effects of *P.mirifica* on blood clinical biochemistry parameters were also determined so as to preliminarily investigated the subchronic toxicity and lipid-lowering effects of the compounds in this plant.

Hypothesis

P.mirifica demonstrated an induction and/or inhibition effects on hepatic CYPs as well as blood clinical biochemistry parameters in rats.

Benefit gained from the study

1. A preliminary data of *P.mirifica* whether it possessed an induction and/or inhibition effects on hepatic CYP, especially CYP isoforms involved in a bioactivation of certain classes of drugs, chemicals and environmental pollutants resulting in reactive metabolites.
2. A preliminary subchronic toxicity data as well as the potentially lipid-lowering effect of *P.mirifica* in normal and high cholesterol diet conditions.

Study design and process

Experimental design: *ex vivo*, and *in vitro* study.

The following processes were performed:

1. An *ex vivo* study
    1.1 Animal dosing for 90 days
    1.2 Blood collecting
    1.3 Preparation of liver microsomes
    1.4 Determination of blood clinical biochemistry parameters
    1.5 Determination of hepatic microsomal CYP activities
2. An *in vitro* inhibition study
    2.1 Induction of CYP by various inducers such as phenobarbital and acetone
    2.2 Preparation of liver microsomes
    2.3 *In vitro* inhibition studies
3. Data collecting and analysis
4. Writing a thesis

CHAPTER II

LITERATURE REVIEWS

PUERARIA MIRIFICA

Pueraria mirifica Airy Shaw and Suvatabandhu, known locally as " Kwao Keur ", is a Thai climbing plant found in the forests of northern Thailand and Burma. *P.mirifica* is in the family Leguminosae, a family in which plants comprise abundant of isoflavonoids. This plant is well known as a rejuvenating medicine suggested for old men and women but not recommended for young people. Dried tuberous root of this plant is combined with honey in a 1:1 ratio and prepared in the form of tiny pills. Several indications of this plant were suggested for a traditional purpose such as skin enrichment, thickening and blackening hair, a relief of weakness and increasing an appetite, treatment of insomnia and breast enlargement in women, etc. (หลวงอนุสารสุนทร, 2474). These uses of *P.mirifica* in traditional medicine and folklore may be ascribed to its estrogenic properties (Caine, 1960; Murkies et al, 1998).

Several previous studies demonstrated that this plant possessed various compounds including phytoestrogens, the plant compounds with estrogen-like biological activity. Phytoestrogens in *P.mirifica* include miroestrol (Caine, 1960), kwakhurin (Tahara et. al, 1987), puerarin (Ingham et. al, 1986a), coumestrol, daidzin, daidzein, mirificin (Ingham et. al, 1986b), genistein, genistin (Ingham et. al, 1989) and deoxymiroestrol (Chansakaow et. al, 2000a). Besides phytoestrogens, this plant also comprises other nonestrogenic compounds such as puemiricarpene (Joshi and Kamart, 1973), mirificoumestan (Ingham et. al, 1988) and isomiroestrol (Chansakaow et. al, 2000b) etc..

Deoxymiroestrol was recently isolated from *P.mirifica* by Chansakaow and collaborates in 2000 (Chansakaow et al, 2000a). They found that deoxymiroestrol was easily converted to miroestrol and isomiroestrol by air oxidation during the isolation. Therefore, known miroestrol may be an artifact. Moreover, deoxymiroestrol was found to possess the strongest growth promoting effect on MCF-7 human breast cancer cells compared to those of other phytoestrogens in this plant. Miroestrol also possesses high

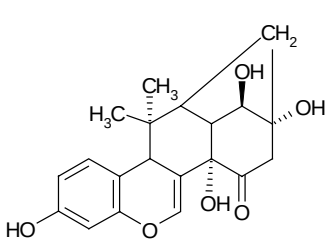
estrogenic activity, almost as strong as deoxymiroestrol. Coumestrol and genistein possess moderate estrogenic activity whereas daidzein and kwakhurin possess a weaker estrogenic acitivity (Table 1)(Chansakaow et al., 2000b).

Natural compounds found in tuberous root of *P.mirifica* can be classified on the basis of their chemical structures as following: (วันชัย ดีเอกนามกูล และชาลี ทองเรือง, 2544)

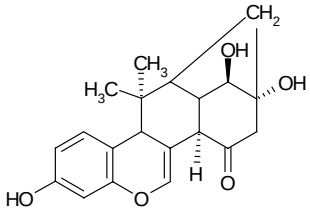
1. **Chromenes**: include
    1.1 Miroestrol
    1.2 Deoxymiroestol
    1.3 Isomiroestrol
2. **Isoflavones**: include
    2.1 Genistein
    2.2 Daidzein
    2.3 Kwakhurin
    2.4 Kwakhurin hydrate
3. **Isoflavone glycosides**: include
    3.1 Genistin
    3.2 Daidzin
    3.3 Mirificin
    3.4 Puerarin
    3.5 Puerarin-6”-monoacetate
4. **Coumestans**: include
    4.1 Coumestrol
    4.2 Mirificoumestan
    4.3 Mirificoumestan glycol
    4.4 Mirificoumestan hydrate
5. **Pterocarpans**: include
    5.1 Tuberosin
    5.2 Puemiricarpene

The chemical structures of these compounds were shown in Figure 1.

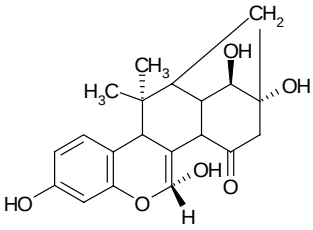
Chromenes



Miroestrol

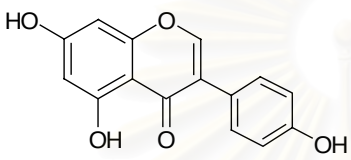


Deoxymiroestrol

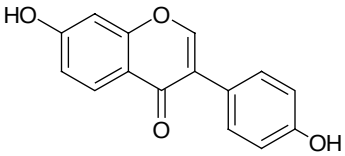


Isomiroestrol

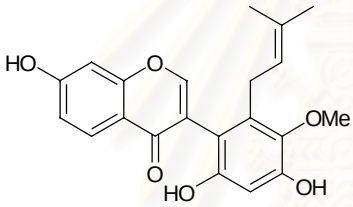
Isoflavones



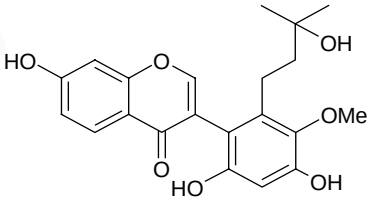
Genistein



Daidzein

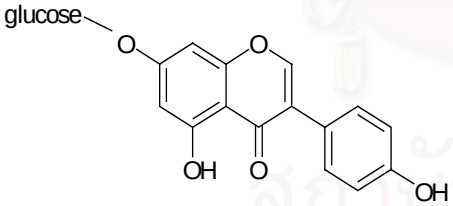


Kwakhurin

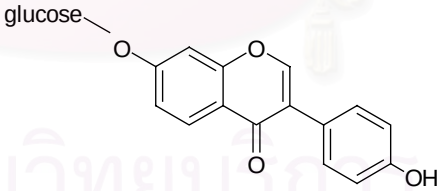


Kwakhurin hydrate

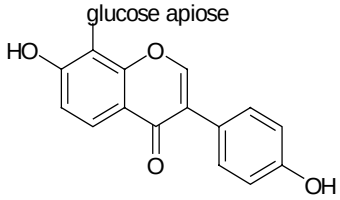
Isoflavone glycosides



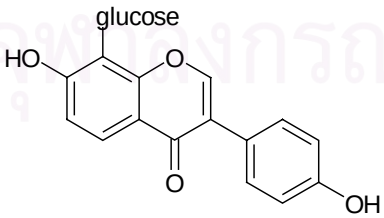
Genistin



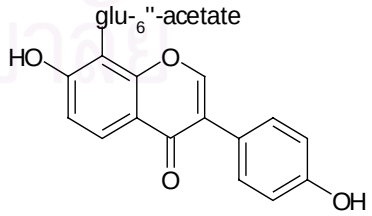
Daidzin



Mirificin



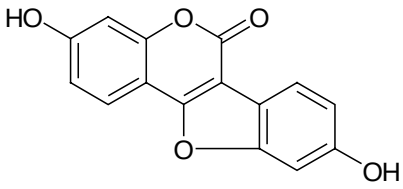
Puerarin



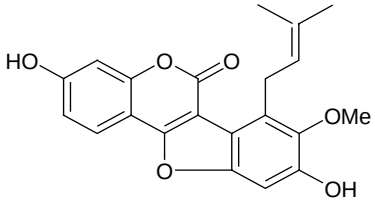
Puerarin-6”-monoacetate

Figure 1 Chemical structures of natural compounds found in tuberous root of *P.mirifica*

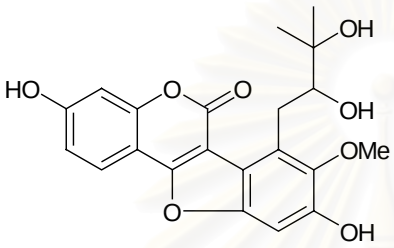
Coumestans



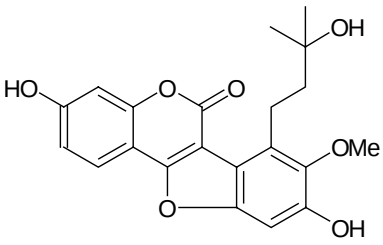
Coumestrol



Mirificoumestan

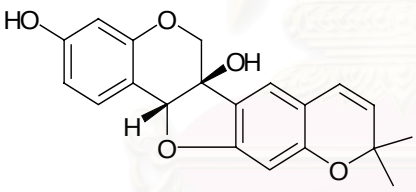


Mirificoumestan glycol

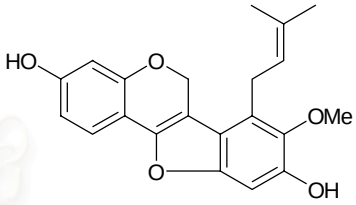


Mirificoumestan hydrate

Pterocarpans



Tuberosin



Puemiricarpene

Figure 1 Chemical structures of natural compounds found in tuberous root of *P.mirifica* (continued)

Table 1 Natural compounds found in tuberous roots of *P.mirifica*. Their contents and growth-promoting effects were based on an effect on MCF-7 human breast cancer cells (Chansakaow et al, 2000a & b)

Compounds	Content (mg/100g powder)	Growth-promoting effects on MCF-7 (Minimal concentration*)
17β-estradiol	-	$<10^{-12}$
Chromenes		
Miroestrol	3.0	10^{-8}
Deoxymiroestrol	2.0	$10^{-10} – 10^{-9}$
Isomiroestrol	2.2	no activity
Isoflavones and glycosides		
Daidzein	46.1	10^{-6}
Genistein	0.6	10^{-7}
Kwahurin	0.6	$>10^{-6}$
Daidzin	8.5	no activity
Genistin	data not shown	data not shown
Coumestan		
Coumestrol	0.07	10^{-7}
Pterocarpens		
Tuberosin	0.3	no activity
Puemiricarpene	1.8	no activity
Acid		
Tetracosanoic acid	15.3	-

* Minimal concentrations of compounds that caused 50% MCF-7 breast cancer cells growth when compared to the control

Pharmacological effects of *P.mirifica*

1. Antifertility

P.mirifica caused an inhibition of egg laying and crowing in female and male quails, respectively. Two weeks after cessation of the compound treatment, these abnormalities returned to normal. *P.mirifica* also caused a reduction of sexual frequency and testis growth in male pigeons as well as an inhibition of egg laying in female pigeons (ยุทธนา สมิตะสิริ, 2541). Male rats receiving *P.mirifica* at a dosage of 100 and 200 mg/kg significantly decreased sperm synthesis and movement resulted in a reduction of fertilization and embryo implantation. Therefore, the number of neonatal rats fertilized from these sperms were decreased but their organs were not crippled (ยุพดี ลางคลิจันทร์ และยุทธนา สมิตะสิริ, 2538).

2. Induction of abortion

Administration of *P.mirifica* at a dosage of 100 mg/kg/day for seven days resulted in a complete abortion in pregnant rats but did not induce early birth (ยุทธนา สมิตะสิริ, 2541).

3. Inhibition of lactation

Administration of *P.mirifica* or estrogen to lactating rats resulted in a decrease of mammary gland weight and milk secretion. In addition, weights of the puppies sucking milk from these mammary glands were decreased. These results indicated that *P.mirifica* inhibited mammary gland growth and milk secretion similar to an effect of estrogen. However, these effects can be reversed to normal by prolactin (ยุทธนา สมิตะสิริ และคณะ, 2532).

4. Breast enlargement and reproductive organ growth

P.mirifica promoted mammary duct growth and breast enlargement in both mice and rats similar to estrogen. Female puppies fed with diet supplemented with *P.mirifica* for 26 days demonstrated an increase of size and weight of uterus comparing to the control group (พูลศิลป์ ไวทยะโชติ และคณะ, 2530). Furthermore, size

and number of ovary follicles in quails were increased by *P.mirifica* (นิรันดร์ เมืองเดช และสมบูรณ์ อนันตลาโภชัย, 2528).

5. Cholesterol lowering effects

Cholesterol levels of male rats administered orally with *P.mirifica* at the dosages of 10, 100 and 1000 mg/kg/day for 90 consecutive days were significantly lower than those of the control groups. These changes were also observed in female rats given *P.mirifica* at the dosages of 100 and 1000 mg/kg/day for 90 days (ทรงพล ชีวะพัฒน์ และคณะ, 2543). This effect in rats was inconsistent to the results observed in quails that showed an increase of total cholesterol after giving 5% or 10% of *P.mirifica* in diet for 60 days (ปกรณ์ ไทยานันท์ และคณะ, 2535; สมบูรณ์ อนันตภาโภชัย และคณะ, 2532). Triglyceride level in male rats administered with *P.mirifica* orally at a dose of 1000 mg/kg/day was significantly lower than the control group. This change was not significant at any doses given to female rats (ทรงพล ชีวะพัฒน์ และคณะ, 2543).

Toxicity

So far, acute toxicity of *P.mirifica* in animals has not been reported. Median lethal dose (LD_{50}) of this plant in mice was greater than 16g/kg (ทรงพล ชีวะพัฒน์ และคณะ, 2543). A subchronic toxicity study was performed in Wistar rats by administration orally with dried root powder of *P.mirifica* at various doses (10, 100, and 1000 mg/kg/day) for 90 consecutive days. The results revealed that growth rate and food consumption of rat receiving *P.mirifica* at the doses of 100 and 1000 mg/kg/day were significantly lower than the control group. Administration of *P.mirifica* at the dose of 1000 mg/kg/day resulted in decreasing of hematocrit, RBC and hemoglobin and increasing of %reticulocyte in both sexes of animals. Moreover, WBC, %basophil and platelet in male rats were also decreased. Two weeks after a cessation of *P.mirifica* administration, these parameters became normal except for RBC, hemoglobin in female and WBC, platelet in males which were still not recovered. That study concluded that prolonged administration of high doses of *P.mirifica* could affect haemopoetic systems such as anemia in rats. Bilirubin, SCr and uric acid were decreased at the dose of 10, 100 and 1000 mg/kg/day in male rat while no alteration was observed in female rat. ALP was increased in male rat at the dose

of 1000 mg/kg/day whereas ALT was increased in female at the same dose. The uterus of female rats receiving *P.mirifica* at the dose of 100 and 1000 mg/kg/day appeared swollen. The actual uterine weight and % relative uterine weight of these two groups of *P.mirifica* administration were significantly higher than those of the control group. Histopathological examinations indicated that male and female rats receiving the root powder of this plant at the dose of 1000 mg/kg/day demonstrated a significantly higher incidence of testicular hyperemia and kidney tubular cast, respectively compared to their corresponding control groups (ทรงพล ชีวะพัฒน์ และคณะ, 2543).

Male quail receiving 5% and 10% of *P.mirifica* mixed with diet also demonstrated a decrease of RBC count, hemoglobin and lymphocyte (วราภรณ์ พงษ์คำ และคณะ, 2530).

PHYTOESTROGENS

Phytoestrogens are a broad group of plant-derived compounds of nonsteroidal structure that can behave as estrogen mimics (Setchell, 1998).

Phytoestrogens can be classified as following: (Kuiper et al., 1998; Murkies et al., 1998)

1. Flavonoids

   1.1 Isoflavones :

   Isoflavones are found mainly in soy bean and soy products. Genistein and daidzein have been extensively investigated regarding their therapeutic potential, particularly in disease prevention. They are found abundantly in soy, legumes, bean and their products, for example tofu yogurt, soy noodle, soy flour etc. (Murkies et al., 1998). Most isoflavones found in plants are in bound forms as glycosides and are biologically inactive (Knight and Eden, 1996). The glycosides, genistin and daidzin are glucose conjugated forms of their corresponding aglycones, genistein and daidzein, respectively. They are hydrolysed by bacterial enzymes in the

large intestine to release genistein or daidzein. Daidzein is partially further metabolized by bacteria to form the isoflavan, equol (possesses estrogenic effect) and O-desmethylangolensin (O-DMA) (possesses non-estrogenic effect). Genistein is metabolized to the non-estrogenic p-ethyl phenol (Wiseman and Duffy, 2001; Knight and Eden, 1996) and also metabolized by CYPs yielding the isoflavone orobol (Robert-Kirchhoff et al., 1999). Orobol has been reported to possess several effects to the same extent as genistein (Tomonaga et al., 1992; Yamashita et al., 1990; Imoto et al., 1988; Umezawa et al., 1975). Besides undergoing enterohepatic circulation, isoflavones are readily conjugated in livers with glucuronic acid and then excreted in urine. They are also excreted to a lesser extent as sulphate and sulphoglucuronide conjugates (Knight and Eden, 1996).

Genistein and daidzein are also derived from biochanin A and formononetin, respectively, after breaking down by intestinal glucosidases (Murkies et al., 1998). Biochanin A and formononetin are mainly found in clover but rarely found in human diets (Knight and Eden, 1996).

1.2 Flavonol:

For example, quercetin which is rich in olives, onions, and lettuce and kaempferol (Knight and Eden, 1996).

1.3 Flavone:

For example, apigenin and luteolin which are found in celery.

1.4 Flavanone:

For example, naringenin which is found in grapefruit.

1.5 Coumestans:

For example, coumestrol which occurs predominantly during germination of beans sprout and is also found in fodder crops (Murkies et al., 1998).

2. Lignans

Most mammalian lignans are known by the common names of enterodiol and enterolactone, which are converted by gut bacteria from precusors in plants, secisolariciresinol and matairesinol, respectively. These lignan precursors occur in the aleuronic layer of the grain close to the fiber layer.

Lignans occur in high concentration in flaxseed which is also known as linseed. They are found in lesser concentration in whole grain cereals, vegetables, fruits and seeds (Knight and Eden, 1996; Murkies et al., 1998).

3. Others

Chromenes such as miroestrol and deoxymiroestrol are found in *P.mirifica*.

Pharmacological effects

A conspicuous feature of the chemical structure of phytoestrogens is the presence of phenolic rings that, with few exceptions, is a prerequisite for binding to the estrogen receptor (Kuiper et al., 1998; Setchell, 1998; Wiseman and Duffy, 2001). For this reason, phytoestrogens can act either as estrogen agonists or antagonists. Their actions at the cellular and molecular level are influenced by many factors, including receptor subtype, presence or absence of endogenous estrogens, and types of target organ or cell (Setchell, 1998).

Dietary estrogens are weakly estrogenic (10^{-2} to 10^{-3} – fold depending on the system examined) when compared with estradiol or estrone (Setchell, 1998). The preferential binding of nonsteroidal estrogens to the novel estrogen receptor, estrogen receptor β suggests that they may exert their actions through distinct and separate pathway from classical steroidal estrogens (Setchell, 1998; Kuiper et al., 1998). Estrogenic potency of phytoestrogens for both estorgen receptor subtypes is different (Table 2).

Table 2 Ranking of the estrogenic potency of phytoestrogens for both estrogen receptor subtypes (Kuiper et al., 1998)

Estrogen receptor α	Estrogen receptor β
Estradiol >> coumestrol > genistein > daidzein > apigenin = phloretin > biochanin A = kaempferol = naringenin > formononetin = ipriflavone = quercetin = chrysin	Estradiol >> genistein = coumestrol > daidzein > biochanin A = apigenin = kaempferol = naringenin > phloretin = quercetin = ipriflavone = formononetin = chrysin

Miroestrol

Miroestrol possesses high potency of estrogenic activity. Subcutaneous injection to animals, it was found to be equal to 17β-estradiol in mouse uterine growth test, and to have one-quarter of the potency to 17β-estradiol in the rat vaginal cornification test (Schoeller et al., 1940). Given to animals via subcutaneous injection, miroestrol exhibited 70 percent of the activity of 17β-estradiol, in promoting rat mammary duct growth and 2.2 times as active as estrone in a similar test in mice (Benson, in press).

Given orally, miroestrol was approximately three times potent to that of stilbestrol in immature mice uterine growth test and two-thirds of stilbestrol in rat vaginal cornification test (Jones and Pope, 1960).

Clinical study

A clinical study regarding miroestrol was carried out by Dr. P. M. F. Bishop and his collaborates at the Chelsea Hospital for Women, London. Miroestrol was administered at doses of 5 mg or 1 mg daily to ten women suffering from amenorrhea or artificial menopause. Marked estrogen response was noted for both doses during the second or third week of the treatment. When the treatment was discontinued, withdrawal bleeding happened. Actually, these patients had been previously treated with oral estrogen. The withdrawal interval from discontinuing of miroestrol was much longer than that from estrogen. Hot flush was diminished in frequency and severity but recurred by the fifth day after the treatment was stopped. Adverse effects of miroestrol were malaise, headache, nausea, and vomiting (Caine, 1960).

Phytoestrogens and disease relationships

1. Cardiovascular diseases

There is a strong evidence supporting the hypothesis that phytoestrogen consumption contributes to the lower incidence of cardiovascular disease in vegetarian and Asian populations. Thus, phytoestrogens may be cardioprotective (Adlercreutz, 1990).

A study was performed in normolipemic premenopausal women by giving 60 g of soy protein (60 g containing 45 mg isoflavones) daily for one month. The results showed that plasma total cholesterol concentrations of the treated women were significantly decreased by 9.6% compared to the control group (Cassidy et. al, 1994). Consumption of soy resulted in a decrease of total cholesterol as well as an increase of HDL cholesterol in hypercholesterolemic men (Bakhit et al., 1994). The possible mechanism of cholesterol lowering effects of phytoestrogens has been extensively discussed but the conclusion is still not clear. Phytoestrogens may modify plasma lipids and lipoprotein by up regulation of LDL receptor and their antioxidant activity. They may also have effects on arterial walls, either through their inhibitory effect on vascular smooth muscle cell proliferation and

migration or through an effect on vascular reactivity (Tikkanen and Adlercreutz, 2000).

2. Osteoporosis

There is a paucity of data regarding the possible role of phytoestrogen in bone metabolism and the incidence of osteoporosis (Murkies et al., 1998). Dietary soybean prevents significant bone loss in ovariectomized rats (Arjmandi et. al, 1996).

3. Cancer

Epidemiological studies have consistently shown an inverse association between consumption of a phytoestrogen-rich diet, as seen in traditional Asiatic societies, and the lower risk of hormonally dependent and independent human cancers. Therefore, phytoestrogens is especially promising for cancer chemoprevention of which the specific mechanism of action has not been identified. Genistein and daidzein have been extensively studied for anti-breast cancer and anti-prostate cancer because of their estrogenic and antiestrogenic activities. Genistein was shown to inhibit tyrosine specific protein kinases (Akiyama et al., 1987), DNA topoisomerase II (Yamashita et al., 1990), epidermal growth factor-induced phosphatidylinositol turnover (Imoto et al., 1988) and angiogenesis (Fotsis et al., 1993). In addition, phytoestrogens may exert their effects by decreasing the activity of enzymes that activate procarcinogens, such as cytochrome P450 (CYPs). Procarcinogens require metabolism to their fully carcinogenic forms. Metabolism of genistein by CYP1A1, 1A2, 1B and 2E1 may affect the metabolism of other CYP substrates, including procarcinogens via a competitive inhibition mechanism (Roberts-Kirchhoff et al., 1999).

Effect of phytoestrogens on hepatic drug metabolizing enzymes

Several previous studies have investigated the effects of flavonoids on drug metabolizing enzymes (Breinhott et al., 2000; Robert-Kirchhoff et al., 1999; Helsby et al., 1998; Nielsen et al., 1998; Zhai et al., 1998; Helsby et al., 1997; Siess et al., 1995; Li et al., 1994). A variety of flavonoids were shown to influence xenobiotic metabolism by induction

of some CYP isoforms (i.e. CYP1A1, CYP1A2, CYP2B1&2B2) as well as phase II enzymes, GST and UDPGT in an *in vivo* study (Siess et al., 1992). In contrast, inhibition effects of various flavonoids on those CYP isoforms (i.e. CYPs 1A1, 1A2, 2B1, 2B2 and 3A4) were demonstrated in the *in vitro* studies (Zhai et al., 1998; Siess et al., 1995; Li et al., 1994). Isoflavonoids such as genistein, daidzein affected differently on hepatic drug metabolizing enzymes. Genistein and daidzein (or its metabolite, equol) demonstrated no induction effects on CYPs (i.e. CYPs 1A1, 1A2, 2B1&2B2, 2E1 and 3A) as well as the phase II enzymes, glutathione S-transferase (GST) and UDP-glucuronyltransferase (UDPGT) in the *in v*ivo studies (Kishida et al., 2000; Breinholt et al., 1999, Helsby et al., 1997). However, in an *in vitro* study, they exhibited inhibition effects on CYP1A1&1A2 and CYP2E1. The inhibition effects of genistein and daidzein on CYP1A and CYP2E1 offered a possible explanation for their chemopreventive effects against chemical carcinogenesis (Helsby et al., 1998).

XENOBIOTIC METABOLISM

Metabolism is a biological process which converts lipophilic xenobiotics to more hydrophilic metabolites in order to facilitate subsequent renal or fecal excretion. Xenobiotic-metabolizing enzymes occur in many organs such as kidney, lung and gastrointestinal tract with the liver having the largest amount. They are located in the smooth endoplasmic reticulum and cytosol of the liver cells. Metabolic biotransformation reactions of xenobiotics are divided into two phases, both of which are phase I and phase II reactions (Table 3).

Phase I reactions change many xenobiotics to more polar metabolites which are more active, less active or inactive than the parent compounds. The most important reaction in this phase is oxidation, especially using cytochrome P450 monoxygenase enzyme system. Phase II reactions, which are also called conjugation, increase the polarity of the xenobiotics. This phase is generally though to be a detoxification pathway

because after conjugation reaction, the xenobiotics will be more readily excreted from the body. Most substances undergo both phase I and phase II reactions, sequentially.

Table 3 Reactions classed as phase I and phase II metabolism (Gibson and Skett, 1994)

Phase I	Phase II
Oxidation	Glucuronidation/ Glucosidation
Reduction	Sulfation
Hydrolysis	Methylation
Hydration	Acetylation
Dethioacetylation	Amino acid conjugation
Isomeriztion	Glutathione conjugation
	Fatty acid conjugation
	Condensation

CYP functions as a multicomponent electron-transport system responsible for the oxidative metabolism of a variety of endogenous substrates (such as steroids, fatty acid, prostaglandins, and bile acids) and exogenous substrates (xenobiotics), including drugs, carcinogens, insecticides, plant toxins, environmental pollutants, and many other foreign chemicals. This enzyme catalyzes xenobiotic transformation in ways that usually lead to detoxification, but in many cases, they lead to products with greater cytotoxic, mutagenic, or carcinogenic properties. CYP occurs in many forms (isozymes or isoforms) which differ from each other by their amino acid sequences. They also differ in spectral, electrophoretical, and immunological properties as well as different substrate affinities. In addition, these isozymes differ in their regulation and tissue distribution (Mayer, 1996). Due to the fact that liver contains multiple forms of CYP, CYPs involved in drug metabolism were often refered to as having broad and somewhat overlapping substrate specificities.

Xenobiotics, after converted by specific isozymes to more reactive, more electrophilic intermediates, are capable of reacting covalently with biological macromolecules, proteins, nucleic acids or lipids. The binding of xenobiotic metabolites to DNA may cause modification of genetic information, mutation, and consequent possibility of malignant growth.

CYPs in families 1, 2, and 3 play a major role in drug and xenobiotic metabolism. These three families account for about 70% of total CYPs in human livers while CYP4 is family involved in fatty acid and prostaglandins metabolism (Rendic and Di Carlo, 1997). CYP isoforms which play a role in the activation of xenobiotics to toxic metabolites include CYPs 1A1, 1A2, 2B1, 2B2, 2E1 in rats as well as CYPs 1A1, 1A2, 2B6, 2E1, 3A4 in human. An example of rat and human CYPs which can activate some of potential carcinogens/metagens were shown in table 4 and table 5, respectively.

Table 4 Some procarcinogens and other toxins activated by rat CYPs (Soucek and Gut, 1992)

CYPs	Procarcinogens
1A1	Benzo(a)pyrene
	7,12 Dimethylbenz(a)anthracene
	2-Naphthylamine
	Alfatoxin B_1
	2-Acetylfluorene
	o-Aminoazotoluene
	2-Acetylaminofluorene
	3-Methylcholanthrene
	2-Amino-9H-pyrido[2, 3-b]indole
	2-Aminopyridol[1, 2-a: 3', 2'-d]imidazole
	3-Amino-1, 4-dimethyl-5H-pyrido[4,3-b]indole
	3-Amino-1-methyl-5H-pyrido[4,3-b]indole
	1,2,3,4-dibenzathracene
	2-Amino-3-methylimidazo[4,5-f]quinoline
	2-Amino-3,5-dimethylimidazole[4,5-f]quinoline
	2-Amino-3,8- dimethylimidazole[4,5-f]quinoxaline
	4,4'-(bis)methylene chloroaniline
	2-Amino-9H-pyrido[2,3-b]indole
	2-Amino-6-methyldipyridole[1,2-a:3',2'-d]imidazole
	2-Aminodipyridol[1,2-a:3',2'-d]imidazole
	3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
	3-Amino-1-methyl-5H-pyrido[4,3-b]indole
	Aminoanthracene
	Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
2B1	Benzo(a)pyrene
	2-Acetylfluorene
	N-N"-nitrosodimethylamine
	3-Methylcholanthrene
	2-Acetylaminofluorene
	1,2,3,4-Dibenzanthracene
	Aminoanthracene
	4,4'-(bis)methylene chloroaniline
	4-Aminobiphenyl
2B2	4,4'-(bis)methylene chloroaniline
2E1	Nitrosoamine

Table 5 Some procarcinogens and other toxins activated by human CYPs (Soucek and Gut, 1992)

CYPs	Procarcinogens
1A2	Alfatoxin B_1
	2-Amino-3,4,8-trimethylimidazol[4,5-f]quinoxaline
	2-Amino-3-methylimidazo[4,5-f]quinoline
	2-Amino-3,5-dimethylimidazole[4,5-f]quinoline
	2-Amino-3,8- dimethylimidazole[4,5-f]quinoxaline
	2-Amino-6-methyldipyridole[1,2-a:3',2'-d]imidazole
	2-Aminodipyridol[1,2-a:3',2'-d]imidazole
	3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole
	3-Amino-1-methyl-5H-pyrido[4,3-b]indole
	2-Acetylfluorene
	2-Acetylaminofluorene
	2-Aminoanthracene
	4-Aminobiphenyl
2E1	N-N"-nitrosodimethylamine
	N-nitroso-N-benzyl-N-methylamine
	N-nitroso-N-butyl-N-methylamine
	N-nitroso-N-diethylamine
3A4	Alfatoxin B_1
	2-Amino-3-methylimidazo[4,5-f]quinoline
	2-Amino-3,5-dimethylimidazole[4,5-f]quinoline
	2-Amino-3,8- dimethylimidazole[4,5-f]quinoxaline
	Benzo(a)pyrene
	6-Aminochrysene
	Sterigmatocystin
	Tris(2,3-dibromopropyl)-phosphate

CYP1A family of enzymes is responsible for the metabolic activation of some known procarcinogenic environmental chemicals, toxins, and toxic drugs. This family contains enzyme CYP1A1 and CYP1A2. CYP1A1 is expressed in the liver, small intestine, placenta, skin, and lung. CYP1A1 is present at very low level but highly inducible (Gonzalez, 1990). In contrast to CYP 1A1, CYP1A2 is not expressed in extrahepatic tissue. CYP1A1 and CYP1A2 are found in both human and rats. The function of the CYP1A is fairly well conserved across species although there are subtle difference (Parkinson, 1996). For instance, isolated and purified human CYP1A2 enzyme from the liver has been shown to display substrate specificity similar to the rat protein. These isozymes are undoubtedly the most significant in activation of carcinogens since they can activate more than 90% of known carcinogen (Rendic and Di Carlo, 1997), for example, cigarette smoke, charcoal-broiled meat (a source of polycyclic aromatic hydrocarbons), and cruciferous vegetables (a source of various indole) (Parkinson, 1996). Some drugs that are substrates (e.g. caffeine, bufuralol, propanolol and paracetamol), inducers (e.g. omeprazole and lansoprazole), or inhibitors (e.g. cimetidine) also interact with these isozymes.

CYP 2B family has been extensively studied in rats because it can be induced by phenobarbital. CYP2B1 and CYP2B2 are highly similar in nucleotide sequence and have similar substrate specificities. Rat CYP2B1 is analogous to human CYP2B6, which generally exists in small amount. CYP2B6 would be expected to be inducible by phenobarbital, however the levels of isozyme are extremely low even in individuals treated with phenobarbital (Parkinson, 1996). It appears that the ability of phenobarbital to stimulate the biotransformation of xenobiotics in human largely stems from its ability to induce the other CYPs, CYP2C and CYP3A4.

CYP2E1 is expressed constitutively in liver and possibly in extrahepatic tissues, such as kidney, lung, and lymphocytes. CYP2E1 is a major toxicological importance because it is responsible for the formation of reactive metabolites and intermediates from a number of laboratory and environmental chemicals including benzene, aniline, polyhalogenated compounds, urethane, butadiene, chlorofluorohydrocarbons,

fluorohydrocarbons ,etc.. CYP2E1 substrates may also induce CYP2E1 activity; for examples, ethanol, isopropanol, acetone, toluene, and benzene. Isoniazid and imidazole compounds are also potent inducers (Rendic and Di Carlo, 1997). The function and regulation of CYP2E1 are well conserved among mammalian species (Parkinson, 1996).

Mechanism of induction of CYP

Induction is an adaptive response that protects cells from toxic xenobiotics by increasing the detoxification activity. Drugs, environmental chemicals, and many other xenobiotics enhance the metabolism of themselves and/or of other co-ingested/inhaled compounds, resulting in a reduction of pharmacological effects or an increase of toxicity as a result of an increase formation of reactive metabolites. The time course of induction varies with different inducing agents and different isoforms. Also, the induction response is dose-dependent and reversible. Enzyme induction can also enhance the activation of procarcinogens or promutagens. Therefore, enzyme induction is important in interpreting the results of chronic toxicity, mutagenicity, or carcinogenicity and explaining certain unexpected drug interactions in patients. The precise mechanisms of CYP induction are not fully understood, except for the induction of certain CYP1A subfamily by polycyclic aromatic hydrocarbons via the Ah receptor (Whitlock, 1995). The proposed mechanism of induction involves binding of the particular RNA polymerase to the promoter segment of gene causing expression of the respective CYP structural gene with increased transcription of mRNA, resulting in increased CYP isoform synthesis. Moreover, induction of CYP may arise as a consequence of decreased degradation of the protein enzyme or the corresponding mRNA, activation of pre-existing components, or a combination of these two processes (Gibson and Skett, 1994).

Mechanism of inhibition

Many therapeutic drugs and environmental xenobiotics have been reported to inhibit CYP in the liver via different mechanisms. The inhibition effects can take place in several ways including the destruction of pre-existing enzymes, an inhibition of enzyme synthesis, an inactivating of the drug-metabolizing enzymes and a competitive for the enzyme catalytic sites. The inhibition of drug metabolism may result in undesirable elevations in plasma drug concentrations. Thus, the inhibition of CYP is of clinical importance for both therapeutic and toxicological reasons.

Mechanism of CYP inhibition can be divided into three categories (Lin and Lu, 1998; Williams, 1995):

1. **Reversible inhibition**

Reversible inhibition is probably the most common mechanism responsible for the documented drug interactions. Generally, this interaction is the result of competition between inhibitor and substrate at the same CYP active site. The effect of this inhibition will be dissipated after discontinuing the inhibitor.

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

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

3.**Irreversible inhibition**

Irreversible inhibition occurs when a reactive metabolite binds irreversibly to protein or the prosthetic heme of CYP or a combination of both resulting in irreversible inactivation of CYP prior to its release from the active site. This process called mechanism-based inhibition or suicide inhibition.

CHAPTER III

MATERIALS AND METHODS

Animals

Adult male Wistar rats of body weight between 200-250 g were obtained from National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. Rats were housed two per cage at the Faculty of Medicine, Srinakharinwirot University and acclimatized for at least seven days prior to the experimentation. They were maintained at 25 ^{o}C on a 12-hour light/dark cycle and had free access to the diet and water throughout the study. High cholesterol rats had high cholesterol diet containing 1% cholesterol plus 2% sodium choleate. All diets were purchased from C.P.company.

Chemicals

These following chemicals were used in the experimentation:

4-Aminophenol, aniline hydrochloride, benzyloxyresorufin , bovine serum albumin (BSA), cupric sulfate, ethylene diamine tetra acetic acid (EDTA), dimethysulfoxide (DMSO), ethoxyresorufin, Folin&Ciocalteu's phenol reagent, glucose-6-phosphate dehydrogenase (G6PD), methoxyresorufin, nicotinamide adenine dinucleotide phosphate (NADP), pentoxyresorufin, potassium phosphate monobasic anhydrous (KH_2PO_4), resorufin, sodium carbonate (Na_2CO_3), sodium citrate, sodium phosphate dibasic anhydrous (Na_2HPO_4), Trisma ® base (Sigma, U.S.A.)

Glycerol (Carlo Erba, U.S.A.)

Hydrochloric acid (HCl), Magnesium chloride ($MgCl_2$), phenol, potassium chloride (KCl), sodium chloride (NaCl), sodium hydroxide (NaOH), trichloroacetic acid (E.Merck, Germany)

Methanol (HPLC grade) (E.Merck, Germany)

Pentobarbitone sodium (Nembutal®)

Phenobarbital (Gardinal ®, Zuellig)

Sodium dithionite (Fluka Chemical, Japan)

P.mirifica

Tuberous root powder *of P.mirifica* was obtained from Dr.Amphawan Apisariyakul at the department of Pharmacology, Faculty of Medicine, Chiang Mai University, Thailand. Tuberous roots of *P.mirifica* were cultivated at Tumbol Ban Tak, Aumpur Mae Sod, Tak Province during March and April, 2000.

P.mirifica mixture was prepared weekly, by dissolving 6 g of the powder with 100 ml of double distilled water, mixed well, filtered out any remaining fiber with cloth filter and kept in refrigerator.

Instruments

The following instruments were used in the experimentation:

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

Centrifuge (Kokusan, Japan)

Fluorescence spectrophotometer (Jasco, Japan)

Metabolic shaker bath (Heto, Denmark)

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

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

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

Refrigerated ultracentrifuge (Hitachi, Japan)

Sonicator (Elma, Germany)

Spectrophotometer (Jasco, Japan)

Surgical equipments

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

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

Methods

1. An *ex vivo* study

1.1 Animal treatment

Rats were randomly divided into 4 treatment groups. Each treatment group comprised 10 rats.

1. **Normal diet-fed group**: Animals were fed with normal diet and orally administered with double distilled water for 90 days.
2. **Normal diet-fed supplemented with *P.mirifica* group**: Animals were fed with normal diet and orally administered with *P.mirifica* at a dosage of 100 mg/kg/day for 90 days.
3. **High cholesterol diet-fed group**: Animals were fed with high cholesterol diet containing 1% cholesterol plus 2% sodium choleate and orally administered with double distilled water for 90 days.
4. **High cholesterol diet-fed supplemented with *P.mirifica* group**: Animals were fed with high cholesterol diet containing 1% cholesterol plus 2% sodium choleate and orally administered with *P.mirifica* at a dosage of 100 mg/kg/day for 90 days.

During the treatment period, body weight of all rats was recorded at every two weeks.

Four animals were used simultaneously for each experimental period (one rat/each treatment group). On the day after the ninety day of the compound administration, rats were anesthetized with pentobarbitone sodium (Nembutal ®) intraperitoneally. Blood samples were collected by heart puncture. Livers were also removed for preparation of microsomes.

1.2 Determination of blood clinical biochemistry parameters

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

1.2.1 Hematological assays

Whole blood samples were determined for complete blood count (CBC), white blood cell (WBC) count, %differential WBC, platelet count and red blood cell (RBC) morphology.

1.2.2 Blood clinical biochemistry parameters determination

Serum samples were determined for various blood clinical biochemistry parameters using commercial test kit of bioMerieux company (France) as following: glucose, blood urea nitrogen (BUN), serum creatinine (SCr), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), triglyceride, and cholesterol.

The assays mentioned above were performed by Faculty of Allied Health Sciences, Chulalongkorn University.

Determination of total bilirubin and direct bilirubin (using commercial test kit of Merieux Vitex, France), HDL-C and LDL-C (using commercial test kit of Roche company, Germany) in serum were performed by Professional Laboratory Management, Bangkok.

1.3 Preparation of liver microsomes

1.3.1 Reagents

1. 0.1M Phosphate buffer, pH 7.4

   One litre of phosphate buffer, pH 7.4 consisted of 1.78 g of KH_2PO_4, 9.55 g of Na_2HPO_4, and 11.50 g of KCl. The solution was adjusted to pH 7.4 with NaOH or HCl.
2. Phosphate buffer, pH 7.4, containing 20% v/v glycerol.
3. 0.9% w/v NaCl.

1.3.2 Procedure

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

1.4 Determination of protein concentrations.

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

1.4.1 Reagents

1. 2% w/v Sodium carbonate
2. 0.5 M Sodium hydroxide (NaOH)
3. 2% w/v Sodium citrate
4. 1% w/v Cupric sulfate
5. 1 mg/ml BSA in 0.5 M NaOH
6. Folin & Ciocalteu ' s phenol reagent
7. Working protein reagent. The solution was prepared freshly in a sufficient amount for all tubes in the assay (6.5 ml of reagent was required for each tube). This reagent comprised sodium carbonate, sodium hydroxide, sodium citrate, and cupric sulfate solutions in a 100:10:1:1 ratio, respectively.

1.4.2 Procedure

1. 16x125 mm tubes were labeled in duplicate for 7 standards (0, 50, 100, 150, 200, 250, 300 μg) and for each unknown sample.
2. The following reagents were added in μl to each standard solution tube:

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

Each tube was mixed thoroughly after addition of the reagents.

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

1.4.3 Calculations

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

1.5 Spectral determination of total CYP contents

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

1.5.1 Reagents

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

1.5.2 Procedure

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

1.5.3 Calculations

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

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

1.6 Analysis of alkoxyresorufin O-dealkyllation

Rate of hepatic microsomal alkoxyresorufin O-dealkylation was determined according to the method of Burke and Mayer (1974; 1985) and Lubet et al. (1995) with slight modifications. Benzyloxyresorufin and pentoxyresorufin were used as specific substrates of CYP2B1&2B2. Ethoxyresorufin and methoxyresofin were used as specific substrates of CYP1A1 and CYP1A2, respectively.

1.6.1 Reagents

1. 0.1 M Tris buffer, pH 7.4
2. Resorufin & Alkoxyresorufins

   0.5 mM Resorufin (MW 235)

   1.175 mg of resorufin was dissolved with DMSO qs to 10 ml.

   0.5 mM Benzyloxyresorufin (MW 303)

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

   0.5 mM Ethoxyresorufin (MW 241)

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

   0.5 mM Methoxyresorufin (MW 227)

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

   0.5 mM Pentoxyresorufin (MW 283)

   1.415 mg of pentoxyresorufin was dissolved with DMSO qs to 10 ml.
3. NADPH regenerating system

   Glucose 6-phosphate dehydrogenase (G6PD), pH 7.4

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

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

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

   0.1 M NADP, pH 7.4

   0.765 g of NADP was dissolved with 20 mM K_3PO_4 qs to 10 ml, adjusting pH to 7.4 with HCl or NaOH (10 μl contains 1 mmoles of NADP).

0.3 M $MgCl_2$, pH 7.4

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

1.6.2 Procedure

1. Microsomes were diluted with 0.1 M Tris buffer, pH 7.4 to measure out 100 μg of protein for the 1 ml of the reaction mixture.
2. For each ml of the reaction mixture, the following reagents were added
   a. 30 μl of NADPH regenerating system comprised
      10 μl of 0.1 M NADP
      10 μl of 0.5 M G6P
      10 μl of 0.3 M $MgCl_2$
   b. 10 μl of 0.5 mM Alkoxyresorufin
   c. Varied volume of diluted microsomal suspension containing 100 μg of microsomal protein
   d. 0.1 M Tris buffer, pH 7.4 qs to 990 μl.
3. Three tubes were prepared for each microsomal sample. One tube was a sample blank tube and the others were sample tubes. All tubes were preincubated in a 37 ^{o}C shaking water bath for 2 minutes.
4. The reaction was started by the addition of 10 μl of G6PD (1 unit of G6PD / 1 ml of reaction mixture volume). For a sample blank, 10 μl of 0.1 M Tris buffer, pH 7.4 was added instead of G6PD.
5. After a 5 minute incubation, the reaction was stopped by adding 1 ml of methanol (HPLC grade).
6. The absorbance was measured by fluorescence spectrophotometer using an excitation wavelength of 556 and an emission wavelength of 588.
7. A resorufin standard curve was carried out using 8 concentrations of resorufin: 0.025, 0.05, 0.075, 0.1, 0.2, 0.4,0.5, 1.0 nmole/ml.

1.6.3 Calculations

Rate of alkoxyresorufin O-dealkylation was calculated by dividing the amount of resorufin formed by the time of incubation (5 minutes) and an amount of microsomal protein (100 μg) used in the reaction. The units were expressed as pmol/mg protein/min.

1.7 Analysis of aniline 4-hydroxylation

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

1.7.1 Reagents

1. 10 mM Aniline hydrochloride

   93 mg of aniline hydrochloride was dissolved with 100 ml of double distilled water. The solution was stored in a dark brown bottle.
2. 6% w/v Trichloroacetic acid

   60 g of trichloroacetic acid was dissolved with 1 L of double distilled water.
3. 20% w/v Trichloroacetic acid

   200 g of trichloroacetic acid was dissolved with 1 L of double distilled water.
4. 1% w/v Phenol

   20 g of phenol and 40 g of NaOH were dissolved with 2 L of double distilled water.
5. 1 M Na_2CO_3

   212 g of anhydrous Na_2CO_3 was dissolved with 2 L of double distilled water.
6. 10 μM 4-Aminophenol

   36.5 mg of 4-aminophenol was made up to 10 ml with double distilled water. Then 0.1 ml of this aminophenol solution was added to 15 g of tricholroacetic acid and made up to 250 ml with double distilled water.
7. 0.1 M Tris buffer pH 7.4
8. 0.1 M NADP
9. 0.5 M G6P
10. G6PD

11. 0.3 M $MgCl_2$

1.7.2 Procedure

1. Microsomes were diluted with 0.1 M Tris buffer, pH 7.4 so as to be able to measure out 5 mg of protein for the 2 ml of the reaction mixture.
2. For each 2 ml of the reaction mixture, the following reagents were added
    a. 30 μl of NADPH regenerating system comprised
        10 μl of 0.1 M NADP
        10 μl of 0.5 M G6P
        10 μl of 0.3 M $MgCl_2$
    b. 500 μl of 10 mM aniline hydrochloride
    c. Varied volume of diluted microsomal suspension containing 5 mg of microsomal protein
    d. 0.1 M Tris buffer, pH 7.4 qs to 2 ml.
3. Three reaction tubes were prepared for each microsomal sample. One tube was a sample blank tube and the others were sample tubes.
4. All tubes were preincubated in a 37 ^{o}C shaking water bath for 2 minutes. The reaction was initiated by an addition of 20 μl of G6PD. For a sample blank, 20 μl of 0.1 M Tris buffer, pH 7.4 was added instead of G6PD.
5. After a 30 minutes incubation time, the reaction was stopped by adding 1 ml of ice-cold 20% w/v trichloroacetic acid and the tubes were kept on ice for 5 minutes.
6. The solution was then centrifuged at 3,000 rpm for 10 minutes.
7. After 1 ml of the supernatant was transferred to a new tube, 1 ml of 1% w/v phenol and 1 ml of 1 M Na_2CO_3 were added. The solution was mixed well by vortex mixer and kept at room temperature for 30 minutes.
8. The absorbance was measured by spectrophotometer at a wavelength of 630 nm.
9. A standard curve was carried out using 5 concentrations of 4-aminophenol standard solutions (2, 4, 6, 8, 10 μM), following the procedure from step 7 in the same manner as sample.

1.7.3 Calculations

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

2. An *in vitro* study

2.1 Animal treatment

Rats were randomly divided into two treatment groups. Each group comprised four rats.

2.1.1 Phenobarbital treatment group:

Phenobarbital, at a dosage of 80 mg/kg/day, was administered intraperitoneally to rats for three days for an induction of CYP2B1&2B2 (Gibson and Skett, 1994). Liver microsomes prepared from the animals in this treatment group were used for studying the inhibition effect of *P.mirifica* on CYP2B1&2B2.

2.1.2 Acetone treatment group:

Drinking water added with acetone by 1%v/v concentration was allowed for four rats to drink for seven days for an induction of liver CYP2E1 (Helsby et al., 1998). Liver microsomes prepared from the animals in this treatment group were used for studying the inhibition effect of *P.mirifica* on CYP2E1.

At the end of the treatment, rats were anesthetized using light ether vapor. After opening the abdominal cavity, livers were perfused *in situ* with ice-cold 0.9%w/v NaCl until the entire organ became pale. Then the livers were proceeded for preparation of microsomes in the same way as mentioned in the *ex vivo* study (1.3.2).

2.2 Inhibition effect of *P.mirifica* on CYP2B

2.2.1 Procedure

1. Various concentrations of *P.mirifica* were prepared in such a way that equal volume (167μl for 1.5 ml of reaction mixture) of the solutions were used in the reaction. The final concentrations of *P.mirifica* in the reaction mixture were 0, 1, 2.5, 5, 7.5, 10, 15 and 20% w/v.
2. Fifteen microlitre of benzyloxyresorufin was used as a substrate for 1.5 ml reaction mixture.
3. Liver microsomes prepared from rats in the phenobarbital treatment group were used in the reaction.
4. The reactions were performed in the same manner as described in 1.6.

2.3 Inhibition effect of *P.mirifica* on CYP2E1

2.3.1 Procedure

1. Various concentrations of *P.mirifica* were prepared in such a way that equal volume (167μl for 1.5 ml of reaction mixture) of the solution were used in the reaction. The final concentrations of *P.mirifica* in the reaction mixture were 0, 1, 2.5, 5, 7.5 and 10% w/v.
2. Five hundred microlitre of aniline hydrochloride was used as a substrate for 2 ml reaction mixture.
3. Liver microsomes prepared from rats in the acetone treatment group were used in the reaction.
4. The reactions were performed in the same manner as described in 1.7.

3. Statistics

All quantitative data were presented as mean $\pm$ SEM. An independent *t*-test was used for statistical comparisons between two groups at significant level of $p<0.05$.

For estimation of IC_{50} , the % of inhibition was transformed to probit unit. The linear regression method was used to fit a curve between probit unit and dose by using Sigmaplot program. The IC_{50} was calculated from the linear regression equation.

CHAPTER IV

RESULTS

An *ex vivo* study

1.1 General effects of *P.mirifica*

During the experimental period, five rats (accounted for 50% of the total rats in the group) from normal diet-fed supplemented with *P.mirifica* group and four rats (account for 40% of the total rats in the group) from high cholesterol diet-fed supplemented with *P.mirifica* group had hair loss. No rats died at the end of the study.

Body weight gain of rats receiving *P.mirifica* fed with either normal diet or high cholesterol diet was significantly lower than their corresponding control groups (Figure 2). High cholesterol diet feed caused no change of body weight gain compared to the normal diet condition.

High cholesterol diet-fed rats comparing to normal diet fed rats exhibited significantly higher of both liver weight (10.58 ± 0.30 vs. 16.96 ± 0.91; $p<0.05$) and relative liver weight (2.35 ± 0.07 vs. 3.62 ± 0.19; $p<0.05$). *P.mirifica* did not affect liver weight and relative liver weight in normal diet-fed rats. However, in high cholesterol diet condition, *P.mirifica* caused a significant decrease of liver weight. Due to the lower body weight of rats in high cholesterol diet-fed supplemented with *P.mirifica*, relative liver weight of rats in this group was not different from the high cholesterol diet-fed control group (Table 6).

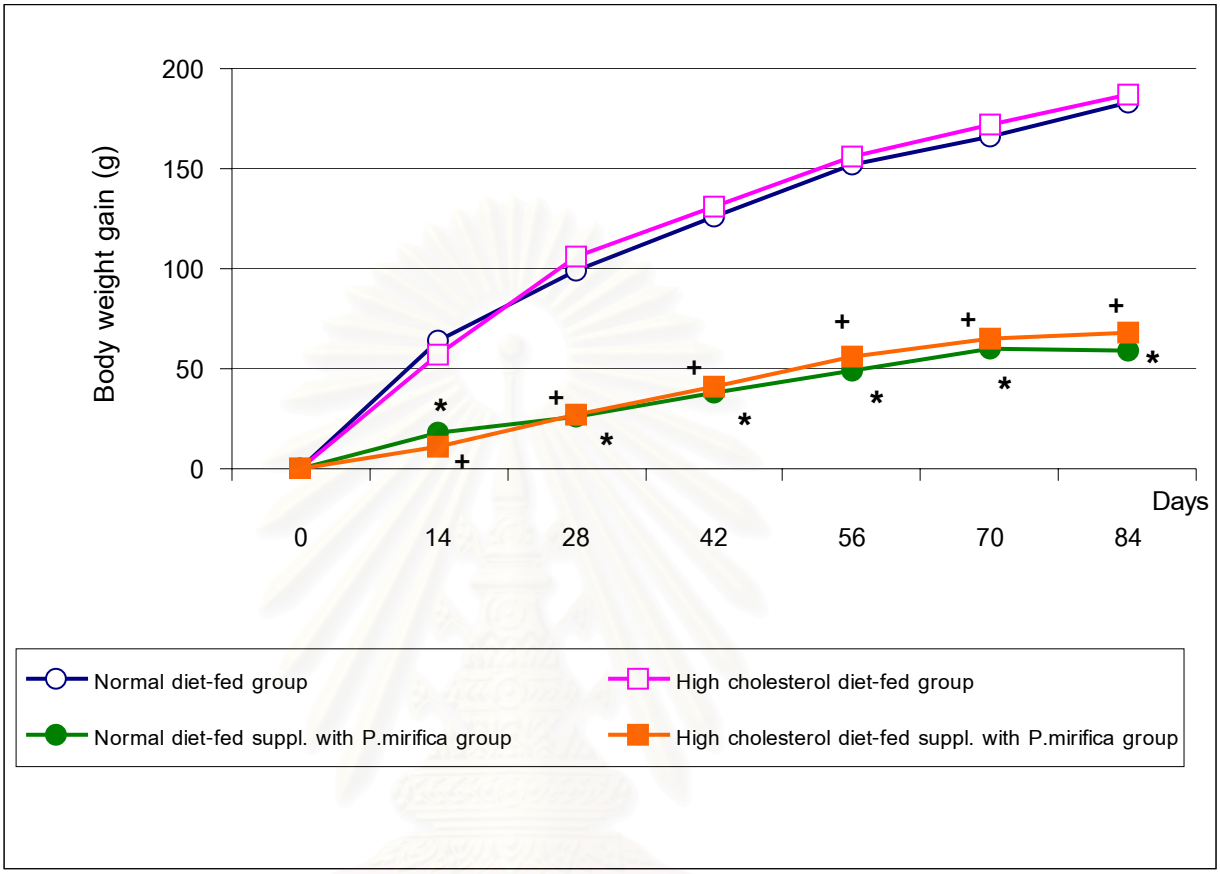


Figure 2 Effect of *P.mirifica* on body weigh gain

Data shown were mean

* P<0.05; normal diet-fed supplemented with *P.mirifica* group vs normal diet-fed group

+P<0.05; high cholesterol diet-fed supplemented with *P.mirifica* group vs high cholesterol diet-fed group

Table 6 Effect of *P.mirifica* on liver weight and relative liver weight

Treatment group	Liver weight (g)	Relative liver weight (g/100 g of body weight)
Normal diet-fed group	10.58±0.30	2.35±0.07
Normal diet-fed supplemented with *P.mirifica* group	9.11±0.39	2.54±0.10
High cholesterol diet-fed group	16.96±0.91	3.62±0.19
High cholesterol diet-fed rats supplemented with *P.mirifica* group	12.60±0.91 +	3.32±0.16

Data shown were mean ± SEM

+P<0.05; high cholesterol diet-fed supplemented with *P.mirifica* group vs high cholesterol diet-fed group

1.2 Effect of *P.mirifica* on blood clinical biochemistry parameters

In both normal diet-fed and high cholesterol diet-fed conditions, *P.mirifica* exhibited no deteriorated effects indicated by these following hematological and blood clinical biochemistry parameters: hemoglobin, hematocrit, WBC count, %differential WBC, RBC morphology, platelet count, glucose, BUN, SCr, total bilirubin, direct bilirubin AST, ALT, and ALP. Interestingly, *P.mirifica* even helped attenuating the liver injury-induced by hypercholesterolemic condition as shown by a significant decrease of AST, ALT and ALP in high cholesterol diet-fed supplemented with *P.mirifica* rats as compared to the corresponding high cholesterol diet-fed rats (Table 7).

Comparing to normal diet-fed rats, high cholesterol diet fed rats demonstrated a significant increase of AST (170.60±10.63 vs. 278.30±24.66; p<0.05), ALT (36.10±1.58 vs. 198.9±39.15; p<0.05), ALP (63.40±3.41 vs. 97.10±6.50; p<0.05).

Table 7 Effect of *P.mirifica* on hematological and blood clinical biochemistry parameters

Hematological parameters	Normal diet-fed group	Normal diet-fed supplemented with *P.mirifica* group	High cholesterol diet-fed group	High cholesterol diet-fed supplemented with *P.mirifica* group
Hemoglogbin (g/dl)	14.64±0.36	14.10±0.30	13.73±0.27	13.94±0.22
Hematocrit (%)	44.00±1.09	42.38±0.93	41.25±0.82	41.89±0.66
WBC count ($x10^9$/l)	1.81±0.36	1.39±0.10	2.05±0.32	1.34±0.19
Neutrophil (%)	27.57±1.78	25.13±3.24	25.75±4.20	22.56±2.59
Lymphocyte (%)	69.71±1.82	71.00±3.51	70.88±4.10	74.67±2.30
Monocyte (%)	2.14±0.51	3.00±1.04	3.00±0.58	2.00±0.33
Eosinophil (%)	0.57±0.43	0.86±0.23	0.75±0.25	0.78±0.32
Basophil (%)	0	0	0	0
RBC morphology	Normal	Normal	Normal	Normal
Platelet ($x10^3$/ul)	339.29±44.61	303.13±34.54	334.38±19.44	322.22±41.76
Blood clinical biochemistry parameters				
Glucose (mg/dl)	129.9±8.16	137.4±11.67	147.9±17.10	138.7±20.48
BUN (mg/dl)	22.05±1.27	20.08±0.60	21.27±1.19	21.81±1.47
SCr (mg/dl)	0.71±0.03	0.67±0.03	0.72±0.03	0.70±0.03
Total Bilirubin (mg/dl)	0.11±0.01	0.10±0.00	0.13±0.02	0.10±0.00
Direct bilirubin (mg/dl)	0.018±0.008	0.029±0.005	0.026±0.006	0.027±0.005
AST (U/l)	170.60±10.63	156.30±19.40	278.30±24.66	174.20±22.12 +
ALT (U/l)	36.10±1.58	29.00±2.85	198.9±39.15	57.1±17.30 +
ALP (U/l)	63.40±3.41	71.40±7.99	97.10±6.50	74.30±3.15 +

Data shown were mean ± SEM

+P<0.05; high cholesterol diet-fed supplemented with *P.mirifica* group vs. high cholesterol diet-fed group

P.mirfica significantly decreased total cholesterol, LDL-C and HDL-C but significantly increased triglyceride in normal diet-fed rats. It also significantly decreased total cholesterol, triglyceride, LDL-C, HDL-C and LDL-C/HDL-C ratio in high cholesterol diet-fed rats (Table 8). High cholesterol diet-fed rats showed a significant increase of total cholesterol (64.40±3.18 vs. 85.60±9.47; $p<0.05$), LDL-C (8.00±0.50 vs. 56.40±9.76; $p<0.05$) and LDL-C/HDL-C ratio (0.10±0.006 vs. 0.78±0.16; $p<0.05$) as compared to the normal diet-fed rats.

Table 8 Effect of *P.mirifica* on serum lipid parameters

Serum lipid parameters	Normal diet-fed group	Normal diet-fed supplemented with *P.mirifica* group	High cholesterol diet-fed group	High cholesterol diet-fed supplemented with *P.mirifica* group
Total cholesterol (mg/dl)	64.40±3.18	32.60±7.10 *	85.60±9.47	39.90±5.05 +
Triglyceride (mg/dl)	72.60±7.80	110.10±10.53 *	54.50±4.07	33.50±3.28 +
LDL-C (mg/dl)	8.00±0.50	5.00±0.67 *	56.40±9.76	17.00±2.64 +
HDL-C(mg/dl)	78.67±3.76	35.50±9.03 *	73.50±5.56	40.50±4.32 +
LDL-C/HDL-C ratio	0.10±0.006	0.18±0.04	0.78±0.16	0.455±0.06 +

Data shown were mean ± SEM

* $P<0.05$; normal diet-fed supplemented with *P.mirifica* group vs. normal diet-fed group

+$P<0.05$; high cholesterol diet-fed supplemented with *P.mirifica* group vs. high cholesterol diet-fed group

1.3 Effect of *P.mirifica* on hepatic CYPs

1.3.1 Effect of *P.mirifica* on hepatic microsomal total CYP contents

P.mirifica significantly decreased hepatic microsomal total CYP content in normal diet-fed rats but not in high cholesterol diet-fed rats as compared to their corresponding diet-fed rats without *P.mirifica* (Figure 3).

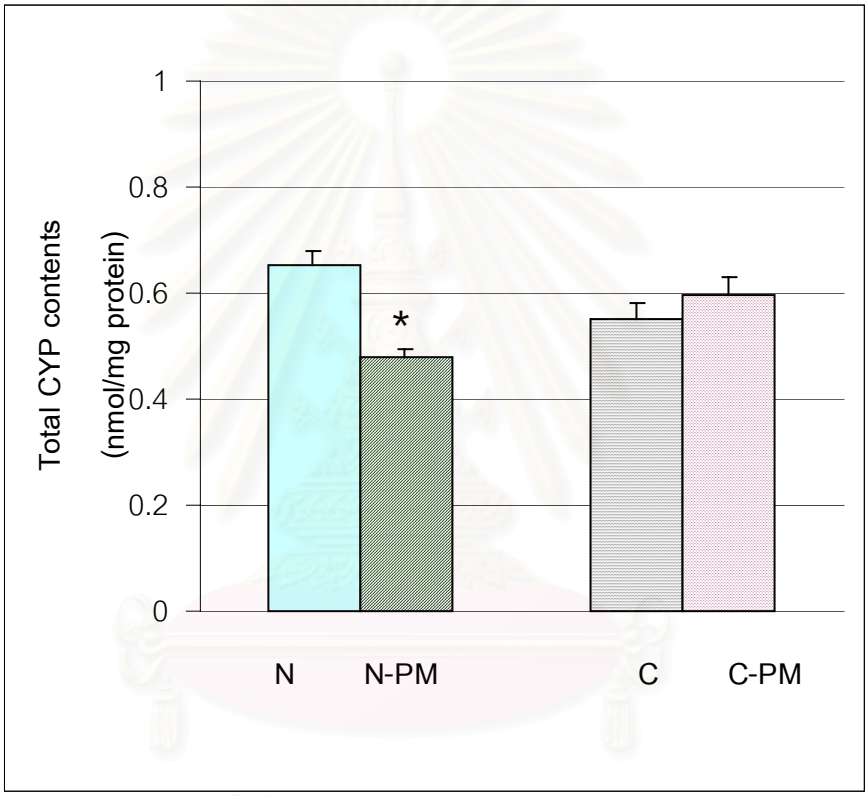


Figure 3 Effect of *P.mirifica* on hepatic microsomal total CYP contents

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

* P<0.05; normal diet-fed supplemented with *P.mirifica* group vs. normal diet-fed group

N = Normal diet-fed group

N-PM = Normal diet-fed supplemented with *P.mirifica* group

C = High cholesterol diet-fed group

C-PM = High cholesterol diet-fed supplemented with *P.mirifica* group

1.3.2 Effect of *P.mirifica* on hepatic microsomal alkoxyresorufin O-dealkylation

P.mirifica did not show any significant effects on the rate of ethoxyresorufin O-dealkylation (EROD; which represented the activities of CYP1A1) in both normal diet and high cholesterol diet conditions (Figure 4). Regarding the effects on CYP1A2, *P.mirifica* exhibited a significant inhibition effect on the rate of methoxyresorufin O-dealkylation (MROD) in normal diet-fed rats but not in high cholesterol diet-fed rats (Figure 5).

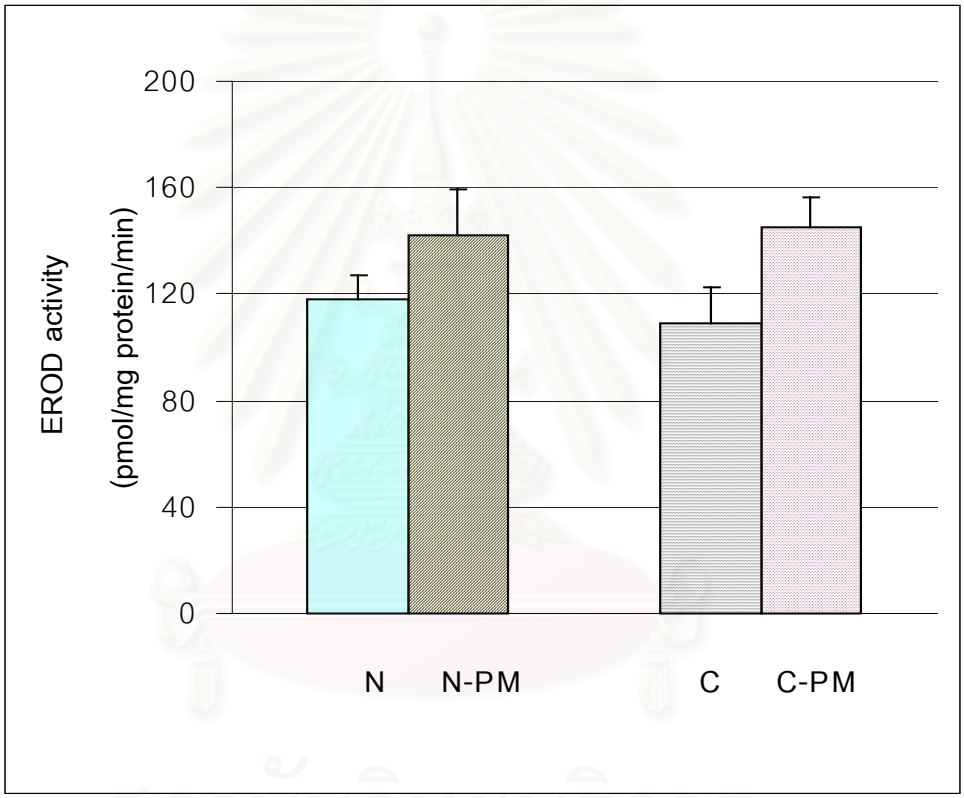


Figure 4 Effect of *P.mirifica* on hepatic microsomal EROD activity
The individual bar represented mean of EROD activity with an error bar of standard error of the mean (n=10).

N = Normal diet-fed group

N-PM = Normal diet-fed supplemented with *P.mirifica* group

C = High cholesterol diet-fed group

C-PM = High cholesterol diet-fed supplemented with *P.mirifica* group

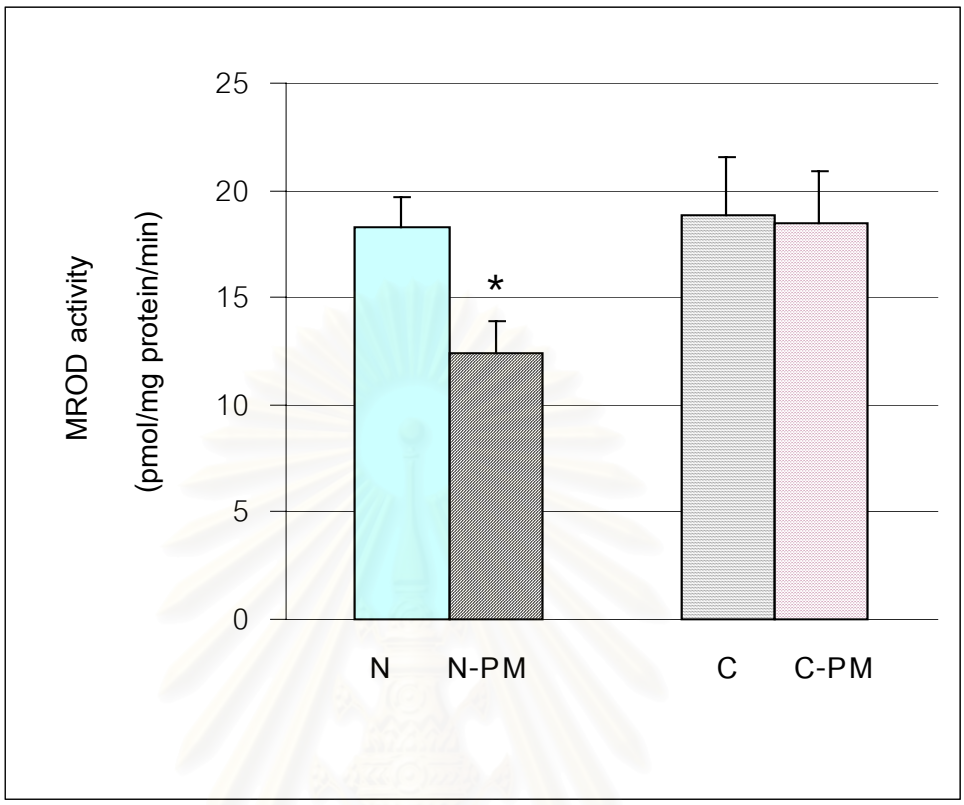


Figure 5 Effect of *P.mirifica* on hepatic microsomal MROD activity

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

* P<0.05; normal diet-fed supplemented with *P.mirifica* group vs. normal diet-fed group

N = Normal diet-fed group

N-PM = Normal diet-fed supplemented with *P.mirifica* group

C = High cholesterol diet-fed group

C-PM = High cholesterol diet-fed supplemented with *P.mirifica* group

Rate of both benzyloxy- and pentoxyresorufin O-dealkylation (BROD and PROD, respectively), which represented the activities of CYP2B1&2B2, were significantly decreased by *P.mirifica* of in both normal diet-fed and high cholesterol diet-fed groups as compared to their corresponding diet-fed control groups (Figure 6 and Figure 7).

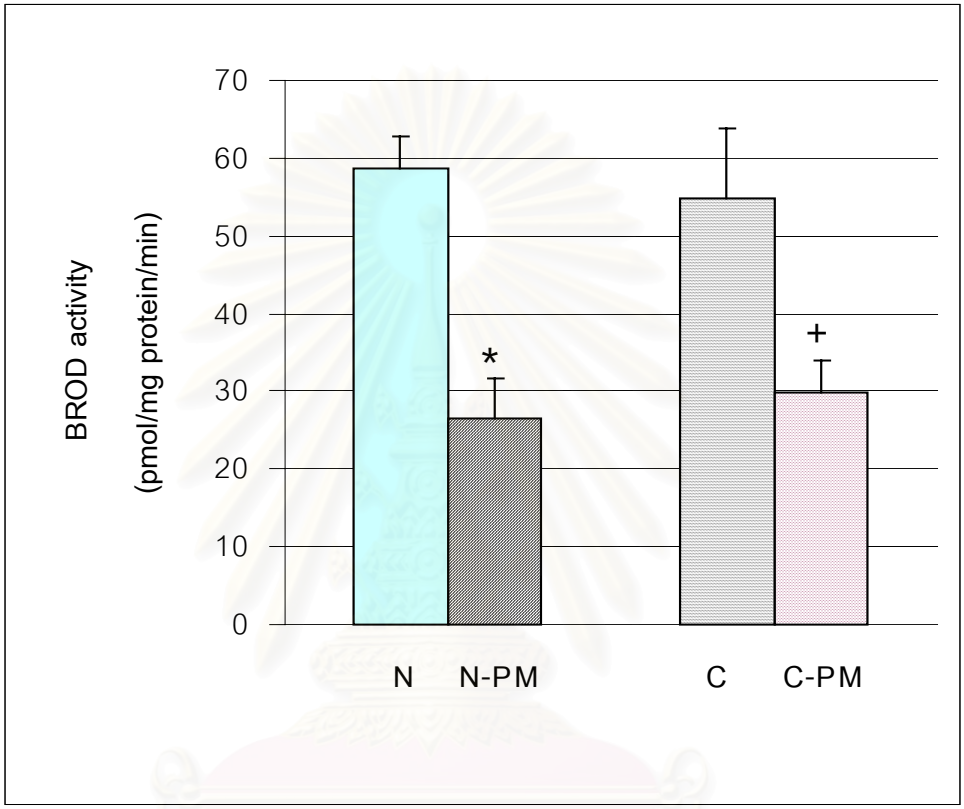


Figure 6 Effect of *P.mirifica* on hepatic microsomal BROD activity

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

* P<0.05; normal diet-fed supplemented with *P.mirifica* group vs. normal diet-fed group

+P<0.05; high cholesterol diet-fed supplemented with *P.mirifica* group vs. high cholesterol diet-fed group

N = Normal diet-fed group

N-PM = Normal diet-fed supplemented with *P.mirifica* group

C = High cholesterol diet-fed group

C-PM = High cholesterol diet-fed supplemented with *P.mirifica* group

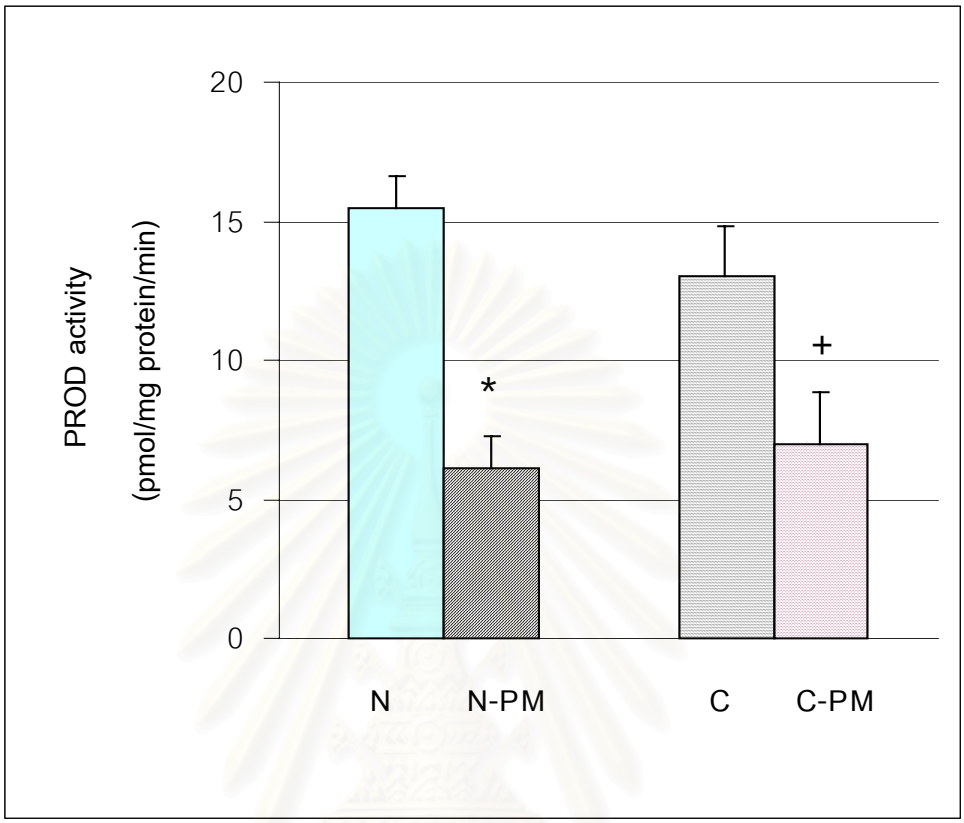


Figure 7 Effects of *P.mirifica* on hepatic microsomal PROD activity

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

* P<0.05; normal diet-fed supplemented with *P.mirifica* group vs normal diet-fed group

+P<0.05; high cholesterol diet-fed supplemented with *P.mirifica* group vs high cholesterol diet-fed group

N = Normal diet-fed group

N-PM = Normal diet-fed supplemented with *P.mirifica* group

C = High cholesterol diet-fed group

C-PM = High cholesterol diet-fed supplemented with *P.mirifica* group

1.3.3 Effect of *P.mirifica* on hepatic microsomal aniline 4-hydroxylation

P.mirifica significantly decreased rate of aniline 4-hydroxylation, which represented the activity of CYP2E1, in normal diet-fed rats but did not affect this reaction in high cholesterol diet-fed rats (Figure 8). High cholesterol diet condition also caused a decreased of CYP2E1 activity as compared to the normal diet condition (0.209±0.02 vs. 0.130±0.02; $p<0.05$).

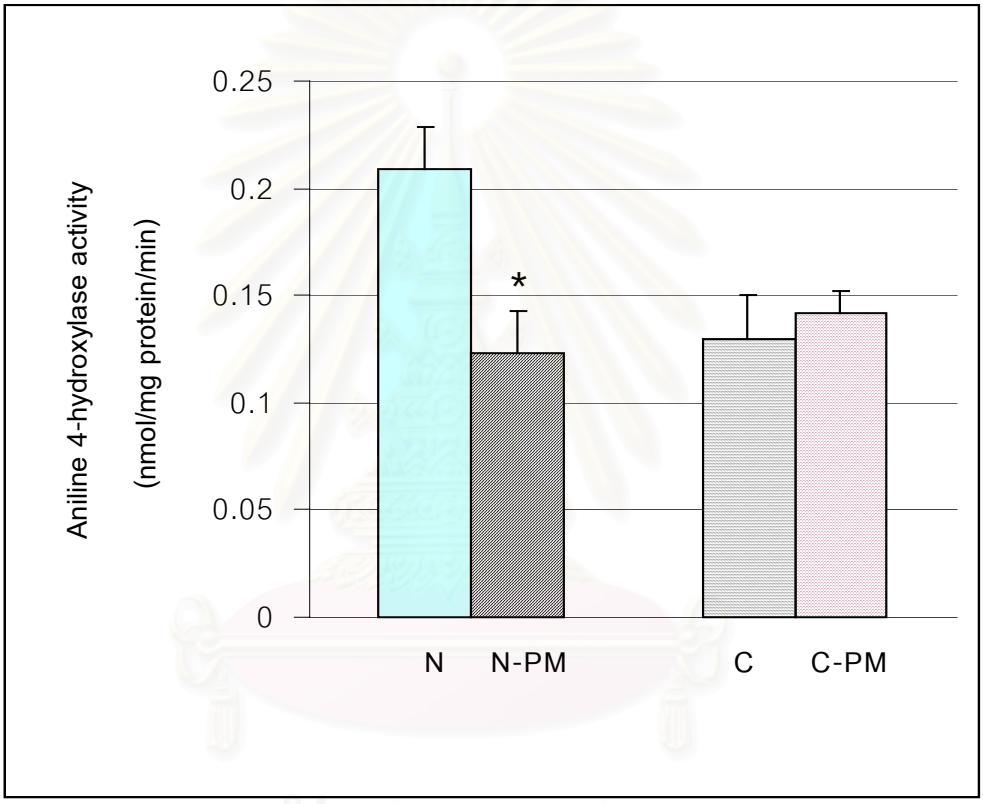


Figure 8 Effect of *P.mirifica* on rate of aniline 4-hydroxylation

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

* $P<0.05$; normal diet-fed supplemented with *P.mirifica* group vs. normal diet-fed group

N = Normal diet-fed group

N-PM = Normal diet-fed supplemented with *P.mirifica* group

C = High cholesterol diet-fed group

C-PM = High cholesterol diet-fed supplemented with *P.mirifica* group

2. An *in vitro* study

2.1 Inhibition effect of *P.mirifica* on CYP2B1&2B2

Consistent to the result found in the *ex vivo* study (Figure 6), *P.mirifica* exhibited an *in vitro* inhibition effect on CYP2B1&2B2 in a dose-dependent manner with a median inhibition concentration (IC_{50}) of 23.09 %w/v (Figure 9).

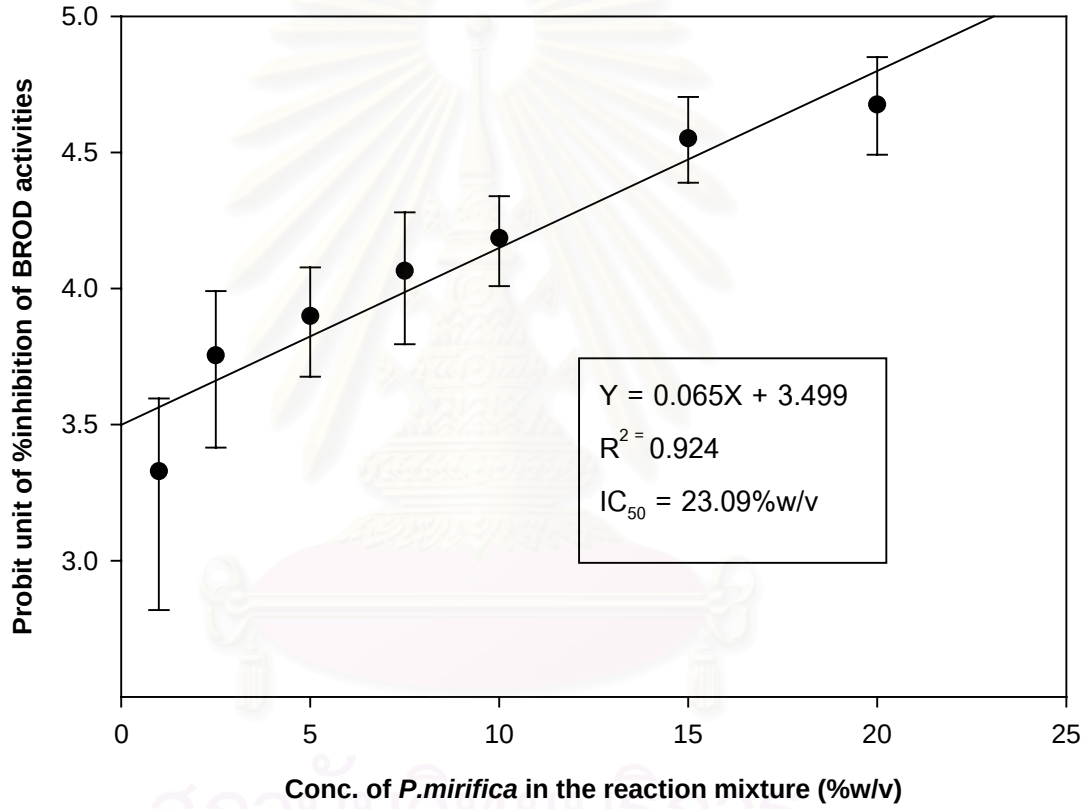


Figure 9 *In vitro* inhibition effect of *P.mirifica* on CYP2B1&2B2

Data shown were mean ± SEM (n=4)

2.2 Inhibition effect of *P.mirifica* on CYP2E1

Consistent to the result found in the *ex vivo* study (Figure 8), *P.mirifica* exhibited an *in vitro* inhibition effect on CYP2E1 in a dose-dependent manner with IC_{50} of 5.18% w/v (Figure 10).

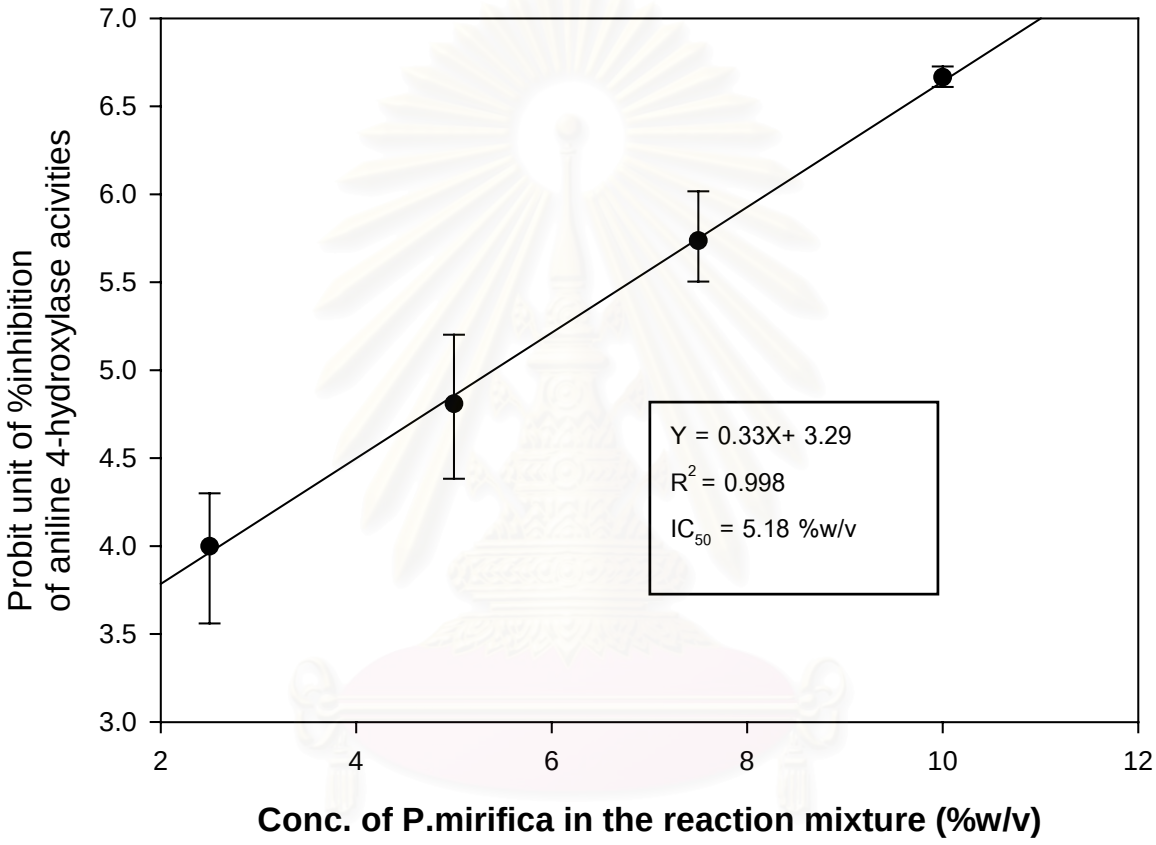


Figure 10 *In vitro* inhibition effect of *P.mirifica* on CYP2E1

Data shown were mean ± SEM (n=4)

CHAPTER V

DISCUSSION AND CONCLUSION

This study primarily investigated subchronic effects of *P.mirifica* on hapatic CYPs involving in various metabolic activations of mutagenic and/or carcinogenic xenobiotics. This would partly give a preliminary information of *P.mirifica* potential either to increase risk of xenobiotic-induced mutagenesis/carcinogenesis or, in the opposite way, to afford antimutagenic/aniticarcinogenic effects against xenobiotic-induced carcinogenesis. Subchronic effects of *P.mirifica* on hematological and blood clinical biochemistry parameters were also investigated. This would confirm the information regarding subchronic effects of *P.mirifica* at the dosage of 100 mg/kg/day, the dosage which was used in this study and was shown to decrease serum cholesterol without any serious toxic effects in the recent study (**ทรงพล ชีวะพัฒน์ และคณะ**, 2543). Due to the well-documented information that cardiovascular advantage of phytoestrogens is attributed to their lipid lowering effects, this study was also performed in hypercholesterolemic rats.

Body weight gains of rats given *P.mirifica* and fed with either normal diet or high cholesterol diet were significantly lower than their corresponding control-diet fed groups. These were consistent to the results reported by Chivapat and collaborates (ทรงพล ชีวะพัฒน์ และคณะ, 2543). In that study, they found that rats receiving *P.mirifica* orally at the doses of 100 and 1000 mg/kg/day for 90 days had body weight gain and food consumption less than the control group. This effects was possibly dued to the effects of some phytoestrogens containing in *P.mirifica*. Miroestrol was shown to cause nausea and vomiting in human (Cain, 1960). Genistein and daidzein were found to suppress food intake and body weight gain in rats (Magee, 1963; Toda et al., 1999). Inhibition of 21-hydroxylase enzymes in adrenal gland cells by both genistein and daidzein resulted in a decreased synthesis of cortisol, the hormone which acted at CNS to stimulate food appetite (Mesiano et al., 1999). Effects of estrogens on the growth and body weight of rodents are well documented. Both synthetic and natural estrogens decrease growth rate in rats and mice via acting centrally at the hypothalamus to decrease food consumption

(Gibson et al., 1967; Heywood and Wadsworth, 1980; Hart, 1990; Biegel et al., 1998). Dose-dependent growth retardation and decrease in food consumption have been reported in long-term studies with most estrogens (Biegel et al., 1998; Gibson et al., 1967). Weights of the livers were not affect by *P.mirifica* as shown by the undifferent relative liver weight of rats given *P.mirifica* fed with either normal or high cholesterol diet comparing to their corresponding control groups. In contrast, high cholesterol diet increased both liver weight and relative liver weight as comparing to the normal diet condition. This increment might be due to an accumulation of fat in the liver.

Hair loss occurred in the *P.mirifica* treated rats. This effect induced by chronic estrogen treatment has been reported (Biegel et al., 1998; Gibson et al., 1967). Although less studies were performed on estrogens than on androgens, prolonged intraperitoneal, subcutaneous implant or oral administration of estrogens has been shown to block hair growth in rats and mice (Smart et al., 1999; Biegel et al., 1998; Gibson et al., 1967). Topical ICI 182 780, a pure estrogen receptor antagonist, stimulates hair regrowth in male mice (Smart et al., 1999). Hair follicle is a complex structure that is influenced by systemic factors including androgens, glucocorticoids and estrogens. The estrogen receptor pathway within dermal papilla regulates the telogen-anagen transition of the hair follicle in CD-mice (Oh and Smart, 1996).

Results from this study showed that *P.mirifica* given orally at the dose of 100 mg/kg/day for 90 days did not cause any toxic effects to the hematopoietic system of male rats. In addition, there were no effects of *P.mirifica* at this dose on serum glucose as well as the functions of liver and kidney. These results corresponded to the result of Chivapat and collaborates (ทรงพล ชีวะพัฒน์ และคณะ, 2543). From that study, *P.mirifica* affected blood parameters only when the compound was given at 1000 mg/kg/day. Toxic effects of estrogens on blood system have been shown in animal studies. Ninety day feeding rats with diet contained 10 and 50 ppm of 17β-estradiol demonstrated mild anemia with the mean value of hematocrit, RBC count and hematocrit lower than the control group (Biegel

et al, 1998). Administration of diethystilbestrol, a synthetic estrogen, in the diet for two years caused a slight reduction in hemoglobin and hematocrit in both sexes of Sprague-Dawley rats (Gibson et al., 1967). A favorable effect of *P.mirifica* on the liver was demonstrated while this compound was given to high cholesterol diet rats. High cholesterol diet-fed condition caused a significant increase of serum hepatic parenchymal enzymes such as AST, ALT as well as the enzyme reflecting cholestasis such as ALP. Accompanying the unpleasant lipid profile with an increase of liver weight in high cholesterol diet rats, it is likely that an accumulation of fat in the liver might be involved in lipid-induced liver injury in this group of animals. *P.mirifica* caused an advantageous effect on lipid profile particularly in high cholesterol diet-fed rats. These findings gave a rational explanation for an attenuating effect of *P.mirifica* on lipid-induced liver injury in high cholesterol diet-fed rats.

To investigate the effects of *P.mirifica* on hepatic microsomal CYPs which are responsible to carcinogen activation, an *ex vivo* study was performed so as to utilize liver for microsomal preparations and to collect blood for clinical biochemistry assays simultaneously. The results showed that in normal diet-fed rats, *P.mirifica* decreased total CYP contents as well as the activities of CYP1A2, CYP2B&2B2 and CYP2E1. CYP1A1&1A2, CYP2B1&2B2 and CYP2E1 are among the CYPs that play a key role in carcinogenic and/or mutagenic activation of many environmental chemicals (Parkinson, 1996, Rendic and Di Carlo, 1997; Gonzalez; 1989). Thus, no induction effect of *P.mirifica* on those isoforms of CYP should be an advantageous feature of this compound regarding a potential increase risk of toxicity from many xenobiotics via metabolic bioactivation. Inhibition effect of *P.mirifica* on CYP1A2, CYP2B1&2B2 and CYP2E1 implied that some constituents in *P.mirifica* were likely to be metabolized by these isoforms of CYP. In the other way, some constituents in this plant possessed an inhibition effect solely on these CYPs without the metabolic involvement. A few studies were performed regarding the inhibition effects of genistein, daidzein on hepatic CYPs (Helbsy et al., 1998) as well as the metabolism of genistein (Robert-Kirchhoff et al., 1999). Both genistein and daidzein (or its metabolite, equol) were found to inhibit CYP1A1&1A2 and CYP2E1 using mice liver microsomes and human specific CYPs (Helbsy et al., 1998). Genistein was shown to be

metabolized by CYP1A1&1A2, CYP1B1 and CYP2E1 (Robert-Kirchhoff et al., 1999). Besides genistein and daidzein, puerarin, another compound which is also found in *P.mirifica* was shown to possess an inhibition effect on CYPs such as CYP2E1, CYP2B1 and CYP3A (Guerra et al., 2000). Therefore, mechanism of these inhibition effects of *P.mirifica* and which constituents in this plant exerted these effects should be further investigated. Effect of *P.mirifica* on other isoforms of CYP that were not assessed in this study such as CYP3A4 which is a major CYP isoform in human and plays a key role in carcinogenic and/or mutagenic activation of many xenobiotics should be further studied.

Effects of *P.mirifica* on hepatic CYPs were somewhat different in high cholesterol diet-fed condition. No effects of *P.mirifica* were found on hepatic total CYP content as well as the activities of CYP1A1&1A2, and CYP2E1. Only CYP2B1&2B2 were inhibited by this compound in high cholesterol diet-fed condition. An interaction between the effect of *P.mirifica* and the effect of high cholesterol diet-fed condition was likely to attribute to these conflicting findings especially for CYP2E1. High cholesterol diet-fed rats demonstrated a decrease of CYP2E1 which was inconsistent to many observations that found an induction of CYP2E1 in rats fed with high fat diet (Raucy et al., 1991) or diet supplemented with either saturated or unsaturated fatty acids containing oils (Ioannides, 1999; Takahashi et al., 1992). However, the inhibition of CYP2E1 was found in hypercholesterolemic rabbits, the animal model which is highly susceptible to dietary cholesterol (McNamara, 2000). High degree of hypercholesterolemic condition induced in high cholesterol diet-fed rats in this study resulted in a liver injury and consequently, the decrease activities some susceptible isoforms of CYPs, the enzymes located in hepatic endoplasmic reticulum membrane.

To preliminarily investigate the inhibition effects of *P.mirifica* on CYP2B1&2B2 and CYP2E1, we further performed an *in vitro* inhibition study. Specific inducing agents were used to induce specific isoforms of CYP on which the inhibition effects of *P.mirifica* would be determined. Phenobarbital was utilized to induce hepatic CYP2B1&2B2 according to the standard regimen generally used (Gibson and Skett, 1994). Acetone was used to induce CYP2E1 according to the method of Helsby et al. (1998). The results showed that

inhibitions of these isoforms of CYP were dose-related. IC_{50} of BROD (or CYP2B1&2B2) and aniline 4-hydroxylase activity (or CYP2E1) were 23.09 and 5.85 %w/v, respectively. These findings indicated that some constituents in *P.mirifica* were substrates of CYP2B1&2B2 and CYP2E1. Further study on the mechanism of inhibition should be elucidated.

In conclusion, subchronic (90 days) exposure of *P.mirifica* given orally at 100 mg/kg/day to male Wistar rats did not show any toxic effects on blood system as well as functions of liver and kidney. *P.mirifica* even attenuated the hepatic injury induced by hypercholesterolemic condition probably due to its beneficial effects on lipid profile especially in high cholesterol diet-fed rats. *P.mirifica* demonstrated no induction effects on CYP1A1&1A2, CYP2B1&2B2 and CYP2E1. In contrast, it demonstrated an inhibition effect on CYP2B1&2B2 in either normal diet or high cholesterol diet-fed rats but an inhibition effect of CYP1A2 and CYP2E1 was found only in normal diet-fed rats. Inhibitions of CYP2B1&2B2 and CYP2E1 were also found *in vitro* with an IC_{50} of 23.09 and 5.85 %w/v, respectively. Effects of *P.mirifica* at various doses, long-term uses as well as mechanism of effects should be further investigated. Effects of this compound on other isoforms of CYP should also be explored.

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APPENDIX

Table 9 Body weight of individual rat in normal diet-fed group

Rat number	Body weight (g)							
	Day0	Day14	Day28	Day42	Day56	Day70	Day84	Day91
1	206	297	360	395	444	459	495	495
2	269	323	363	388	424	435	468	459
3	282	366	413	427	471	466	508	485
4	213	312	366	370	411	428	463	466
5	331	379	405	452	464	494	488	488
6	257	327	354	375	388	409	415	403
7	271	348	371	411	432	446	449	435
8	284	332	339	371	392	410	417	400
9	316	344	387	409	440	448	446	446
10	376	417	442	466	463	485	488	454

Table 10 Body weight of individual rat in high cholesterol diet-fed group

Rat number	Body weight (g)							
	Day0	Day14	Day28	Day42	Day56	Day70	Day84	Day91
1	220	313	385	437	489	500	512	512
2	282	340	382	395	438	452	480	468
3	275	377	426	436	472	486	506	489
4	338	368	421	427	465	491	520	520
5	305	353	384	417	442	460	465	464
6	259	315	351	389	406	403	425	417
7	273	337	368	416	435	445	445	444
8	307	353	406	425	421	435	444	440
9	341	391	451	471	502	520	536	536
10	339	370	423	439	428	469	472	413

Table 11 Body weight of individual rat in normal diet-fed supplemented with *P.mirifica* group

Rat number	Body weight (g)							
	Day0	Day14	Day28	Day42	Day56	Day70	Day84	Day91
1	220	266	274	299	310	337	319	319
2	260	281	280	243	303	354	309	329
3	259	300	296	297	311	331	339	304
4	288	291	303	309	321	330	327	348
5	350	325	340	359	377	374	373	373
6	276	308	316	333	355	357	355	352
7	305	323	302	334	346	359	359	346
8	336	356	335	383	380	386	389	376
9	312	315	344	352	359	362	374	374
10	314	303	310	333	340	348	355	498

Table 12 Body weight of individual rat in high cholesterol diet-fed supplemented with *P.mirifica* group

Rat number	Body weight (g)							
	Day0	Day14	Day28	Day42	Day56	Day70	Day84	Day91
1	231	263	270	274	304	310	324	324
2	314	302	339	340	353	373	371	369
3	284	292	323	338	349	355	358	350
4	308	257	309	321	320	338	350	342
5	313	258	323	341	367	377	364	379
6	279	281	295	338	356	351	361	362
7	305	333	350	366	377	399	394	385
8	315	355	399	417	419	429	436	432
9	315	298	329	324	363	333	348	348
10	314	205	292	334	334	362	356	483

Table 13 Liver weight of individual rat rat in normal diet-fed group

Rat number	Liver weight (g)
1	10.32
2	9.07
3	12.65
4	11.07
5	10.57
6	10.71
7	10.65
8	10.12
9	9.79
10	10.89

Table 14 Liver weight of individual in high cholesterol diet-fed group

Rat number	Liver weight (g)
1	19.98
2	13.92
3	19.04
4	14.30
5	16.40
6	13.73
7	22.08
8	17.02
9	18.55
10	14.53

Table 15 Liver weight of individual rat in normal diet-fed supplemented with *P.mirifica* group

Rat number	Liver weight (g)
1	7.09
2	9.30
3	7.96
4	7.72
5	10.10
6	9.61
7	9.30
8	11.07
9	8.77
10	10.26

Table16 Liver weight of individual rat in high cholesterol diet-fed supplemented with *P.mirifica* group

Rat number	Liver weight (g)
1	9.30
2	9.18
3	12.78
4	9.90
5	13.49
6	13.85
7	12.82
8	18.33
9	10.99
10	15.32

Table 17 Blood clinical biochemistry parameters of individual rat in normal diet-fed group

Blood clinical biochemistry	Rat number									
	1	2	3	4	5	6	7	8	9	10
Glucose (mg/dl)	176	115	154	136	127	127	80	112	128	144
BUN (mg/dl)	26	21.7	23.6	26	22.3	16.7	16.3	17.7	23.1	28.1
SCr (mg/dl)	0.7	0.8	0.6	0.7	0.8	0.6	0.6	0.7	0.8	0.8
Total cholesterol (mg/dl)	71	49	63	60	66	53	81	57	76	68
Triglyceride (mg/dl)	58	49	98	42	94	61	108	45	76	95
SGOT=AST(U/L)	105	171	173	121	187	199	211	195	179	165
SGPT=ALT (U/L)	33	30	41	26	41	39	36	40	37	38
ALP (U/L)	82	56	59	59	70	65	46	76	67	54
HDL-C (mg/dl)	91	63	-	75	87	65	96	72	81	78
LDL-C (mg/dl)	9	6	-	7	9	9	10	6	9	7
LDL-C/HDL-C ratio	0.10	0.10	-	0.09	0.10	0.14	0.10	0.08	0.11	0.09
Total bilirubin (mg/dl)	0.1	0.1	-	0.1	0.2	0.1	0.1	0.1	0.1	0.1
Direct bilirubin (mg/dl)	0.00	0.00	-	0.01	0.04	0.00	0.00	0.06	0.05	0.00
Hematology										
Hb (g/L)	clotted	12.67	15.3	15.3	15	clotted	15.3	14.3	clotted	14.6
Hct (%)	clotted	38	46	46	45	clotted	46	43	clotted	44
WBC count ($x10^9$/L)	clotted	1	3.45	1.5	1.35	clotted	0.85	1.85	clotted	2.65
Differential (%)										
- Neutrophil	clotted	32	21	27	32	clotted	22	27	clotted	32
- Lymphocyte	clotted	66	76	70	66	clotted	76	70	clotted	64
- Monocyte	clotted	2	3	3	1	clotted	2	0	clotted	4
- Eocinophil	clotted	0	0	0	1	clotted	0	3	clotted	0
- Basophil	clotted	0	0	0	0	clotted	0	0	clotted	0
RBC morphology	clotted	normal	normal	normal	normal	clotted	normal	normal	clotted	normal
Platelet ($x10^3$/uL)	clotted	500	400	275	325	clotted	350	400	clotted	125

Table 18 Blood clinical biochemistry parameters of individual rat in high cholesterol diet-fed group

Blood clinical biochemistry	Rat number									
	1	2	3	4	5	6	7	8	9	10
Glucose (mg/dl)	254	78	124	120	108	175	212	161	101	146
BUN (mg/dl)	30.1	16.9	18.8	22.9	23.6	19.3	22.4	19.3	19.3	23.1
SCr (mg/dl)	0.7	0.6	0.6	0.6	0.8	0.7	0.7	0.7	0.8	0.7
Total cholesterol (mg/dl)	50	114	68	105	104	120	120	56	73	46
Triglyceride (mg/dl)	-	46	-	42	68	53	69	38	56	60
SGOT=AST(U/L)	326	394	268	171	257	398	311	222	194	242
SGPT=ALT (U/L)	275	379	207	41	213	391	199	109	136	39
ALP (U/L)	138	100	109	70	93	111	106	70	84	90
HDL-C (mg/dl)	64	108	72	50	80	73	97	61	70	60
LDL-C (mg/dl)	26	79	33	99	67	82	92	25	45	16
LDL-C/HDL-C ratio	0.41	0.73	0.46	1.98	0.84	1.12	0.95	0.41	0.64	0.27
Total bilirubin (mg/dl)	0.1	0.1	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.2
Direct bilirubin (mg/dl)	0.00	0.00	0.02	0.03	0.06	0.01	0.05	0.02	0.04	0.03
Hematology										
Hb (g/L)	clotted	clotted	12.67	14.3	12.7	14.6	13.3	14	14	14.3
Hct (%)	clotted	clotted	38	43	38	44	40	42	42	43
WBC count ($x10^9$/L)	clotted	clotted	2.85	1.45	1.6	1.3	1.9	1.9	3.9	1.5
Differential (%)										
- Neutrophil	clotted	clotted	35	20	51	22	25	14	20	19
- Lymphocyte	clotted	clotted	61	78	47	74	70	80	76	81
- Monocyte	clotted	clotted	3	1	2	2	5	5	3	0
- Eosinophil	clotted	clotted	1	1	0	0	0	1	1	0
- Basophil	clotted	clotted	0	0	0	0	0	0	0	0
RBC morphology	clotted	clotted	normal	normal	normal	normal	normal	normal	normal	normal
Platelet ($x10^3$/uL)	clotted	clotted	250	325	300	325	325	400	325	425

Table 19 Blood clinical biochemistry parameters of individual rat in normal diet-fed supplemented with *P.mirifica* group

Blood clinical biochemistry	Rat number									
	1	2	3	4	5	6	7	8	9	10
Glucose (mg/dl)	209	149	103	92	172	126	100	158	116	149
BUN (mg/dl)	20.9	18.9	20.5	18.4	19.9	15.9	21.9	21.5	21	21.9
SCr (mg/dl)	0.7	0.7	0.6	0.6	0.6	0.6	0.7	0.7	0.9	0.6
Total cholesterol (mg/dl)	15	28	25	21	33	32	19	34	25	94
Triglyceride (mg/dl)	63	77	106	127	172	124	124	115	126	67
SGOT=AST(U/L)	70	167	163	134	110	179	302	174	146	118
SGPT=ALT (U/L)	30	26	32	21	19	23	50	28	20	25
ALP (U/L)	86	137	66	68	60	59	69	48	68	53
HDL-C (mg/dl)	16	32	18	23	35	32	15	40	31	113
LDL-C (mg/dl)	<3	<3	6	3	6	3	7	6	4	9
LDL-C/HDL-C ratio	0.19	0.09	0.33	0.13	0.17	0.09	0.47	0.15	0.13	0.08
Total bilirubin (mg/dl)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Direct bilirubin (mg/dl)	0.02	0.00	0.01	0.04	0.05	0.04	0.03	0.04	0.03	0.03
Hematology										
Hb (g/L)	clotted	clotted	13.33	14.3	14	15.6	13.6	13	14	15
Hct (%)	clotted	clotted	40	43	42	47	41	39	42	45
WBC count ($x10^9$/L)	clotted	clotted	1.45	1.45	0.75	1.25	1.45	1.6	1.55	1.65
Differential (%)										
- Neutrophil	clotted	clotted	18	33	32	32	7	23	32	24
- Lymphocyte	clotted	clotted	79	65	64	64	92	66	66	72
- Monocyte	clotted	clotted	2	2	3	2	1	10	1	3
- Eosinophil	clotted	clotted	1	0	1	2	0	1	1	1
- Basophil	clotted	clotted	0	0	0	0	0	0	0	0
RBC morphology	clotted	clotted	normal	normal	normal	normal	normal	normal	normal	normal
Platelet ($x10^3$/uL)	clotted	clotted	150	300	325	350	400	425	300	175

Table 20 Blood clinical biochemistry parameters of individual rat in high cholesterol diet-fed supplemented with *P.mirifica* group

Blood clinical biochemistry	Rat number									
	1	2	3	4	5	6	7	8	9	10
Glucose (mg/dl)	182	143	99	129	132	152	55	133	73	289
BUN (mg/dl)	17.6	17.8	19.8	23.9	32.2	19.4	20.6	19.7	19.7	27.1
Cr (mg/dl)	0.6	0.8	0.6	0.8	0.8	0.6	0.6	0.7	0.8	0.7
Total cholesterol (mg/dl)	13	37	49	40	21	60	34	39	40	66
Triglyceride (mg/dl)	28	45	35	46	20	37	24	20	33	47
SGOT=AST(U/L)	61	184	144	277	102	130	228	272	172	172
SGPT=ALT (U/L)	21	27	37	203	51	33	27	89	42	41
ALP (U/L)	81	68	53	82	76	82	65	73	77	86
HDL-C (mg/dl)	30	41	46	37	22	58	35	43	40	113
LDL-C (mg/dl)	8	10	20	20	11	28	20	16	28	<3
LDL-C/HDL-C ratio	0.27	0.24	0.43	0.54	0.50	0.48	0.57	0.37	0.70	0.03
Total bilirubin (mg/dl)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Direct bilirubin (mg/dl)	0.03	0.03	0.00	0.03	0.04	0.04	0.04	0.02	0.00	0.04
Hematology										
Hb (g/L)	clotted	15	14	13.3	13.3	14.3	14	14	13	14.6
Hct (%)	clotted	45	42	40	40	43	42	42	39	44
WBC count ($x10^9$/L)	clotted	0.3	2.25	0.8	1.15	1.5	1.2	1.75	1.35	1.75
Differential (%)										
- Neutrophil	clotted	33	17	28	22	18	13	32	27	13
- Lymphocyte	clotted	65	80	70	74	80	84	66	72	81
- Monocyte	clotted	2	1	1	2	2	3	2	1	4
- Eosinophil	clotted	0	2	1	2	0	0	0	0	2
- Basophil	clotted	0	0	0	0	0	0	0	0	0
RBC morphology	clotted	normal	normal	normal	normal	normal	normal	normal	normal	normal
Platelet ($x10^3$/uL)	clotted	475	125	325	350	350	400	450	300	125

Table 21 Serum lipid parameters of individual rat in normal diet-fed group

Serum lipid parameters	Rat number									
	1	2	3	4	5	6	7	8	9	10
Total cholesterol (mg/dl)	71	49	63	60	66	53	81	57	76	68
Triglyceride (mg/dl)	58	49	98	42	94	61	108	45	76	95
HDL-C (mg/dl)	91	63	-	75	87	65	96	72	81	78
LDL-C	9	6	-	7	9	9	10	6	9	7
LDL-C/HDL-C ratio	0.10	0.10	-	0.09	0.10	0.14	0.10	0.08	0.11	0.09

Table 22 Serum lipid parameters of individual rat in high cholesterol diet-fed group

Serum lipid parameters	Rat number									
	1	2	3	4	5	6	7	8	9	10
Total cholesterol (mg/dl)	50	114	68	105	104	120	120	56	73	46
Triglyceride (mg/dl)	-	46	-	42	68	53	69	38	56	60
HDL-C (mg/dl)	64	108	72	50	80	73	97	61	70	60
LDL-C (mg/dl)	26	79	33	99	67	82	92	25	45	16
LDL-C/HDL-C ratio	0.41	0.73	0.46	1.98	0.84	1.12	0.95	0.41	0.64	0.27

Table 23 Serum lipid parameters of individual rat in normal diet-fed supplemented with *P.mirifica* group

Serum lipid parameters	Rat number									
	1	2	3	4	5	6	7	8	9	10
Total cholesterol (mg/dl)	15	28	25	21	33	32	19	34	25	94
Triglyceride (mg/dl)	63	77	106	127	172	124	124	115	126	67
HDL-C (mg/dl)	16	32	18	23	35	32	15	40	31	113
LDL-C (mg/dl)	3	3	6	3	6	3	7	6	4	9
LDL-C/HDL-C	0.19	0.06	0.33	0.13	0.17	0.09	0.47	0.15	0.13	0.08

Table 24 Serum lipid parameters of individual rat in high cholesterol diet-fed supplemented with *P.mirifica* group

Serum lipid parameters	Rat number									
	1	2	3	4	5	6	7	8	9	10
Total cholesterol (mg/dl)	13	37	49	40	21	60	34	39	40	66
Triglyceride (mg/dl)	28	45	35	46	20	37	24	20	33	47
HDL-C (mg/dl)	30	41	46	37	22	58	35	43	40	70
LDL-C (mg/dl)	8	10	20	20	11	28	20	16	28	3
LDL-C/HDL-C	0.27	0.24	0.43	0.54	0.50	0.48	0.57	0.37	0.70	0.03

Table 25 Hepatic microsomal total CYP content of an individual rat

Rat number	Treatment group			
	Normal diet-fed group	High cholesterol diet-fed group	Normal diet-fed suppl. with *P.mirifica* group	High cholesterol diet-fed suppl. with *P.mirifica* group
1	0.800	0.387	0.506	0.709
2	0.560	0.569	0.478	0.756
3	0.695	0.618	0.528	0.495
4	0.687	0.629	-	0.588
5	0.668	0.516	0.495	0.506
6	0.725	0.379	0.470	0.640
7	0.599	0.626	0.418	0.508
8	0.604	0.654	0.539	0.547
9	0.643	0.549	0.481	0.497
10	0.547	0.585	0.407	0.728

Unit expressed as nmol/mg protein.

Table 26 Hepatic microsomal EROD activity of an individual rat

Rat number	Treatment group			
	Normal diet-fed group	High cholesterol diet-fed group	Normal diet-fed suppl. with *P.mirifica* group	High cholesterol diet-fed suppl. with *P.mirifica* group
1	93	49	206	132
2	154	187	92	115
3	122	142	260	193
4	166	146	160	195
5	90	52	100	107
6	101	96	91	116
7	123	122	120	110
8	93	81	132	133
9	140	98	137	166
10	102	119	124	186

Unit expressed as pmol/mg protein/min.

Table 27 Hepatic microsomal MROD activity of an individual rat

Rat number	Treatment group			
	Normal diet-fed group	High cholesterol diet-fed group	Normal diet-fed suppl. with *P.mirifica* group	High cholesterol diet-fed suppl. with *P.mirifica* group
1	21	9	21	15
2	26	35	11	15
3	16	24	11	27
4	21	22	12	17
5	16	8	11	11
6	11	13	4	18
7	15	28	8	14
8	20	14	14	11
9	22	16	14	22
10	15	19	18	35

Unit expressed as pmol/mg protein/min.

Table 28 Hepatic microsomal BROD activity of an individual rat

Rat number	Treatment group			
	Normal diet-fed group	High cholesterol diet-fed group	Normal diet-fed suppl. with *P.mirifica* group	High cholesterol diet-fed suppl.with *P.mirifica* group
1	45	20	21	15
2	76	99	19	28
3	75	99	63	26
4	72	63	46	18
5	59	17	14	26
6	66	64	11	32
7	42	45	20	25
8	53	44	18	44
9	57	45	24	24
10	41	53	30	60

Unit expressed as pmol/mg protein/min.

Table 29 Hepatic microsomal PROD activity of an individual rat

Rat number	Treatment group			
	Normal diet-fed group	High cholesterol diet-fed group	Normal diet-fed suppl. with *P.mirifica* group	High cholesterol diet-fed suppl. with *P.mirifica* group
1	12	6	4	3
2	22	22	6	8
3	16	21	16	0
4	12	14	1	3
5	18	5	1	6
6	18	17	2	9
7	15	10	8	6
8	18	11	6	11
9	14	10	8	4
10	10	14	9	20

Unit expressed as pmol/mg protein/min.

Table 30 Hepatic microsomal aniline 4-hydroxylase activity of an individual rat

Rat number	Treatment group			
	Normal diet-fed group	High cholesterol diet-fed group	Normal diet-fed suppl. with *P.mirifica* group	High cholesterol diet-fed suppl. with *P.mirifica* group
1	0.172	0.054	0.092	0.106
2	0.203	0.113	0.153	0.122
3	0.095	0.048	0.300	0.084
4	0.299	0.155	0.133	0.146
5	0.246	0.051	0.084	0.142
6	0.263	0.288	0.084	0.197
7	0.230	0.108	0.105	0.134
8	0.211	0.167	0.088	0.143
9	0.209	0.111	0.073	0.142
10	0.158	0.206	0.119	0.203

Unit expressed as nmol/mg protein/min.

Table 31 BROD acitivities of individual rat at various concentrations of *P.mirifica* in the reaction mixture

Concentration of *P.mirifica* in the reaction Mixture (%w/v)	BROD activities (pmol/mg protein/min)			
	Rat number			
	1	2	3	4
0	3880	2380	3138	3596
1	3748	2390	3100	3080
2.5	3302	2366	2996	2784
5	3364	2312	2690	2742
7.5	3128	2522	2420	2600
10	3048	2200	2226	2698
15	2718	1932	2000	1952
20	2426	1946	1726	1856

Table 32 Aniline 4-hydroxylase activities of individual rat at various concentrations of *P.mirifica* in the reaction mixture

Concentration of *P.mirifica* in the reaction Mixture (%w/v)	Aniline 4-hydroxylase activities (pmol/mg protein/min)			
	Rat number			
	1	2	3	4
0	0.2	0.33	0.31	0.41
1	0.25	0.26	0.31	0.31
2.5	0.23	0.22	0.3	0.3
5	0.17	0.09	0.26	0.14
7.5	0.09	0.06	0.06	0.04
10	0.01	0.02	0.01	0.02

VITAE

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