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ในการศึกษาแบบนอกกาย

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ปีการศึกษา 2558

IN VITRO STUDY OF THE HEPATOPROTECTIVE EFFECT AND HERB-
DRUG INTERACTION OF PHIKUD NAVAKOT EXTRACT

Mr. Abhiruj Chiangsom

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Pharmacology and Toxicology

Department of Pharmacology and Physiology

Faculty of Pharmaceutical Sciences

Chulalongkorn University

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Thesis Title	*IN VITRO* STUDY OF THE HEPATOPROTECTIVE EFFECT AND HERB-DRUG INTERACTION OF PHIKUD NAVAKOT EXTRACT
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อภิรุจ เชียงโสม : ฤทธิ์ปกป้องตับและอันตรกิริยาระหว่างสมุนไพรกับยาของสารสกัดพิกัดนวโกฐใน การศึกษาแบบนอกกาย (*IN VITRO* STUDY OF THE HEPATOPROTECTIVE EFFECT AND HERB-DRUG INTERACTION OF PHIKUD NAVAKOT EXTRACT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ภญ. พ.ต.ท. หญิง ดร.สมทรง ลาวัณย์ประเสริฐ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ภญ. ดร.รวิวรรณ มณีรัตนโชติ, 153 หน้า.

พิกัดนวโกฐเป็นเครื่องยาที่มีองค์ประกอบของพืชสมุนไพร 9 ในสัดส่วนที่เท่าๆกันเครื่องยานี้นิยมใช้ใน การรักษากลุ่มอาการทางระบบไหลเวียนโลหิต การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของสารสกัดพิกัดนวโกฐและ สารออกฤทธิ์เบต้าซิโตสเตียรอลในการปกป้องตับจากภาวะเครียดออกซิเดชัน รวมทั้งศึกษาผลของสารสกัด พิกัดนวโกฐต่อเอนไซม์ไซโตโครมพี 450 ในตับมนุษย์โดยการศึกษาแบบนอกกาย เพื่อศึกษาผลปกป้องตับของสาร สกัดพิกัดนวโกฐและเบต้าซิโตสเตียรอลทำการศึกษาโดยบ่มเซลล์เพาะเลี้ยงชนิดเฮพจี 2 กับสารสกัดพิกัดนวโกฐ (0-1 มก./มล.) หรือเบต้าซิโตสเตียรอล (0-20 ไมโครโมลาร์) แล้วเหนี่ยวนำให้เกิดภาวะเครียดออกซิเดชันด้วยไฮโดรเจน เปอร์ออกไซด์ ศึกษาผลปกป้องตับในการป้องกันการตายของเซลล์จากภาวะดังกล่าวด้วยวิธี MTT assay และวัด ระดับอนุมูลอิสระที่เกิดขึ้นภายในเซลล์ด้วยวิธี DCHF-DA assay วัดสมรรถนะของเอนไซม์และสารต้านออกซิเดชัน ภายในเซลล์คือ SOD, CAT, GPx, GR, total GSH, GSH และ GSH/GSSG ratio และผลต่อการแสดงออกของ mRNA และโปรตีนที่เกี่ยวข้องกับการขจัดอนุมูลอิสระภายในเซลล์ได้แก่ Nrf2 และ HO-1 ผลการศึกษาพบว่าสาร สกัดพิกัดนวโกฐและเบต้าซิโตสเตียรอลมีผลในการป้องกันการตายของเซลล์เพาะเลี้ยงเฮพจี 2 และป้องกันการเพิ่ม ระดับของอนุมูลอิสระภายในเซลล์ที่เหนี่ยวนำโดยไฮโดรเจนเปอร์ออกไซด์ เพิ่มสมรรถนะเอนไซม์และสารต้าน ออกซิเดชัน (CAT, GPx, GR, total GSH, GSH และ GSH/GSSG) แต่ลดระดับ GSSG สารสกัดดังกล่าวมีผลเพิ่มการ แสดงออกของ Nrf2 และ HO-1 ในขณะที่เบต้าซิโตสเตียรอลมีผลเฉพาะต่อการเพิ่มการแสดงออกของ HO-1 ผล การศึกษานี้แสดงให้เห็นว่าสารสกัดพิกัดนวโกฐมีฤทธิ์ในการปกป้องตับและฤทธิ์ดังกล่าวเกี่ยวข้องกับการขจัดอนุมูล อิสระของสารออกฤทธิ์ที่เป็นองค์ประกอบในพิกัดนวโกฐ เช่น เบต้าซิโตสเตียรอล เพื่อศึกษาผลของสารสกัด พิกัดนวโกฐต่อเอนไซม์ไซโตโครมพี 450 ในตับมนุษย์ทำการศึกษาโดยใช้ไมโครโซมที่เตรียมได้จากตับมนุษย์และ สับสเตรทที่จำเพาะในการศึกษาฤทธิ์ยับยั้งเอนไซม์ และใช้เซลล์เพาะเลี้ยงตับมนุษย์ในการศึกษาฤทธิ์เหนี่ยวนำ เอนไซม์ ผลการศึกษาพบว่าสารสกัดพิกัดนวโกฐมีผลในการยับยั้งสมรรถนะของเอนไซม์ CYP1A2, CYP2C9, CYP2D6 และ CYP3A4 โดยความเข้มข้นของสารสกัดพิกัดนวโกฐที่ยับยั้งสมรรถนะของเอนไซม์ 50% คือ 13, 62, 67 และ 88 มคก./มล.ตามลำดับ การยับยั้งเอนไซม์ CYP ของสารสกัดพิกัดนวโกฐเป็นแบบไม่ขึ้นกับระยะเวลาและ สามารถผันกลับได้ โดยมีค่าคงที่ของการยับยั้งของ CYP1A2, CYP2C9, CYP2D6 และ CYP3A4 คือ 34, 80, 12 และ 150 มคก./มล. ตามลำดับ สารสกัดพิกัดนวโกฐไม่มีผลต่อการยับยั้งสมรรถนะของเอนไซม์ CYP2C19 และ CYP2E1 รวมถึงไม่มีผลต่อการเหนี่ยวนำสมรรถนะของเอนไซม์ CYPs ดังนั้นการใช้ตำรับยาดังกล่าวในการ บำบัดรักษาอาการต้องมีข้อควรระวังในการใช้ร่วมกับยาที่เป็นสับสเตรทของเอนไซม์ CYPs ดังกล่าว

ภาควิชา	เภสัชวิทยาและสรีรวิทยา	ลายมือชื่อนิสิต ....
สาขาวิชา	เภสัชวิทยาและพิษวิทยา	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม ....

ABHIRUJ CHIANGSOM: *IN VITRO* STUDY OF THE HEPATOPROTECTIVE EFFECT AND HERB-DRUG INTERACTION OF PHIKUD NAVAKOT EXTRACT. ADVISOR: ASSOC. PROF. POL. LT. COL. SOMSONG LAWANPRASERT, Ph.D., CO-ADVISOR: RAWIWAN MANIRATANACHOTE, Ph.D., 153 pp.

Phikud Navakot (PN), a Thai traditional remedy recommended for treatment of cardiovascular symtomps, comprises an equal weight proportion of nine herb species. This study aimed to investigate the effect of hydroethanolic extract of PN, and one of its constituents, β-sitosterol against H_2O_2-induced oxidative stress in HepG2 cells to assess it hepatoprotective effect. Effect of this extract on human cytochrome P450 (CYP) were also investigated in vitro for assessing its herb-drug interaction potential. For the first aimed, cells were treated with different concentrations of PN (0-1 mg/mL) or β-sitosterol (0-20 µM) prior to incubation with H_2O_2. Cell viability and ROS generation were assessed by MTT and DCFH-DA assays, respectively. Oxidative defense mechanisms were determined by measuring glutathione levels and the activities of antioxidant enzymes. Expression levles of Nrf2 and HO-1 mRNA and proteins were investigated by qRT-PCR and western blot analyses, respectively. The results demonstrated that PN extract (0.001-0.1 mg/mL) and β-sitosterol (1-20 µM) significantly improved cell viability and prevented ROS generation induced by H_2O_2 in HepG2 cells. PN and β-sitosterol also increased the activities of antioxidant enzymes (CAT, GPx, GR and SOD), total GSH, GSH and GSH/GSSG ratio, but decreased GSSG. In addition, pretreatment of PN reversed Nrf2 and HO-1 protein while β-sitosterol affected only HO-1 protein. It was concluded that PN extract possessed hepatoprotective action associated with the antioxidant effects which may be attributed from it constituents such as β-sitosterol. Regarding herb-drug interaction study, selective substrates of CYPs were used to investigate the inhibitory effects of PN using human liver microsomes. Primary human hepatocytes were used to assess the inductive effect of PN using P450 Glo^{TM} CYP assay and western blot analysis. The results showed that, PN inhibited the activities of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 with the maximal inhibitory concentration (IC_{50}) values of 13, 62, 67 and 88 µg/mL, respectively. Inhibition of PN on these CYPs was not a time dependent type but a reversible inhibition with the Ki of 34, 80, 12 and 150 µg/mL for CYP1A2, CYP2C9, CYP2D6 and CYP3A4, respectively. Meanwhile, it had no effect on the activities of CYP2C19 and CYP2E1 (IC_{50} > 1 mg/mL). PN did not have an induction effect on CYP1A2, CYP2C9, CYP2C19 and CYP3A4 in primary human hepatocytes. Thus, PN may cause herb-drug interactions via inhibition of CYP1A2, CYP2C9, CYP2D6 and CYP3A4, and precautions should be taken when PN is coadministered with drugs that are metabolized by these CYP enzymes.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AhR	= Aryl hydrocarbon receptor
ALT	= Alanine transaminase
ARE	= Antioxidant response element
AST	= Aspartate aminotransferase
AUC	= Area under the curve
CAR	= Constitutive androstane-receptor
CAT	= Catalase
CCl_4	= Carbon tetrachloride
C_{max}	= Maximum serum concentration
CO	= Carbon monoxide
CYP	= Cytochrome P450
DCF	= Dichlorodihydrofluorescein
DCFH-DA	= Dichloro-dihydro-fluorescein diacetate
DDIs	= Drug-drug interactions
DMEM	= Dulbecco's modified Eagle's medium
DMSO	= Dimethylsulfoxide
DNA	= Deoxyribonucleic acid
DPPH	= 2,2-diphenyl-1-picrylhydrazyl
ECV304	= Human umbilical vein endothelial cell
EDTA	= Ethylene diamine tetra-acetic acid
EGTA	= Ethylene glycol tetra-acetic acid
et al.	= And others
etc.	= And other things
FA	= Ferulic acid
FBS	= Fetal bovine serum

Fig.	= Figure
GA	= Gallic acid
GLC	= Glutamate cysteine ligase
GPx	= Glutathione peroxidase
GR	= Glutathione reductase
GSH	= Glutathione
GSSG	= Oxidized glutathione
GST	= Glutathione S-transferase
H_2O_2	= Hydrogen peroxide
HCl	= Hydrochloric acid
HLMs	= Human liver microsomes
HO-1	= Heme oxygenase-1
HPLC	= High liquid performance chromatography
i.e.	= id est
i.p.	= Intra-peritoneal
Kg	= Kilogram
Ki	= Dissociation constant for inhibitor
Km	= Michaelis constant
MDA	= Malondialdehyde
mg	= Milligram
mL	= Millilitre
mM	= Millimolar
MTT	= 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
Na_3VO_4	= Sodium orthovanadate

NAC	= *N*-acetylcysteine
NaCl	= Sodium chloride
NADPH	= Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NaF	= Sodium fluoride
NAPQI	= *N*-acetyl-p-benzoquinone imine
NF-κB	= Nuclear factor kappa-light-chain-enhancer of activated B cells
nm.	= Nanometre
NO$^{\cdot}$	= Nitric oxide
NOAEL	= No observed adverse effect level
Nrf2	= Nuclear factor-erythroid 2- related-factor 2
O$_2{}^{\cdot-}$	= Superoxide
OD	= Optical density
PAGE	= Polyacrylamide gel electrophoresis
PN	= Phikud Navakot
PXR	= Pregnane X receptor
RLMs	= Rat liver microsomes
ROS	= Reactive oxygen species
RXR	= Retinoid X receptor
SDS	= Sodium dodecyl sulphate
SOD	= Superoxide dismutase
TNF-α	= Tumor necrosis factor-α
TST	= Testosterone
UV	= Ultraviolet

Vmax	= Maximal velocity
XRE	= Xenobiotic-responsive element
˙HO	= Hydroxyl radical
μL	= Microliter
μM	= Micromolar

CHAPTER I
INTRODUCTION

Traditional medicine is increasingly important in healthcare worldwide since it is easily accessible to local communities. Therefore, development of herbal products requires more formal scientific investigations of quality, efficacy and safety. Phikud Navakot (PN) is a Thai traditional remedy that has been used for hundreds of years. It was approved for clinical use by the Thai Ministry of Public Health in 2006 [1]. PN comprises an equal weight proportion of ingredients from various parts (i.e. root, rhizome, aerial part, and gall) of nine herb species (Table 1). The main active constituents of PN assessed by HPLC showed many common antioxidant compounds which found in herbal ingredients of PN such as gallic acid, vanillic acid, rutin and ferulic acid. Many other compounds these herbs are also found in PN such as imperatorin, β-sitosterol, artemisinin, etc. (Table 2). PN is generally used in powder form and recommended for treatment of cardiovascular symptoms and circulatory disorders such as dizziness and syncope [1]. One of the underlying mechanisms is the action of PN on vasorelaxation, as found in isolated rat aorta [2]. The extract also possesses antioxidant effects through scavenging of reactive oxygen species such as superoxide, hydroxyl and nitric oxide radicals, as well as hydrogen peroxide in human endothelial ECV304 cells [3]. These findings support the use of PN for the treatment of circulatory disorders such as dizziness and fainting. For the point of safety concern, the extract is relatively nontoxic for acute and sub-chronic (90 days) administration in Sprague-Dawley rats. In acute toxicity test, limited dose of 2,000 mg/kg did not cause any mortality of all rats. Treatment-related toxicity was not observed in sub-chronic test. However, increased plasma uric acid was detected in rats following treatment with high dose PN (1,000 mg/kg/day) for 90 days [4]. A chronic toxicity study in male and female Sprague-Dawley rats for 12 months revealed that high dose of PN (1,000

mg/kg/day) was associated with mesengiolysis, which involved the damage of endothelial cells within glomeruli. The NOAEL was shown to be 100 mg/kg/day [5]. These findings indicate the caution of using PN at high dose for a long period of time, especially in patients with gout or kidney diseases.

Despite the limited data available regarding pharmacological effects of PN, many studies demonstrated antioxidant and cytoprotective effects of the extracts of herbs which are constituents in PN. The extracts from *A. dahurica* [6], *P. kurrooa* [7], *S. lappa* [8], *A. lancea* [9] and *A. annua* [10] exhibited hepatoprotective effects associated with the antioxidant properties. Antioxidant effects of *T. chebula* on rat heart tissues [11], *L. chuanxiong* on endothelial cells [12], and *N. Jatamansi* on C6 glioma cells [13] were also demonstrated while *A. sinensis* possessed antioxidant properties *in vivo* in human [14]. These findings indicate the high possibility that PN will possess hepatoprotective effect, providing additional beneficial effect as it is traditionally used for circulatory symptoms in elderly who usually receive multiple medications. Most drugs which undergo phase I metabolism via cytochrome P450 enzyme system, always cause detrimental effect to liver cells due to reactive oxygen species (ROS) occurring during the catalytic reactions [15]. Thus, this study aimed to investigate the hepatoprotective effects of PN extract against H_2O_2-induced oxidative stress in hepatocellular carcinoma (HepG2) cells and assess the involved mechanism such as regulation of antioxidant enzymes and cellular defense proteins (Nrf2 and HO-1). These data will provide additional useful information of PN extract against oxidative stress in the liver, an organ that has a major role in xenobiotic metabolism.

PN is widely used in the elderly, who are more likely to take multiple medications for treatment of various complications. Co-administration of herbal components with other medicines may cause herb-drug interactions and drug toxicity, which are the point of safety concerns. In addition, herb-drug interactions involving

PN are unknown. Therefore, the aim of this study was to examine the effects of PN on cytochrome P450 (CYP) enzymes *in vitro*, in accordance the U.S. FDA Guidance for Industry (2012) [16], as a basis for understanding potential pharmacokinetic drug interactions of PN. Inhibition of six major CYP isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) by PN was examined in human liver microsomes, and induction of CYP1A2, CYP2C9, CYP2C19 and CYP3A4 by PN was investigated in primary human hepatocytes. These data will provide important information for safe co-administration of PN with other medicines and essential labeling recommendations for clinical use.

Table 1. Herbs contained in Phikud Navakot

Plant name	Part	Scientific name	Family
Kot Soa	Root	*Angelica dahurica* Benth & Hook f.	Umbelliferae
Kot Khamao	Rhizome	*Atractylodes lancea* (Thunb.) DC.	Compositae
Kot Chula-lumpa	Aerial part	*Artemisia pallens* L.	Compositae
Kot Chiang or Dang Gui	Root	*Angelica sinensis* (Oliv.) Diels.	Umbelliferae
Kot Hua Bua	Rhizome	*Ligusticum chuanxiong* Hort.	Umbelliferae
Kot Jatamansi	Root, Rhizome	*Nardostachys jatamansi* (D. Don) DC.	Valerianaceae
Kot Kan Prao	Rhizome	*Picrorhiza kurrooa Royle ex Benth.*	Scrophulariaceae
Kot Kradook	Rhizome	*Saussurea costus (Falc.) Lipsch.*	Compositae
Kot Phung Pla	Gall	*Terminalia chebula* Retz.	Combretaceae

Table 2. Compounds found in each plant

Plant	Ferulic acid[3,17]	Vanillic acid[3,18]	Gallic acid[3]	Rutin[3,19]	Imperatorin[20]	Ligustilide[21]	β-Eudesmol[22]	β-Sitosterol[23]	Isosafrole[23]	Artemisinin[23]	β-Maaliene[24]	Picroside II[25]
Kot Soa	√				√			√				
Kot Khamao						√						
Kot Chula-lumpa			√	√						√		
Kot Chiang or Dang Gui	√	√				√		√	√			
Kot Hua Bua	√	√				√						
Kot Jatamansi								√			√	
Kot Kan Prao	√	√						√				√
Kot Kradook				√								
Kot Phung Pla			√									

Objectives of the study

1. To investigate the protective effect of PN extract against H_2O_2-induced oxidative stress in HepG2 cells and elucidate the involved mechanisms.

2. To investigate the effect of PN extract on CYPs both the inhibition and induction aspects using *in vitro* studies and elucidate the mechanism as well as the potent of modulation.

Hypothesis to be tested

PN extract possesses protective effect against H_2O_2-induced oxidative stress in HepG2 cells. In addition, PN may cause herb-drug interactions via inhibition or induction of CYPs.

Research Design

Experimental research

Scope of the study

This study was performed *in vitro* focusing on the protective effect of PN extract against oxidative stress in HepG2 cells. Firstly, cytotoxicity of PN extract on HepG2 cells was investigated to find out the non-toxic concentrations of the extract. Then, oxidative stress was induced by H_2O_2 pretreatment of PN was performed to assess the protective effect of the extract against H_2O_2-induced cell viability as well as against H_2O_2-induced intracellular ROS in HepG2 cells. To evaluate the protective mechanism of PN extract, activities of various antioxidant enzymes namely catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and glutathione (GSH) were assessed. The molecular mechanism of antioxidant effect of PN by assessing Nrf2 and HO-1 activation. Effects of PN on both inhibition and induction of CYP enzyme activities *in vitro* were investigated. Inhibition of six major CYP isoforms (CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4) by PN was examined using human liver microsomes, and induction of CYP1A2, CYP2C9, CYP2C19 and CYP3A4 by PN was investigated using primary human hepatocytes. The conceptual framework and experimental design are shown in Figure 1, and Figure 2-3, respectively.

Expected benefits and application

This study will provide the information regarding the hepatoprotective effect of PN extract against oxidative stress and the involved mechanism as well as the safety information regarding drug-herb interaction. Both pharmacological and safety profile of PN *in vitro* gained from this study will provide useful information for the decision of further performing the clinical study in human.

Conceptual framework
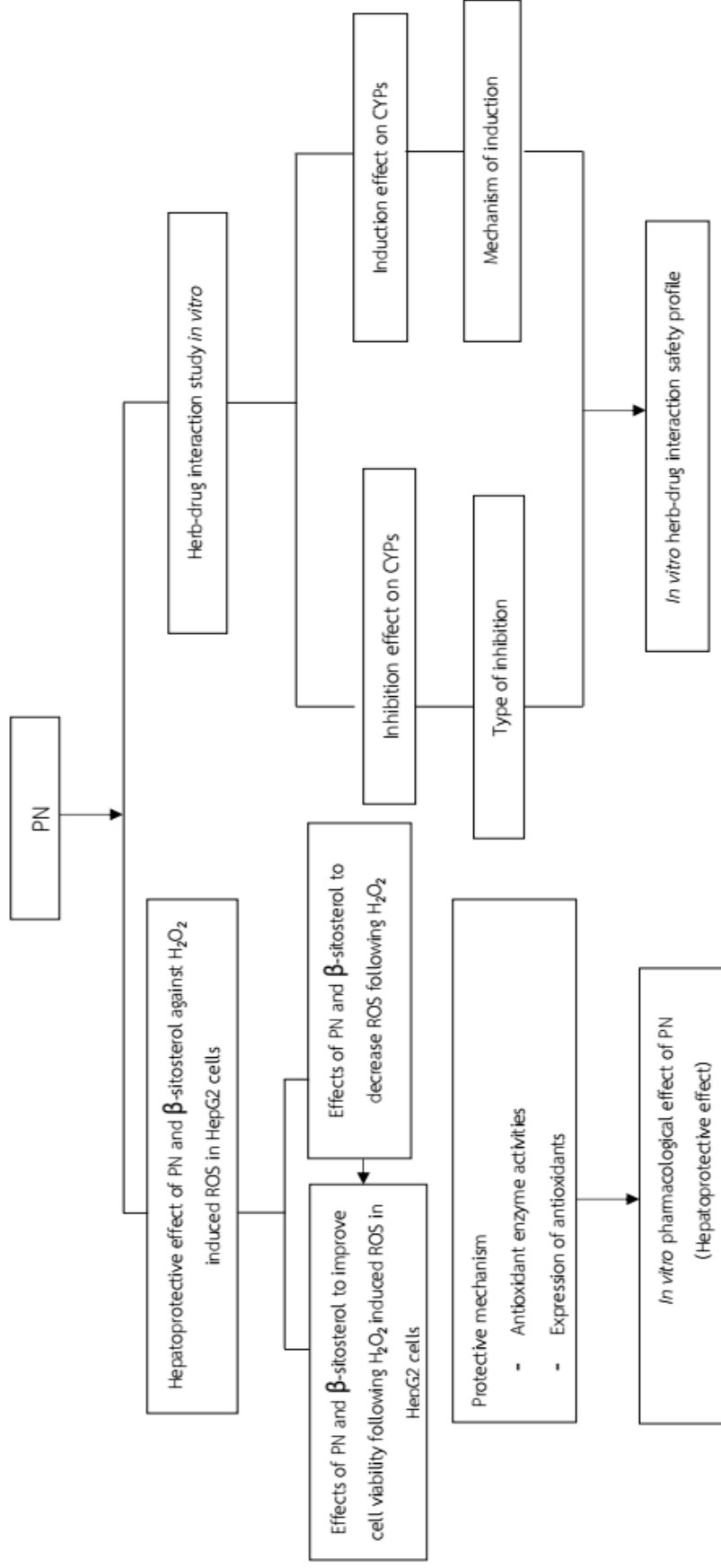
Figure 1. Conceptual framework

PN = Phikud Navakot extract

Experimental design
Part I Hepatoprotective effect of PN extract

HepG2 cells

Various concentrations of H_2O_2 and determine cell viability using MTT

Various concentrations of PN and β-sitosterol and determine cell

HepG2 cells

Concentration of H_2O_2 at IC_{50}

Non-toxic concentrations of PN and some active constituents

Toxic concentrations of PN and β-sitosterol

Effect of PN and β-sitosterol to improve cell viability following treatment with H_2O_2 using MTT assay

Effect of PN and β-sitosterol to decrease intracellular ROS following treatment with H_2O_2 using DCFH-DA assay

Effect of PN on the generation of intracellular ROS in HepG2 cells using DCFH-DA assay

Possible responsive mechanism of PN and β-sitosterol on intracellular ROS

Real time RT-PCR

HO-1 mRNA

Nrf-2 mRNA

Western blot analysis

HO-1 protein

Nrf-2 protein

Effect of PN and β-sitosterol on the intracellular level of antioxidants

GSH

GSSG

GSH/GSSG ratio

Effect of PN and β-sitosterol on the activities of enzymatic antioxidants

SOD

CAT

GPx

GR

Figure 2. Experimental design part I Hepatoprotective effect of PN

Experimental design

Part II Inhibition and induction effect of PN extract on CYPs
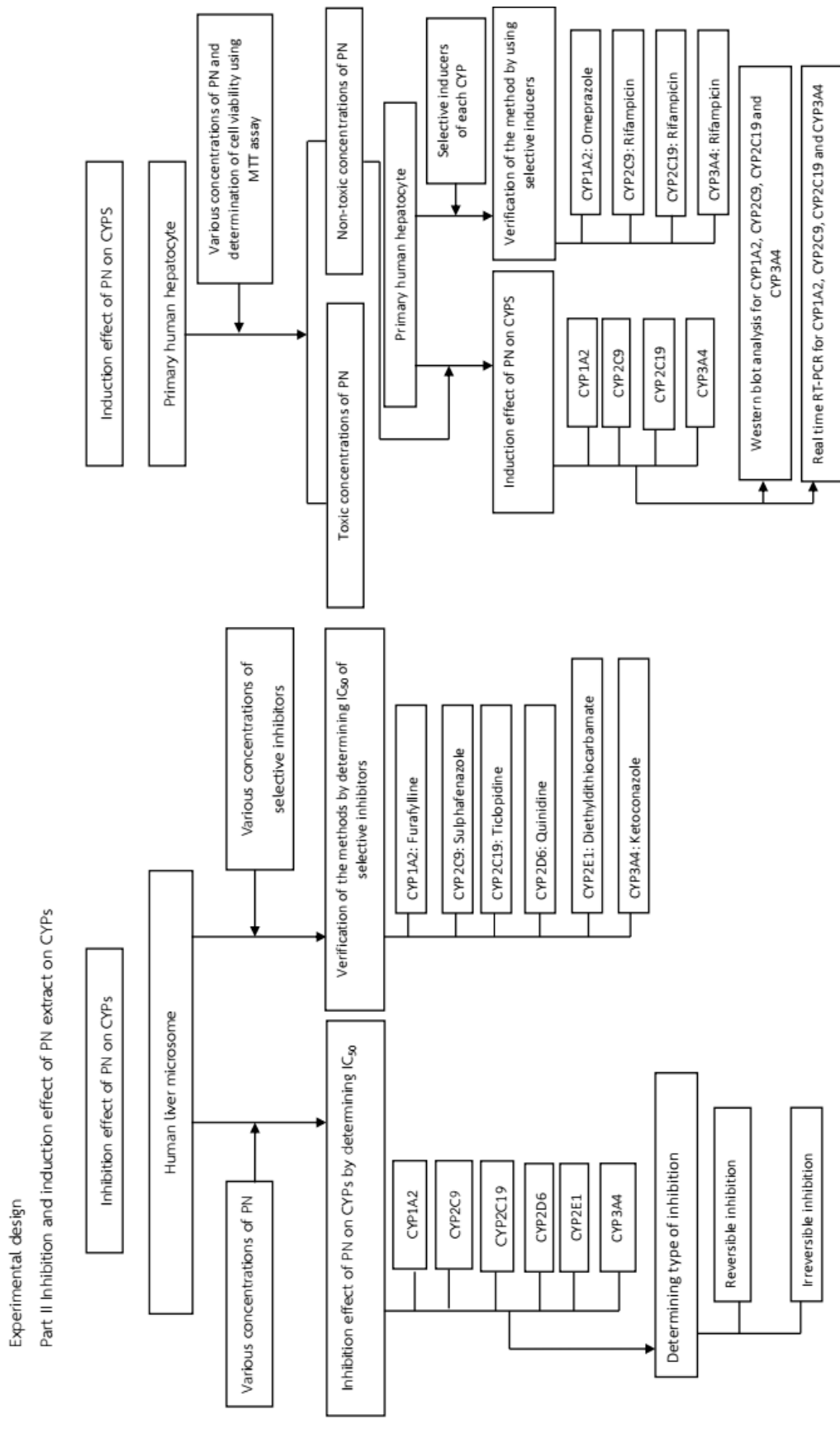
Figure 3. Experimental design part II Inhibition and induction effect of PN extract on CYPs

CHAPTER II

LITERATURE REVIEWS

Cytochrome P450

Cytochrome P450 (CYP) is a family of isozymes responsible for the biotransformation of endogenous and exogenous compounds such as drugs and chemicals. CYPs are embeded on the smooth endoplasmic reticulum with the highest concentrations in the liver cells (hepatocytes) and small intestine. These enzymes are primarily responsible for the oxidative (Phase I) metabolism of a large numbers of compounds, including drugs, xenobiotics and endogenous compounds [26]. CYP catalyzed oxidative reaction requires substrate (R), CYP enzyme, molecular oxygen, NADPH, and NADPH-P450 reductase which can be shown as follows:

$$RH + O_2 + NADPH + H^+ \longrightarrow ROH + NADP^+ + H_2O$$

The catalytic cycle of CYP involves electron transferring steps, where electrons are supplied from NADPH via NADPH-P450 reductase. The overall effect of the reaction is the addition of one atom of oxygen to the substrate (drug) to form a hydroxylated metabolite while the other atom of oxygen is converted to water [27]. The major human CYP isoforms involved in xenobiotic metabolism included CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. Among these isoforms, CYP3A are the most important one in xenobiotic metabolism. CYP3A4 and CYP3A5 collectively represent the most abundant approximately 30% of hepatic CYP isoforms, followed by approximately 20% of CYP2C (Figure 4). In terms of the isoforms involved in xenobiotic metabolism, CYP3A are known to be associated in the metabolism of the most number of xenobiotic are use clinically approximately

50%, followed by 25% of CYP2C [28]. Metabolism of drugs via the CYP system has appeared as an important determinant in the occurrence of various drug interactions that can result in drug toxicities, reduced pharmacological effect, and adverse drug reactions. Investigation whether the drugs or herbs act as enzyme substrates, inducers, or inhibitors of this enzyme can prevent clinically significant interactions. Several widely used drugs can inhibit or induce CYP to cause clinically significant changes in the concentrations of other drugs. The substrates, inhibitors and inducers of CYP which are common are shown in Table 3. Avoiding co-administration and adjusting a patient's drug regimen during in the course of therapy can provide optimal response with minimal adverse effects. Some drugs undergo biotransformation to pharmacologic active agents while in some cases the metabolites can be toxic, carcinogenic or teratogenic. Most drugs can exhibit decreased efficacy due to rapid metabolism, while drugs with active metabolites can exhibit increased drug effect and/or toxicity due to enzyme induction.
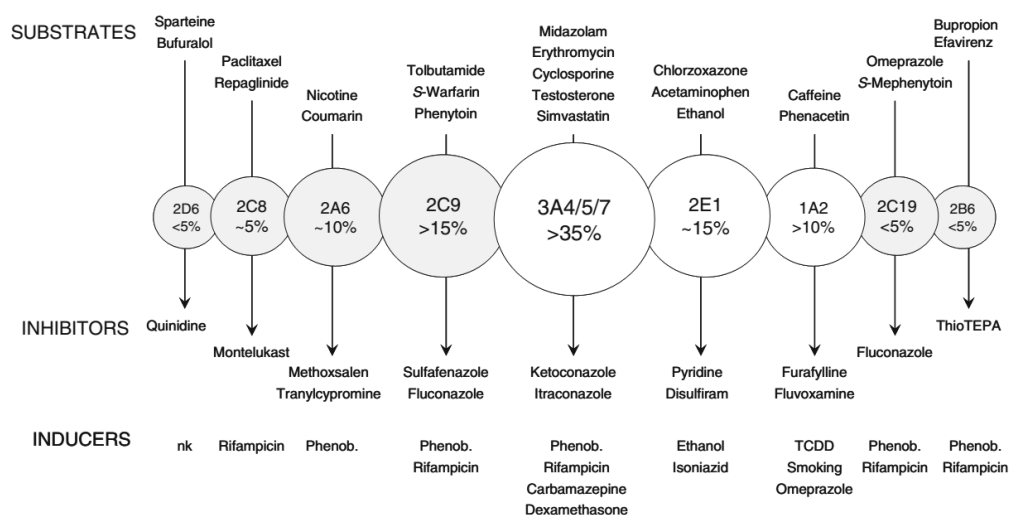
Figure 4. Relative abundance of individual CYP forms in the liver and some examples of substrates, inhibitors and inducers [29].

Induction and inhibition

Induction of CYP

Induction can cause marked increases in CYP quantity and chemical detoxification or bioactivation. As a result, induction can increase tolerance to some toxicants while enhancing the toxicity of others. In addition, induction can decrease the therapeutic effect of drugs by increasing the rate of metabolism. Xenobiotics can induce enzymes that play a major or no role in their biotransformation. The induction of CYP can be classified by at least 5 different mechanisms. Induction of CYP enzyme is mostly mediated by intracellular receptors such as aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR) [30].

Induction of CYPs 1A1/1A2 and 1B1

Polycyclic aromatic hydrocarbons (such as tobacco smoke, charcoal grilled meat or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) bind to the AhR which then migrate to the nucleus. After AhR-ligand complex enters the nucleus, it heterodimerizes with the nuclear protein aryl hydrocarbon nuclear receptor translocator (ARNT) and the complex binds to specific DNA sequences (xenobiotic-responsive elements, XRE) upstream of the CYP1A1/1A2 and 1B1 genes [31].

Induction of CYP2B6 2C8/2C9/2C19 and 3A4

The mechanism of induction of these CYP isoforms are controlled by transcription factors which are usually found in the nucleus, thus they are termed as nuclear receptors. They are classified as following

1. Constitutive androstane receptor (CAR): This receptor mediated control of CYP2 series and CYP3A4. Normally, CAR is conjugated with co-repressor protein (SCR-1). This complex bind the inducer ligand inside the nucleus, and the inducer-receptor complex forms a heterodimer with the retinoid X receptor (RXR). This heterodimer binds to a DNA response element, enhances DNA transcription and finally protein synthesis [30]. Phenobarbital a known CYP2C inducer, binds to CAR and increases the expression of CYP2B6, CYP2C8, CYP2C9 as well as CYP3A4.

2. Pregnane X receptor (PXR): A large number of endogenous and exogenous chemicals, such as rifampicin, phenobarbital, dexamethasone, St. John's wort and steroids, are known to induce CYP3A4. Upon ligand binding, it forms a heterodimer with the RXR and binds to hormone response elements on DNA which elicits expression of gene products. One of the primary targets of PXR activation is the induction of CYP3A4, an important phase I oxidative enzyme that is responsible for the metabolism of overall clinically drugs [30].

It is apparent that the main inducible CYPs such as, CYP1A, CYP2C and CYP3A, employ similar systems to regulate their ability to respond to increases in drug concentration. The exception to this rule seems to be CYP2E1, which appears to have a unique system of induction. Ethanol selectively induces CYP2E1 primarily by stabilizing the enzyme protein [32].

Inhibition of CYP

Enzyme inhibition occurs when two compounds undergoes metabolism via the same CYP by competition for the same enzyme binding site. The more potent inhibitor will exceed, resulting in decreased metabolism of the contesting drug. For most drugs, this can lead to increased serum levels of the non-metabolized drug, leading to a greater potential for toxicity. For drugs whose pharmacological activity

requires biotransformation from a prodrug, inhibition can lead to decreased efficacy [33]. Therefore, drug interaction study is required during the drug developmental process so as to evaluate the potential drug interactions. The recent FDA drug interaction guidance recommends the study using *in vitro* and *in vivo* systems [16].

The mechanism of CYP inhibition can be classified into 3 categories: reversible inhibition, quasi-irreversible inhibition and irreversible inhibition. Reversible inhibition is possibly the more common mechanism associated to drug interaction.

Reversible inhibition can occur through four main processes: competitive, non-competitive, uncompetitive and mixed type inhibition.

1. Competitive inhibition: This is the simplest form of inhibition, where the substrate and the inhibitor are very similar in structure and have similar affinities for the same active site. Thus, inhibitor and substrate compete for the same active site. Mathematically, if a Lineweaver-Burk double reciprocal plot is made of this type of inhibition, the Michaelis constant (*Km*) changes, but the maximal velocity (*Vmax*) does not; in other words, the enzyme will still run at a maximum rate if enough substrate is used, but affinity decrease [34].

2. Non-competitive inhibition: There is called the allosteric site, which is distant from the active site. Upon a ligand binding, conformation of the active site is automatically changed and it becomes less likely to bind the substrate thus decrease rate of product formation. Because the enzyme cannot run at maximal rate, the Lineweaver-Burk plot will show a decrease in *Vmax* but *Km* does not change, that is, the affinity of the substrate in the active site is unchanged [34].

3. Uncompetitive inhibition: this is an uncommon form of inhibition. The inhibitor binds only to the enzyme-substrate complex, resulting in encouraging enzyme-substrate complex formation so increasing affinity lead to decrease the *Km*

meanwhile the enzyme-substrate-inhibitor complex is non-functional, thus the *Vmax* also decrease [34].

4. Mixed type inhibition: This similar to non-competitive inhibition except that the inhibitor may bind to the enzyme whether or not the enzyme has already bound the substrate but has a greater affinity for one state or the other. The change in binding affinity is included in the chemical equation by the term of the equilibrium dissociation constant for the inhibitor (*Ki*). For this reason, the apparent *Vmax* changes, because the inhibitor is capable of preventing catalysis regardless of whether the substrate is bound to the enzyme. With mixed inhibitors, the change in apparent *Km* varies, depending on the relative values of *Kia* (the *Ki* for binding to the free enzyme) and *Kib* (the *Ki* for binding to the ES complex). Thus, depending on the relative values of the *Ki*, the apparent *Km* may decrease [34].

Quasi-irreversible inhibition

This type of inhibition usually involves the same initial steps as a competitive inhibitor, but then the CYP catalytic cycle proceeds, in that the drug substrate is metabolically activated to a reactive or metabolic intermediate (MI), which then occupies the P450 active site and inactivates the CYP by binding to the heme, apoprotein, or both. Compounds that bind to the heme do so after oxidative metabolism into reactive intermediates that form a complex with the CYP heme, a stable MI-heme complex is formed leading to the enzyme catalytically nonfunctional. However, MI-heme complex can be reversed and the catalytic function of ferric CYP can be restored by *in vitro* incubation with highly lipophilic compounds that displace the metabolic intermediate from the active site [33].

Irreversible inhibition

The inhibitors of this type of inhibition are called mechanism based inhibitors or suicide substrate. The drug substrate is metabolically activated to a reactive intermediate which then covalent binding or quasi-irreversible noncovalent tight binding of reactive intermediate metabolite to the CYP that catalyzes its formation as a result in the loss of CYP activity [34].

Pharmacokinetic drug-drug interactions involving CYP enzymes

Drug-drug interactions (DDIs) are one of the most important concerns in drugs used clinically. Several studies demonstrated that 2.4-6.5% of hospital admissions involve drug adverse effects [35], and about 7% of hospitalized patients experience serious adverse drug effects [36]. Most of the adverse drug events found in hospitalized patients are dose-dependent. Pharmacokinetic interactions leading to increased drug exposure might have serious events. As CYP associated metabolism represents a major route of xenobiotic elimination and as many drugs used in clinic are metabolized by the same CYP isoforms, they have an important role in pharmacokinetic DDIs. The result of CYP inhibition is an increase in the plasma concentration of parent drug and a reduction in its metabolite. When a drug is metabolized by a particular CYP enzyme, inhibition causes to prolonged pharmacological effect. If the drug has the narrow therapeutic window, there is an increased likelihood of adverse drug effects. If a drug has many metabolic pathways, the inhibition of CYP can many times be compensated by unaffected pathways, thus, the increase in the plasma concentration of the parent drug remains small. In the past years, inhibition of CYP mediated metabolism of a drug has led to the removal of several drugs from the market. In contrast, CYP induction may attenuate the pharmacological effect of a drug due to the sub-therapeutic plasma concentrations

of the drug. In the case of prodrug, which requires CYP to catalyse it to become an active metabolite, the CYP inhibition might cause a decreased of the active metabolite while an induction causes an increase of drug effect. Enzyme inhibition *in vivo* can be predicted using *in vitro* data. A simple and well accepted rule is the [I]/*Ki* ratio, where [I] is the inhibitor concentration at the CYP enzyme and *Ki* is the inhibition constant that can be experimentally determined *in vitro*. A ratio of [I]/*Ki* > 1 suggests that an interaction is highly likely, whereas ratios of 0.1< [I]/*Ki* < 1 or [I]/*Ki* < 0.1 indicates that an interaction is likely or not likely, respectively. Meanwhile, enzyme induction generally demonstrates reduced AUC, C_{max}, through concentrations and half-life ($t_{1/2}$) of the victims as a reflection of increased clearance [34].

Table 3. Examples of substrates, inhibitors, and inducers of CYP enzymes [37].

Substrates					
CYP1A2	CYP2C*	CYP2C19	CYP2D6	CYP2E1	CYP3A
Amitriptyline	Celecoxib	Amitriptyline	Amitriptyline	Acetaminophen	Amiodarone
Clomipramine	Diazepam	Citalopram	Carvedilol	Aniline	Amlodipine
Clozapine	Diclofenac	Clopidogrel	Chlorphenamine	Chlorzoxazone	Aripiprazole
Imipramine	Fluoxetine	Diazepam	Chlorpromazine		Atorvastatin
Theophylline	Fluvastatin	Lansoprazole	Clomipramine		Buspirone
R-Warfarin	Glibenclamide	Omeprazole	Codeine		Ciclosporin
Caffeine	Glimepiride	Pantoprazole	Dextromethorphan		Clarithromycin
	Glipizide	Proguanil	Donepezil		Dexamethasone
	Ibuprofen	Propanolol	Duloxetine		Diazepam
	Irbesartan	*R*-Warfarin	Fluoxetine		Diltiazem
	Losartan		Haloperidol		Domperidone
	Meloxicam		Imipramine		Erythromycin
	Naproxen		Metoclopramide		Estradiol
	Phenytoin		Metoprolol		Felodipine
	S-Warfarin		Ondansetron		Fentanyl
			Oxycodone		Finasteride
			Paroxetine		Hydrocortisone
			Propranolol		Indinavir
			Tamoxifen		Lercanidipine
			Timolol		Methadone
			Tramadol		Nelfinavir
			Venlafaxine		Nifedipine
					Progesterone
					Ritonavir
					Saquinavir
					Sildenafil
					Simvastatin
					Tacrolimus
					Testosterone
					Verapamil
					R-Warfarin
Inhibitors					
Amiodarone	Amiodarone	Chloramphenicol	Amiodarone	Clomethiazole	Cimetidine

Cimetidine Ciprofloxacin Fluvoxamine	Fluconazole Isoniazid	Cimetidine Ketoconazole Lansoprazole Omeprazole Oxcarbazepine Pantoprazole	Bupropion Celecoxib Cimetidine Citalopram Clomipramine Duloxetine Escitalopram Fluoxetine Levomepromazine Paroxetine Quinidine Sertraline	Diallyldisulfide Diethyldithiocarbamate	Clarithromycin Diltiazem Erythromycin Fluconazole Indinavir Itraconazole Ketoconazole Nelfinavir Ritonavir Saquinavir Verapamil Grapefruit Juice
Inducers					
Broccoli Brussels sprouts Cigarette smoking Omeprazole	Rifampicin Secobarbital	Carbamazepine Rifampicin	Rifampicin	Ethanol	Carbamazepine Efavirenz Nevirapine Oxcarbazepine Phenobarbital Phenytoin Pioglitazone Rifampicin St John's Wort

Reactive oxygen species

Reactive oxygen species (ROS) are molecules or ions formed by the incomplete one-electron reduction of oxygen. Oxygen molecule has two unpaired electrons in separate orbits in its outer electron shell. This electron structure makes oxygen vulnerable to radical establishing. The sequential abatement of oxygen through enhances of the electrons leads to the formation of a number of ROS including superoxide ($O_2^\bullet$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^\bullet HO$), and nitric oxide ($NO^\bullet$) etc. ROS are formed through the following mechanism: the hemolysis of covalent bond (A : B $\longrightarrow$ A$^\bullet$ + B$^\bullet$); one electron adding to the electrically neutral

atom ($A + e^- \longrightarrow A^{-\bullet}$); and loss of one electron form electrically neutral atom $A \longrightarrow$ $A^{+\bullet} + e^-$

The production of reactive radicals is poisonous to all aerobic species. These molecules, generated as outgrowths via the process of mitochondrial electron transport of respiration or by oxidoreductase enzymes, the metabolism of drug and substances via the CYP enzyme system as well as metal catalyzed oxidation. It was initially thought that only phagocytic cells were liable for ROS production as their part in host cell defense mechanisms. Regularly, cell defense themselves against oxidative stress damage by several antioxidant and detoxification enzymes namely superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione. In addition, low molecular weight antioxidants such as ascorbic acid (vitamin C), tocopherol (E) also play an important role for cellular antioxidant. However, throughout the environmental stress such as cell injuries, infection from viral or bacterial, UV, chemical and heat exposure etc., ROS levels can accumulate apparently. Accumulation of ROS is known as oxidative stress. This affects significant damage too many components of the cell for example protein, nucleic acid and DNA, leading to pathological changes including cardiovascular disease, neurological degenerations (e.g., Alzheimer's disease, ischemic stroke), cancer, as well as the normal ageing processes. Oxidative damage to cellular DNA causes mutation and may play an important role in the initiation and progression of several steps of carcinogenesis [38].

ROS associated liver pathology

Liver play an important role in the metabolism of xenobiotics, nutrients, drugs, hormones, and body metabolic waste products, thereby maintaining body homeostasis. Cytochrome P450 system are the major enzyme system involved in drug metabolism. Metabolism of a drug and several substances may bring about a

reactive intermediate that can deduct molecular oxygen straight to generate ROS. Acetaminophen, which is well known for analgesic and antipyretic, is metabolized by CYP to N-acetyl-p-benzoquinone imine (NAPQI) which reacts with GSH leading to the reduction of hepatic GSH till as much as 90%, NAPQI reacts to intracellular proteins with covalent bond as an acetaminophen-cysteine adducts. Accumulation of NAPQI leads to the depletion of GSH and the formation of covalent adduct. In addition, liver cells contain a large number of mitochondria. Production of ROS by this organelle is due to the reduction of molecular oxygen by an electron that leaks from the ubiquinone semiquinone anion that is established in redox cycling of ubiquinone presented in mitochondrial complex III. Alike, complex I of the mitochondrial electron transport chain may also perform superoxide anions during the re-oxidation of the flavin mononucleotide of NADH–ubiquinone reductase or reversion of iron–sulfur centers [39]. Since liver contains various transition metal (Fe^+, Zn^+), it is believed that these atoms catalyze the Fenton reaction which is continuously generated ROS. Ischemia-reperfusion injury is a multicomponent procedure which is triggered when the liver or other organs are provisionally handled to reduced blood supply followed by reperfusion. It has been shown that ROS is produced during this procedure and may indicate essential mediators of the reassuring pathological complications. During hepatic ischemia-reperfusion procedure causes activation of monocytes and neutrophils. Adhesion of these cells to hepatocytes results in the production and release of ROS, such as hydrogen peroxide, by activated leukocytes [40]. Furthermore, ischemia reperfusion injury involves directly on the liver cells viability, especially during the surgery of the liver and liver transplantation. Decreases of blood supply activate Kupffer cells which are the main origin of ROS during the reperfusion period. Moreover, xanthine oxidase is an enzyme that catalyzes the oxidation of hypoxanthine to xanthine, afterward producing uric acid. This catalytic process generates ROS. Normally, liver cells contain many of detoxification enzymes

and anti-oxidants which defense themselves against the accumulation of ROS. However, the imbalance of the excess ROS levels results from various factors and depletion of intracellular detoxification enzymes and anti-oxidants, lead to oxidative stress condition which may implicate to several pathological states such as diabetes mellitus, liver cirrhosis, hepatitis and hepatocellular carcinoma [38].

Antioxidants

Antioxidants are substances that involve in the prevention of cellular damage caused by unstable molecules known as free radicals. Antioxidant defenses of the body are composed of non-enzymatic antioxidant molecules and enzymatic antioxidants.

Endogenous antioxidant

Intracellular compounds can be classified as detoxification enzymes and non-enzymatic antioxidants. The main enzymatic antioxidants directly involved in the neutralization of ROS such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR). SOD, the first affection of defense against free radicals, catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. The H_2O_2 is then changed into water and oxygen by CAT and GPx. In addition, H_2O_2 is removed by using GPx to oxidize reduced GSH into oxidized glutathione (GSSG). Glutathione reductase, a flavoprotein enzyme catalyse the reduction of GSSG in the presense of NADPH which is oxdised to $NADP^+$ (Figure 5). GPx also reduces lipid or non-lipid hydroperoxides while oxidizing glutathione [41]. Non-enzymatic antioxidants belong to substances that are produced via metabolism in the body, such as glutathione, L-arginine, coenzyme Q10, melatonin, metal-chelating proteins, transferrin, etc.
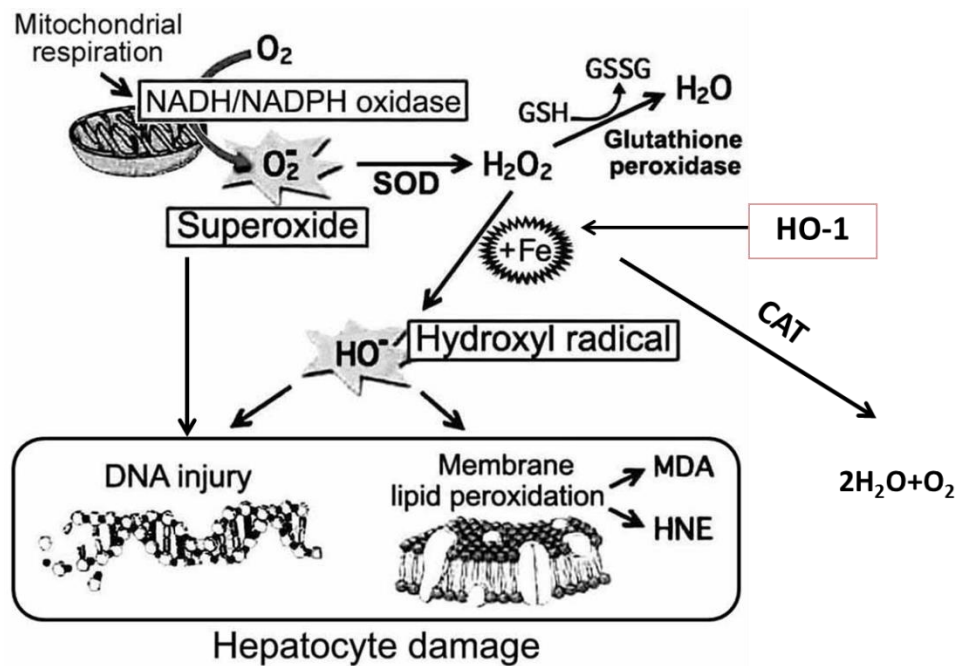
Figure 5. Defense mechanism on oxidative stress induced cell injury [42]

Exogenous antioxidant

Nutrients and phytochemical antioxidants belonging to exogenous antioxidants are compounds which cannot be created in the body and must be received through foods or dietary supplements, such as vitamin E, vitamin C, carotenoids, trace metals (selenium, manganese, and zinc), flavonoids etc. [41].

Nuclear factor (erythroid-derived 2)-like 2) (Nrf2)

Nrf2 is the primary cellular defense against the cytotoxic effects of oxidative stress. Nrf2 acts as a redox sensor and its down regulation may affect the balance between the oxidant and antioxidant system. Generally, Nrf2 is localized in the cytoplasm under unstimulated state bound to its inhibitor protein called Kelch-like ECH-associated protein 1 (Keap1). The complex is disrupted by exposure to several stimuli, and free Nrf2 translocates to the nucleus to form heterodimers with small oncogene family proteins. This leads to the selective recognition of the antioxidant

response element (ARE) on target genes, resulting in the regulation of gene expression of phase II detoxifying enzymes such as glutamate cysteine ligase (GCL), glutathione *S*-transferase (GST), heme oxygenase-1 (HO-1) and NAD(P)H:quinone acceptor oxidoreductase 1(NQO1) [43] (Figure 6).
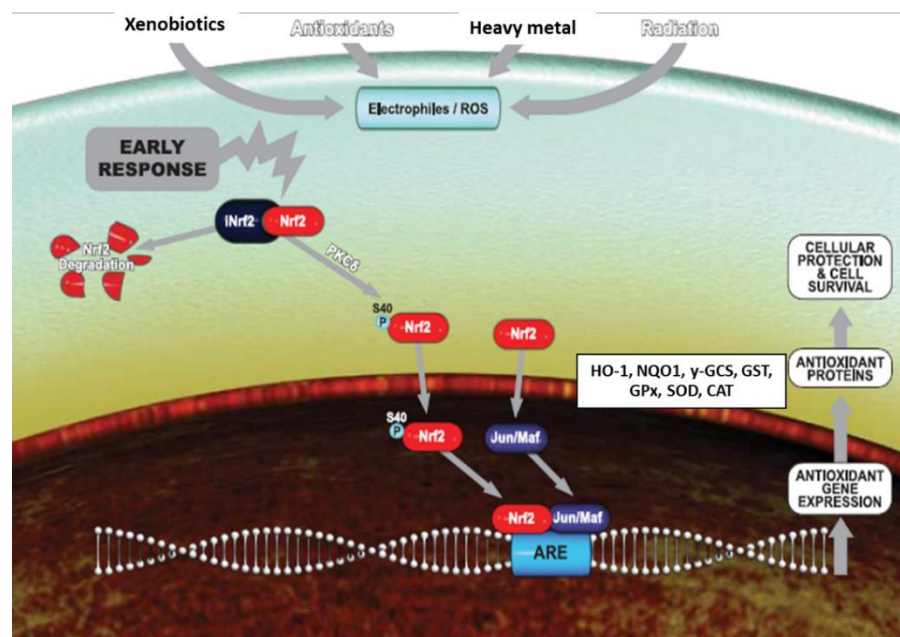
Figure 6. Schematic illustration of regulation of the Nrf2 and antioxidant response pathway [44]

Heme oxygenase-1 (HO-1)

HO-1 is the rate limiting step enzyme that catalyzes the degradation of heme, a process leading to the formation of biliverdin, iron, and carbon monoxide (CO). This enzyme is a stress response protein that is highly inducible under various conditions, including oxidative or heat stress. Increased HO-1 activity attenuate cell apoptosis and decreases superoxide formation. Regulation of HO-1 gene expression occurs on multiple levels and is inducer-specific. At the transcriptional level, HO-1 is mediated by the transcription factor Nrf2. Protective agents such as flavonoid, resveratrol or

sulforaphane have shown beneficial effects against neuronal diseases associated with the activation of Nrf2/HO-1 antioxidant pathway [45]. The protective mechanism of HO-1 is associated to the by-products of HO-1catalyzed heme cleavage, i.e., iron, bilirubin and CO. Biliverdin and bilirubin are potent antioxidants, which scavenge peroxy radicals and inhibit lipid peroxidation [46]. CO shares some properties with NO, such as its effects on intracellular signaling processes, including anti-inflammatory, anti-proliferative, anti-apoptotic, and anti-coagulative effects. Overexpression of HO-1 attenuated the reduction of inner retinal thickness in ischemia-reperfusion injury rats and ameliorated retina apoptosis. The mechanism on the overexpression of HO-1 was associated with inhibition of caspase-3, p53, NF- **K**B, iNOS and with an increase expression of anti-apoptotic protein Bcl-xL. Meanwhile, the anti-inflammatory effect of HO-1 was related to reduction in the recruitment of macrophage infiltration [47] (Figure 7).
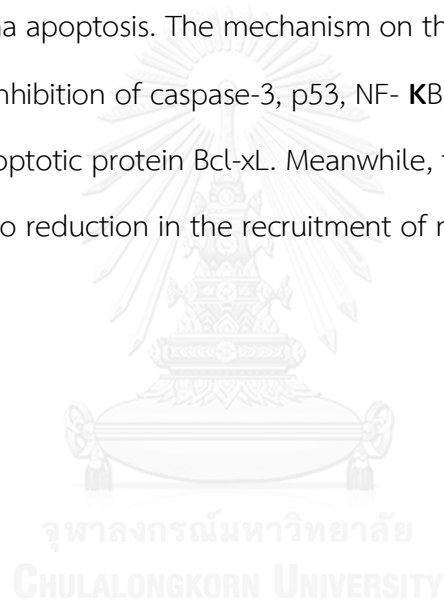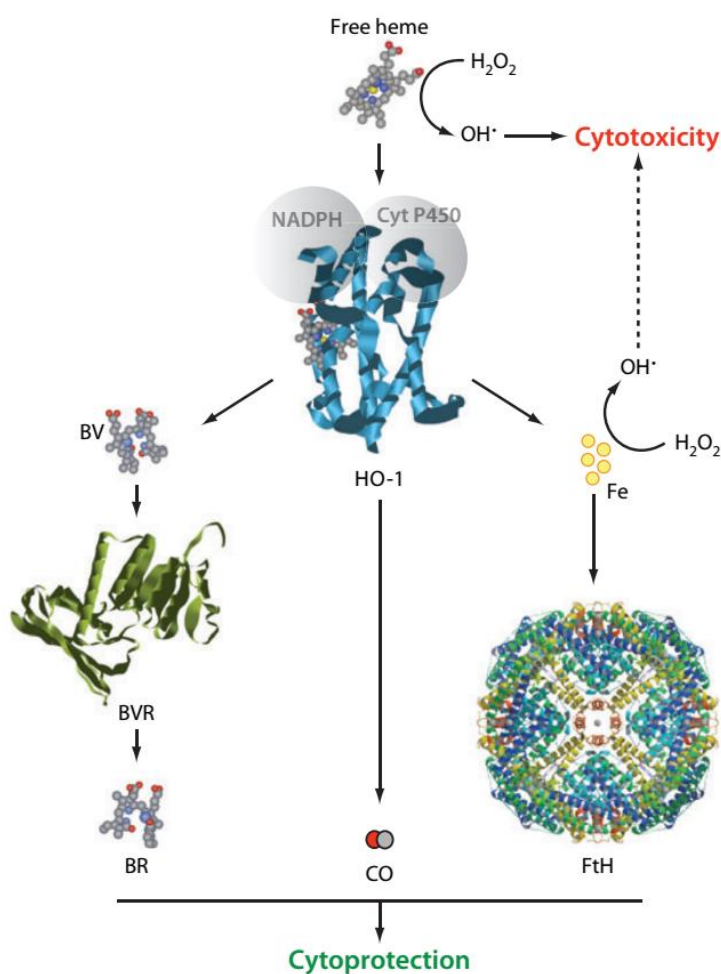
Figure 7. Schematic on OH-1 protective mechanisms pathway [48]

Phikud Navakot (PN)

PN is a major ingredient of "Yahom Navakot" which has been used in Thai traditional medicine in the form of powder. PN is composed of nine herbs in an equal portion by weight. Equal portion by weight of each herb is combined according to the similar effects for treatment of diseases and non-conflict of medicine properties. In the Thai traditional medicine, this preparation is recommended for treating the symptoms of circulatory system; dizziness and syncope which are especially prevalent among Thai elderly. These following are nine Thai herbs that comprise of an equal portion by weight in PN (Figure 8).

Angelica dahurica	*Atractylodes lancea*	*Angelica sinensis*
Artemisia pallens	*Ligusticum sinense*	*Nardostachys jatamansi*
Picrorhiza kurrooa	*Saussurea lappa*	*Terminalia chebula*

Figure 8. Thai herbs comprises in Phikud Navakot (PN)

1. **Kot Soa** (*Angelica dahurica* Benth) Its synonyms are *Callisace dahurica* Franch & Sav., *Angelica macrocarpa* H.Wolff*., Angelica porphyrocaulis* Nakai & Kitag. It is classified in the order of Apiales, and family of Umbelliferae. Its important chemical constituents are coumarins such as byakangelicin, byakangelicol, imperatorin, oxypeucedanin, phellopterin, polyacetylenic, ferulic acid, β-sitosterol etc. Its roots were used in Thai traditional medicine to relieve symptoms of bronchitis, cough, fever and skin lesion. It also used in Chinese traditional medicine for purgation the body from any abnormal body temperature such as heat, clamminess, dryness, and cold on the skin. This herb is also used in female for the treatment of leucorrhea [49].

2. **Kot Khamao** (*Atractylodes lancea* Thunb. DC.) Its synonyms are *Atractylis lancea* DC., *Atractylis ovate* Thunberg, *Atractylis chinensis* Bunge DC and *Acana chinensis* Bunge. It is classified in the family of Compositae. The main chemical constituents consist of 3.5-5.6% of volatile oil such as β-eudesmol, atractylodin, hinesol, elemol, atractylon, polyacetylene, coumarin, etc. (Thai crude drug database, Faculty of Pharmaceutical Sciences, Ubon Rajathani University, 2008). This herb is widely used in Thai and Chinese folk medicine for treatment of arthritis, bone and joint pain, diuretic, flatulent, common cold and diarrhea [49].

3. **Kot Chiang or Dang Gui** (*Angelica sinensis* Oliv. Diels) Its synonyms are *Angelica polymorpha* Maxim. var. and sinensis Oliv.. It is classified in the family of Umbelliferae. The major chemical components consist of 0.4-0.7% volatile oil such as safrole, isosalfrole, carvacrol; and other substances i.e. alkylphthalides, ligustilide, ferulic acid, N-valerophenone-o-carboxylic acid, terpenoid, phenylpropanoid and coumarinpolyacetyline. Thai traditional medicine use this plant for the treatment of several pain such as bone and joint pain, post-operative pain, stomach pain while traditional Chinese medicine widely uses this plant for the treatment of female symptoms for example dysmenorhea, hot flush and amenorrhea [49].

4. **Kot Chula-lumpa** (*Artemisia vulgaris* L.) Its synonyms are *Artemisia chamomilla* C. Winkler, *Artemisia stewartii* C.B. Clark, *Artemisia wadei* Edgeworth, It is in the family Compositae. The major chemical constituents consist of sesquiterpene lactone such as qinghaosu or artemisinin. This compound is effective against the resistant malarial *Plasmodium falciparum* and *Plasomdium vivax*. Recently, this compound and its semi-synthetic derivatives have been used in the treatment of malaria in China and many countries. Moreover, this plant also contains flavonoids such as casticin, cirsilineol, chysoplenol-D, chrysoplenetin, gallic acid and rutin, which possess synergistic effect with artimisinin against malaria. Traditional Thai and Chinese

medicine also use this herb for antipyretic, relieve cough, sweaty agent and in the treatment of hemorrhoids [49].

5. **Kot Hua Bua** (*Ligusticum chuanxiong* Hort) Its synonyms are *Ligusticum sinense* Oliv., *Ligusticum wallichii* auct.non Franch, in the family of Umbelliferae. The main chemical components consist of 2% volatile oil such as, cnidium lactone, cnidic acid and phthalide group namely ligustilide, neocni-dilide, wallichilide, 3-butylidine-7-hydroxyphthalide, senkyunolide A, butylidenephthalide, butylpthalide, crysophanol, ferulic acid, perlolyrine, sedanonic acid, spathulenol, sedanonic acid, tetramethylpyrazine and vanillic acid. Thai traditional medicine uses rhizomes of this plant for carminative, headache, bone and joint pain, tooth ache, dysmenorrhea and amenorrhea [49].

6. **Kot Jatamansi or Kot Chularos** (*Nardostachys jatamansi* DC.) Its synonyms are *Valeriana jatamansi* Wall, *Patrinia jatamansi* Wall, *Fedia grandiflora* Wall and *Nardostachys jatamansi* DC., It is in the family of Valerianaceae. The major chemical constituents consist of angelicin, jatamansic acid, jatamansin, jatamansinol, jatamansone, jatamol A, jatamol B, patchouli alcohol, β-maaliene and β-sitosterol. Thai folk medicine usually uses this plant for the pus removal, detoxification, carminative, anthelmintics and the abscess of male congenital organs [49].

7. **Kot Kan Prao** (*Picrorrhiza kurroa* Benth.) Its synonyms are *Veronica lindleyana Wall* and *Picrorrhiza lindleyana* (Wall.) Steud. It is in the family of Scrophulariaceae. The major chemical components consist of monocyclic phenolic compound namely vanillic acid and apocyanin, glycoside such as picein, picroside-II and androsin; and aromatic acid for example cinnamic acid, vanillic acid, ferulic acid and β-sitosterol. Thai traditional medicine often uses this herb for chronic fever, fever with hick up and vomiting. In Indian aryurvedic medicine, low dose of this herb

is often used as an appetizing agent and laxative while in high dose it is often used as stimulant [49].

8. **Kot Kradook** (*Saussurea costus*) Its synonyms are *Aucklandia lappa* Dcne*, Aucklandia costus* Falconer*, Aplotaxis lappa* Decne*, Aplotaxis auriculata* DC., and *Saussurea lappa* Clarke. It is in the family of Compositae. Its main chemical constituents are volatile oils with sesquiterpene lactone structure, alkaloids, flavonoids such as rutin and anthraquinone. This herb has a bitter taste and aromatic flavor. In Thai folk medicine, it is widely used to relieve the symptom of fatigue, nausea, flatulent, etc [49].

9. **Kot Phung Pla** (*Terminalia chebula* Retz.) Its synonyms is *Myrobalan chebula* Gaertn., in the family Combretaceae. The main chemical components consist of tannins such as chebulinic acid, gallic acid, tannic acid, etc (Thai crude drug database, Faculty of Pharmaceutical Sciences, Ubon Rajathani University, 2008). This herb is used traditionally for treatment of dysentery diarrhea, gastritis, antipyretic, abscess and ulcerative infection. It is also used as astringent [49].

Previous studied on biological effects of PN

1. Acute and sub-chronic oral toxicity of PN was investigated in Sprague Dawley rats. Rats were randomly into 3 groups. Six rats were used for acute test, 110 rats for sub-chronic test, while five rats were used as sentinel animal to indicate environmental status in long term study. PN was administered to three fasted female rats at the dose of 2,000 mg/kg for limit test and then observed at 0.5, 4, 8, 12 and 24 h after dosing and continuing observed for 14 days. In the sub-chronic test, rats were administered daily oral doses of 10, 100, 1,000 mg/kg/day for 90 days, and 1,000 mg/kg for 90 days with 14 days recovery period while the control group received water at the same volume of the PN. The results demonstrated that, for the

acute toxicity test the limit dose of 2,000 mg/kg did not cause any mortality or symptoms of toxicity in all rats during the observation period. In the sub-chronic tests, PN did not show any treatment–related effect of toxicity. However, increased plasma uric acid was detected in rats receiving high dose of PN (1,000 mg/kg/day) for 90 days [4].

2. Effect of PN on chronic ingestion of PN was tested in Sprague Dawley rats. Rats (10 rats/group) were administered daily oral doses of 10, 100, and 1,000 mg/kg/day of PN for 12 months while the control group was given water at the same volume as the PN treated group. It was found that PN does not produce marked physiological, behavioral, hematological and anatomical adverse effects in these rats. However, chronic adverse effect of a high dose of PN was (1,000 mg/kg/day) reported and this reaction was associated with mesangiolysis, which involves damage to endothelial cells within glomeruli, and PN has a NOAEL of 100 mg/kg/day [5].

3. Nusuetrong et al. (2012) investigated the effect of PN on vascular reactivity in the isolated rat aorta. The results showed that pre-treatment of PN significantly decreased endothelium-dependent relaxations to carbachol [2].

4. Nalinratana et al. (2014) investigated the antioxidant effect of PN on H_2O_2-induced stress in human endothelial ECV304 cells. The results showed that PN attenuated H_2O_2-induced stress in human endothelial cells in a concentration dependent manner [3].

PN and the anti-oxidative stress effects

Recently, hepatoprotective effects of PN are still remain unreported. However, hepatoprotective effects of Thai herbals which are the components of PN were reported in several aspects as follows.

1. Oh et al. (2002) have reported imperatorin and (+)-byakangelicin, the MeOH extract of Kot Soa exhibited strong hepatoprotective activities against tacrine induced oxidative stress in HepG2 cells [6].

2. Hepatoprotective effect of Kot Kan Prao was performed on mouse liver slice culture and ethanol was used to generate oxidative stress. The results clearly showed that aqueous extract of this herb with high antioxidant activity, as demonstrated using different radical scavenging assays, was effective in suppressing the deleterious effects of ethanol. In addition, aqueous extract was treated along with ethanol restored the activities of antioxidant enzymes (SOD and CAT) and significantly reduced lipid peroxidation [7].

3. Antihepatotoxic activity of Kot Kradook was performed on D-galactosamine (D-GalN) and lipopolysaccharide (LPS)-induced hepatitis mice. Pretreatment of mice with aqueous-methanol extract of this plant significantly prevented the increased in plasma levels of ALT and AST in a dose-dependent manner. Moreover, it also significantly restricted the progression of hepatic damage induced by D-GalN and LPS. These data indicate that this aqueous-methanol extract exhibits hepatoprotective effect in mice and this study rationalize the traditional use of this plant in liver disorders [8].

4. Mahesh et al. (2007) have reported administration of aqueous extract of Kot Phung Pla to aged rats prevented the depletion of antioxidants enzymes (SOD, CAT, GPx) activities and GSH, vitamin C and E contents and also decreased the level of MDA content in heart tissues. This result showed that

the aqueous extract modulates the activities of antioxidants and lipid peroxidation through the management of oxidant/antioxidant imbalance in rat heart tissues [11].

5. Protective Effects of Kot Khamao was performed on liver ischemia–reperfusion injury rats. Treatment with the extract of Kot Khamao significantly reduced the elevated expression of markers of liver dysfunction and the hepatic morphologic changes in ischemia–reperfusion injury rats. The extract also markedly inhibited ischemia–reperfusion injury induced lipid peroxidation and altered the activities of the antioxidant enzyme SOD and MDA levels. In addition, pretreatment with this extract suppressed the expression of interleukin-1β and NF-kB [9].

6. Hepatoprotective activity of the methanolic extract of Kot Chula-lumpa was performed by orally administered of the extract of Kot Chula-lumpa to D-galactosamine and lipopolysaccharide induced liver injury mice. Pretreatment with different concentrations of the extract (150–600 mg/kg) significantly and dose-dependently prevented chemically or immunologically induced increase in serum levels of hepatic enzymes (AST and ALT) [50].

7. Antioxidative stress of Kot Chiang was performed on free radical generation and lipid peroxidation and its modulatory effect on immunity activity in middle-aged women subjects at dosage levels of 125 mg/kg. Antioxidant activities such as SOD, CAT, GPx and GSH were significantly enhanced. In addition, lipid peroxidation levels were reduced compared to the control group [14].

8. Protective effect of Kot Hua Bua was reported by Hou et al (2004). Human umbilical vein endothelial cells (ECV304) were treated with the aqueous extract of Kot Hua Bua followed by H_2O_2 were given to induce cells damage. Kot Hua Bua was exhibited to protect ECV304 cells against H_2O_2 induced cells

damage by enhancing the antioxidative ability via increased activities of SOD, CAT and GPx, activating ERK and eNOS signaling pathway [12].

9. Cytoprotective effect of Kot Jatamansi was performed on C6 glioma cells against hydrogen peroxide induced oxidative damage. C6 glioma cells were pretreated with water and methanolic extract of Kot Jatamansi for 24 h then cells were pretreated for 24 h with H_2O_2 to induce oxidative damage. The level of antioxidant enzymes, CAT, Cu/ZnSOD, GPx and GSH, significantly increased following pretreatment with the extract of Kot Jatamansi. In addition, lipid peroxidation was also significantly decreased in pretreated cultures [13].

PN and the activities of CYP

Effects of PN on the activities of CYP are never reported in the recent time. However, inhibition of CYP activities by Thai herbs that composed of PN were reported in several aspects as follows.

1. The inhibitory effect of gallic acid (GA) on the activity of CYP3A was performed in human liver microsomes (HLMs) and using androstenedione 6β-hydroxylase activity reaction. It was found that GA inhibits androstenedione 6β-hydroxylase activity (*Ki* 70 µM). Moreover, the pre-incubation of GA (100 µM) with HLMs in the absence of NADPH, as compared with the presence of NADPH, before assay of androstenedione 6β-hydroxylase activity significantly increased the inhibitory effects of the GA [51].

2. Effects of Kot Chula-lumpa, Kot Khamao, Kot Hua Bua and Kot Phung pla, which are comprises of PN on the activity of CYP3A were assessed *in vitro* using testosterone (TST) as a specific CYP3A probe substrate in pooled enzymes rat liver microsomes (RLMs). The amount of 6β-hydroxy

testosterone (6β-TST), major metabolite of TST, obtained from each metabolic reaction with or without herbal crude extracted was determined using High Performance Liquid Chromatography (HPLC) technique, thereafter converted into % inhibition. Results revealed that the aqueous extracts of 3 herbs were affected the activity of CYP3A in different degree of inhibition as follows; Kot Phung pla 25.76%, Kot Chula-lumpha 11.92%, and Kot Hua Bua 11.48%. The alcoholic extracts of and Kot Chula-lumpa strongly inhibited the activity of CYP3A with % inhibition of 91.03% and 90%, respectively whereas the extract of Kot Khamao has no inhibitory effect [52].

3. Effects of Kot Kradook and Kot Soa on the activity of CYP3A were determined in *in vitro* using pooled enzyme RLMs and testosterone a selective CYP3A probe substrate. The amount of 6β-hydroxytestosterone (6β-TST), major metabolite of TST, obtained from each metabolic reaction was detected using HPLC technique then converted into % inhibition. Result demonstrated that the aqueous extracts were affected CYP3A activity in different degree of inhibition as follows; 24.9% of Kot Kradook and 44.7% of Kot Soa. On the other hand, the alchoholic extracts of Kot Kradook and Kot Soa inhibited the activity of CYP3A with 35.50% and 41.16%, respectively [53].

4. Effect of the hydroalcoholic extract of fruit pulp of Kot Phung Pla was evaluated for its safety through CYP activities assay using RLMs. The results demonstrated that both extract and fraction of Kot Phung Pla also showed a linear concentration-dependent inhibition of CYP3A. In addition, result showed more inhibition of fraction when compared to the extract and gallic acid (GA), a major component found in this extract However, the inhibition showed by fraction is less when compared with Ketoconazole a known CYP3A inhibitor. Thus, this study indicated the *in vitro* cytochrome P450 inhibition potential of Kot Phung Pla one herbal comprise of PN [54].

Some major components found in PN

Several studies reported the protective effect of plant that comprises in PN. In general, more than one compound is found in each of the plants and mostly phenolic compound with antioxidant properties, thus, protective effects reported of these plants might be resulted from this compound. In addition, synergistic or additive effect can occur due to more than one compound comprises in each of the plants or preparation. There are several compounds which are found in each plant that comprises in PN such as ferulic acid, gallic acid, imperatorin, rutin etc.

Ferulic acid

Ferulic acid (FA), a ubiquitous natural phenolic compound found in seeds, leaves, both in its free form and covalently conjugated to the plant cell wall polysaccharides, glycoproteins, polyamines, lignin and hydroxy fatty acids. The content of FA in commelinid plants ranged from 1-15 mg/g with the highest quantity being found in 0.2-240 mg/0.1 kg with the highest quantity being found in bamboo shoot [55]. A wide variety of biological activities of FA reported were associated with antioxidant, antiinflammatory, antimicrobial, antiallergic, anticarcinogenic as well as hepatoprotective [55]. Previous studies on protective effect of FA reported are as follows;

1. Protective effect of FA was examined in alloxan induced diabetic mice. FA 10 mg/kg was given orally once a day to mice for 15 days after mice were received 75 mg/kg of alloxan intra peritoneal (i.p.) for 15 days and basic biochemical parameters, enzymatic as well as non-enzymatic activities, lipid peroxidation and immunohistochemical were assessed. The results showed that FA significantly prevented the increased of basic biochemical markers

and lipid peroxidation and the level of TNF-**α**. In addition, FA restored the activities of enzymatic and non-enzymatic antioxidant activities [56].

2. Sompong et al. (2015) reported the protective effect of FA on high glucose-induced protein glycation, lipid peroxidation and membrane ion pump activity in human erythrocytes. Erythrocytes from 6 healthy volunteer were incubated with high glucose concentration and FA at 0.1, 1, 10 and 100 μM were added into the reaction mixtures for 24 h and protein glycation, lipid peroxidation and membrane ion pump activity were measured. The results showed that FA significantly decreased the levels of HbA1c, inhibited lipid peroxidation in erythrocytes exposed to high concentration of glucose as well as FA reversed a reduction in Na^+/K^+-ATPase activity in the erythrocyte plasma membrane which induced by high glucose concentration [57].

Gallic acid

Gallic acid (GA) is an organic acid found in a variety of foods and herbs that are well known as powerful antioxidants. Natural source of GA include bearberry leaves, hazelnuts, grape seed, apples, flax seed, green tea and evening primrose [58]. Generally, antioxidant effect of phenolic compounds is related to the hydroxyl molecules containing in their structure and the number of such molecules changes their binding position to aromatic ring activities. GA is a phenolic compound that contains three hydroxyl molecules in its structure it was reported that this compound counteracts superoxide, hydroxyl, nitric oxide and peroxy nitrite radicals. In addition, protective effect of GA was reported associated with modulation of GSH and enzymatic antioxidants but the molecular mechanism are still remain unclear. Previous studies on the protective effect of GA are reported as follows;

1. Hepatoprotective effect of GA was evaluated in acetaminophen induced liver damage in mice. GA 100 mg/kg was given to mice via i.p. 30 min after a dose of 900 mg/kg acetaminophen intraperitoneally was given to mice. After 4 h, liver marker enzymes (AST, ALT) and inflammatory mediator tumor necrosis factor-alpha (TNF-α) were estimated in serum, while the lipid peroxidation and antioxidant status (CAT, GPx, GR, GST, GHS and SOD) were determined in liver homogenate of mice. It was found that activities of liver marker enzymes, TNF-α and lipid peroxidation levels were elevated in mice exposed to acetaminophen, whereas the antioxidant status was found to be depleted as compared with control group. However, GA treatment significantly reverses all above changes by its antioxidant action as compared with the acetaminophen-challenged mice [59].

2. An orally gavage of 50 mg/kg of GA was given to female Spraque-dawley rats once a day for 7 days before 45 min ischemia and 60 min reperfusion were performed. Blood chemical analysis and histological analysis were used to evaluated the protective effect of GA. The results demonstrated that serum AST and ALT was found to be significantly lower in rats pre-treated with GA as compared with control group and histological findings are in line with these results [60].

Imperatorin

Imperatorin is a naturally furanocoumarin which widely distributed in selected herbal medicines, namely in the roots and fruits of *A. dahurica* (Kot Soa) and *A. archangelica* from the family of Umbelliferae. Due to its widespread occurrence, imperatorin was employed in several pharmacological studies. It was found that this compound exerted an anxiolytic effect, improved different stages of memory

processes and protected against memory impairments induced by scopolamine, possessing antioxidant properties, anti-inflammatory, antiproliferative, antibacterial and anticoagulant [61]. The biological effects of imperatorin associated with antioxidant properties and protective effects were reported as follows;

1. Effects of imperatorin on nicotine-induced anxiety and memory related responses and oxidative stress was investigated in mice. Imperatorin 10 and 20 mg/kg was intraperitoneally given to impair anxiogenic mice induced by nicotine (0.1 mg/kg, s.c.). Anxiety tested using the elevated plus maze test (EPM), cognition using passive avoidance (PA) procedures were used. Oxidative stress was evaluated by determination of antioxidant enzymes (GPx, GR, SOD activities as well as the concentration of malondialdehyde (MDA) in the whole brain, hippocampus and prefrontal cortex. The results demonstrated that imperatorin improved different stages of memory processes (both acquisition and consolidation) when injected in combination with non-active dose of nicotine in the PA task. Furthermore, imperatorin prevented nicotine-induced decreases in enzymatic antioxidants activities indicators of oxidative stress, especially in the hippocampus and the cortex [61].

2. Effects of imperatorin were evaluated in scopolamine-induced cognitive impairment and oxidative stress mice. Imperatorin was given at the doses of 5 and 10 mg/kg (i.p.) before 1 mg/kg of scopolamine was given. The PA paradigm, an animal model of Alzheimer disease (AD) was used for memory deficit measured in mice. It was demonstrated that imperatorin improved memory acquisition and consolidation impaired by scopolamine. Moreover, imperatorin increased the activity of enzymatic antioxidants and attenuated the increased of MDA, an indicator of lipid peroxidation level [62].

Rutin

Rutin is a bioflavonoid, flavonol glycoside comprised of the quercetin and the disaccharide rutinose (rhamnose and glucose). It is found in many plants, fruits and vegetables with the highest source in buckwheat and also found in citrus fruits, noni, black tea, apple peel. During digestion, much of the rutin is metabolized to its aglycone and quercetin. Rutin possesses a wide variety of pharmacological effects such as antioxidant, anti-inflammatory, anti-tumor, anti-mutagenic as well as protective effect [63]. Protective effects of rutin were reported as follows;

1. Protective effect of rutin was investigated in CCl_4 and acetaminophen induced hepatotoxicity in male Wistar rats. Rutin 20 mg/kg was given to rats every 12 h for 4 consecutive intervals and hepatic injury rats was induced by 640 mg/kg of acetaminophen or 1.5 ml/kg of CCl_4 1 h after the last dose of rutin. Thereafter, basic blood chemical (AST and ALT) were analysed. It was found that rutin prevented the raised of serum enzyme AST and ALT in acetaminophen and CCl_4-induced hepatic injury [64].

2. Khan et al. (2012) reported the protective effect of rutin against CCl_4-induced hepatotoxicity in rat. Hepatotoxicity was induced by 3 ml/kg of 30% CCl_4, 2 times a week for 4 consecutive weeks and rutin at a dose of 50 and 70 mg/kg were given via intragastric after the 48 h of CCl_4 treatment. Then serum enzyme level, lipid profile, activities of antioxidant enzymes and molecular markers were investigated. The results showed that rutin significantly prevented the increased of serum enzyme AST and ALT levels. Rutin increased the activities of enzymatic antioxidants. Moreover, the decreased of p53 and CYP2E1 expression induced by CCl_4 was restored with treatment of rutin [63].

β-Sitosterol

β-Sitosterol is a plant derived-sterol also known as a phytosterol. This compound is white, waxy powders with a characteristic odor, hydrophobic and soluble in alcohols. It is widely distributed in the plant kingdom and found in vegetable oil, nuts, avocados and prepared foods, such as salad dressings. It possesses several pharmacological effect such as inhibit the absorption of cholesterol, cancer-cell growth, angiogenesis, invasion and metastasis [65]. Previous study associated with antioxidant and protective properties of β-Sitosterol as followings;

1. Antioxidant effects of β-sitosterol was investigated in 1,2-dimethylhydrazine-induced colon carcinogenesis in rats. It was reported that β-sitosterol possessed a ROS scavenger. It modulated the elevation in enzymatic and non-enzymatic antioxidants as well as the GSH and GSH/GSSG ratio [66].

2. Protective effects of β-sitosterol were investigated in CCl_4 induced hepatotoxicity and gentamicin induced nephrotoxicity in rats. β-sitosterol were administered intragastrically with at daily doses of 35 and 350 μg/kg for 14 consecutive days followed by a dose of 1 mL/kg of CCl_4 after the last dosing of β-sitosterol to induced hepatotoxicity or a daily dose of 150 mg/kg of gentamicin from day 9 to day 14 during the course of β-sitosterol treatment (6 doses) to induced nephrotoxicity. Basic liver enzymes (AST, ALT), BUN and plasma creatinine level were measured as a marker of liver and nephrotoxicity. The results revealed that pretreated with β-sitosterol attenuated CCl_4-induced liver injury but not gentamicin induced renal toxicity in rats. The hepatoprotective effect of β-sitosterol was associated with the modulation of mitochondrial GSH redox status,

presumably through the glutathione reductase-mediated enhancement in mitochondrial GSH redox cycling [67].

CHAPTER III

MATERIALS AND METHODS

Chemicals

The following chemicals and reagents were used in the study:

1. Acetaminophen (Wako Pure Chemicals, Osaka, Japan)

2. Acetonitrile (Kanto Chemical, Tokyo, Japan)

3. *N*-Acetylcysteine (NAC) (Sigma-Aldrich, St. Louis, MO, USA)

4. Aminopyrine (Wako Pure Chemicals, Osaka, Japan)

5. Aniline hydrochloride (Wako Pure Chemicals, Osaka, Japan)

6. Ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA)

7. Avidin (Gibco, Grand Island, NY, USA)

8. Biotin-HRP (Gibco, Grand Island, NY, USA)

9. (+/−)-Bufuralol hydrochloride (Ultrafine-Chemicals, Manchester, UK)

10. Caffeine anhydrous (Wako Pure Chemicals, Osaka, Japan)

11. Catalase assay kit (Cayman Chemical, Ann Arbor, MI, USA)

12. Clonazepam (Ultrafine-Chemicals, Manchester, UK)

13. Cryopreserved hepatocyte recovery medium (Gibco, Grand Island, NY, USA)

14. 3,3′-Diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA)

15. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA)

16. *N,N*-Diethyldithiocarbamate trihydrate (Wako Pure Chemicals, Osaka, Japan)

17. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA)

18. 2,2-Diphenyl-1-picrylhydrazyl (Sigma-Aldrich, St. Louis, MO, USA)

19. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA)

20. Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA)

21. Ethylene diamine tetra-acetic acid (EDTA) (Gibco, Grand Island, NY, USA)

22. Ethylene glycol tetra-acetic acid (EGTA) (Sigma-Aldrich, St. Louis, MO, USA)

23. Ferulic acid (Tokyo Chemical Industry, Tokyo, Japan)

24. Fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA)

25. Furafylline (Funakoshi, Tokyo, Japan)

26. Gallic acid (Wako Pure Chemicals, Osaka, Japan)

27. Glutamine (Gibco, Grand Island, NY, USA)

28. Glutathione peroxidase (GPx) (Cayman Chemical, Ann Arbor, MI, USA)

29. Glutathione reductase (GR) (Cayman Chemical, Ann Arbor, MI, USA)

30. Hydrogen peroxide (H_2O_2) (Sigma-Aldrich, St. Louis, MO, USA)

31. 1'-Hydroxybufuralol maleate (Ultrafine-Chemicals, Manchester, UK)

32. 6-Hydroxychlorzoxazone (Ultrafine-Chemicals, Manchester, UK)

33. (+/−)-4'-Hydroxymephenytoin (Ultrafine-Chemicals, Manchester, UK)

34. Imperatorin (Sigma-Aldrich, St. Louis, MO, USA)

35. Ketoconazole (LKT Laboratories, St. Paul, MN, USA)

36. *S*-(+)-Mephenytoin (Ultrafine-Chemicals, Manchester, UK)

37. Methanol (Kanto Chemical, Tokyo, Japan)

38. Midazolam (Wako Pure Chemicals, Osaka, Japan)

39. 1'-OH-Midazolam (Ultrafine-Chemicals, Manchester, UK)

40. β-NADPH (Oriental Yeast, Tokyo, Japan)

41. Neutravidin biotin-binding protein (Gibco, Grand Island, NY, USA)

42. Omeprazole (Sigma-Aldrich, St. Louis, MO, USA)

43. Phenacetin (Wako Pure Chemicals, Osaka, Japan)

44. Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA)

45. Protease inhibitor (Sigma-Aldrich, St. Louis, MO, USA)

46. Quinidine (Wako Pure Chemicals, Osaka, Japan)

47. Rifampicin (Sigma-Aldrich, St. Louis, MO)

48. Rutin (Tokyo Chemical Industry, Tokyo, Japan)

49. β-Sitosterol (Merck Millipore, Darmstadt, Germany)

50. Sodium chloride (NaCl) (Merck Millipore, Darmstadt, Germany)

51. Sodium fluoride (NaF) (Merck Millipore, Darmstadt, Germany)

52. Sodium deoxycholate (Sigma-Aldrich, St. Louis, MO, USA)

53. Sodium dodecyl sulfate (SDS) (Bio-Rad, Hercules, CA, USA)

54. Sodium orthovanadate (Na_3VO_4) (Sigma-Aldrich, St. Louis, MO, USA)

55. Sulfaphenazole (Daiichi Pure Chemicals, Tokyo, Japan)

56. Superoxide dismutase assay kit (SOD) (Cayman Chemical, Ann Arbor, MI, USA)

57. Ticlopidine (Wako Pure Chemicals, Osaka, Japan)

58. Tergitol® type NP-40 (Sigma-Aldrich, St. Louis, MO, USA)

59. Total glutathione (GSH) (Cayman Chemical, Ann Arbor, MI, USA)

60. Tris base (Bio-Rad, Hercules, CA, USA)

61. Vanillic acid (Sigma-Aldrich, St. Louis, MO, USA)

62. 7'-OH-Warfarin (Toronto Research Chemicals, Ontario, Canada)

63. *S*-(−)-Warfarin (Cayman Chemicals, MI, USA)

64. William's E medium (Gibco, Grand Island, NY, USA)

Phikud Navakot extract

PN extract was kindly prepared and provided by Dr. Naruporn Sutanthavibul, at the Department of Pharmaceutics and Manufacturing Pharmacy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. In brief, crude drug of nine herbs were purchased from Thai traditional pharmacy in Bangkok, Thailand and identified by Associate Professor Uthai Sothanaphun, Faculty of Pharmacy, Silpakorn University, Nakhon Pathom, Thailand. Voucher specimens (MUS1122-1130) have been deposited

at the Museum of Natural Medicines, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. Equal weights of nine herbs, in coarse powder, were soaked in 10 times by weight of 80% ethanol for overnight and extracted at 100 °C for 3 h, two times each. The extracts were mixed and concentrated. The resulted semi-solid extract was kept at room temperature until the time of analysis. High performance liquid chromatography coupled with photodiode array detector was used to quantitatively determine the analytical markers in 50 mg of PN extract as 0.1589 mg gallic acid, 0.1459 mg vanillic acid and 0.0403 mg ferulic acid [68].

Instruments

1. Beckman Allegra® X-15R (Beckman Coulter, CA, USA)
2. Capcell-Pak C18 UG. 120 (250 x 4.6 mm; Shiseido, Tokyo, Japan)
3. Elmasonic S30H sonicator (Elma, SG, Germany).
4. High Performance Liquid Chromatography model L-2400 UV detector; 245 nm and L-2400 FL detector; Excitation 320 nm, Emission 415 nm (Hitachi, Tokyo, Japan)
5. Microcentrifuge tubes size 1.5 microliter (Biologix, MS, USA)
6. Microplate reader model POWERSCAN 4 microplate reader (DS Pharma Biomedical, Osaka, Japan)
7. Micropipet size 20, 200 and 1,000 microliter (Gilson, WI, USA)
8. Mightysil 18 RP-column (250 x 4.6 mm; Kanto chemical, Tokyo, Japan)
9. Pipette tip size 0.1-10 microliter and 100-1,000 microliter (Corning incorporated, NY, USA)
10. pH/ISE meter (CyberScan pH620, Thermo Sceintific, Beverly, MA, USA)
11. Powerwave XS2 microplate reader (Biotek, VT, USA)

12. Rubber policeman (Thomas Scientific, NJ, USA)

13. SpectraMax M5 microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA)

14. 100 mm Dish (Corning Costar, NY, USA)

15. 96 Well microtiter plate (Corning Costar, NY, USA)

16. 96-well black plates (Corning Costar, NY, USA)

Cell and human liver microsomes

1. Primary human hepatocytes (BD Gentest, Woburn, MA, USA)

2. Human liver hepatocellular carcinoma (HepG2 cell) (ATCC, USA)

3. Human liver microsomes (BD Biosciences, San Jose, CA, USA)

Methods

1 Hepatoprotective effect of PN extract

1.1 DPPH radical scavenging activity of PN and some active constituents in the PN extract

The antioxidant activity of PN and some active constituents (ferulic acid, gallic acid, imperatorin, rutin, vanillic acid and β-sitosterol) were evaluated by DPPH radical scavenging assay according to the method of Sokmen et al (2005) with modification [69].

DPPH (2,2-diphenyl-1-picrylhydrazyl) is considered as a stable radical because of the paramagnetism conferred by its odd electron. The solution in absolute ethanol appears as a deep violet color and show a strong absorption at 515-520 nm. This radical can accept the electron or hydrogen atom to become a stable diamagnetic molecule and has pale violet. If substance for testing antioxidant effect

is mixed with DPPH solution and gives rise to pale violet, it suggests that this substance has antioxidant effect by mechanism of free radical scavenging activity. The assay protocol was prepared as following;

1. PN extract was dissolved in absolute ethanol. Some active constituents (ferulic acid, gallic acid, imperatorin, rutin, vanillic acid and β-sitosterol) were dissolved in methanol.

2. Each sample was diluted for 7 concentrations (performing serial dilution).

3. 0.5 mg/mL of DPPH was prepared in absolute ethanol.

4. Twenty microlitres of each sample were added to 96-well culture plate.

5. The prepared 0.5mg/mL DPPH solution of 180 µL were added into all wells.

6. The plates were covered with plate covers and shaked at room temperature for 30 minutes.

7. The OD was measured at 517 nm using PowerWave XS2 microplate reader

8. Percent inhibition was calculated as following:

   % inhibition = $\dfrac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$

   Dose response relationship was plotted between % inhibition and concentrations of the compounds. Linear regression analysis was carried out for calculating half maximal inhibitory concentration of samples required to scavenge DPPH radical by 50% (IC_{50} value).

9. The experiments were done in triplicate and ascorbic acid; a known natural antioxidant was used as the positive control.

1.2 Cytotoxicity studies

1.2.1 Cytotoxicity of PN in HepG2 cells

Cytotoxicity of PN in HepG2 cells were determined using MTT assay. Briefly, HepG2 cells were seeded in 96-well culture plate (1×10^4 cells/well) and incubated overnight. The cells were then treated with various concentrations of PN extract (0, 0.0001, 0.001, 0.01, 0.1 and 1 mg/mL) for 24 h. At the end of incubation, a 25-μl of 5 mg/ml of MTT reagent in culture medium was added to each well (equal to 1 mg/ml of MTT in a total volume of medium in each well of 125 μl) and the cells were incubated for 3 h at 37 °C. The resulted formazan was dissolved with 100 μl of DMSO. Optical density was measured at 570 nm using a PowerWave XS2 microplate reader. All experiments were performed in 3-independent experiments.

1.2.2 Cytotoxicity of H_2O_2 in HepG2 cells

Cytotoxicity of H_2O_2 in HepG2 cells were determined using MTT assay. Briefly, HepG2 cells were seeded in 96-well culture plate (1×10^4 cells/well) and incubated overnight. The cells were then treated with various concentrations of H_2O_2 (0, 5, 10, 50, 100, 200, 500 and 1,000 μM) for 24 h. At the end of incubation, a 25-μl of 5 mg/ml of MTT reagent in culture medium was added to each well (equal to 1 mg/ml of MTT in a total volume of medium in each well of 125 μl) and the cells were incubated for 3 h at 37 °C. The resulted formazan was dissolved with 100 μl of DMSO. Optical density was measured at 570 nm using a PowerWave XS2 microplate reader. All experiments were performed in 3-independent experiments.

1.2.3 Cytotoxicity of NAC in HepG2 cells

Cytotoxicity of NAC in HepG2 cells were determined using MTT assay. Briefly, HepG2 cells were seeded in 96-well culture plate (1×10^4 cells/well) and incubated overnight. The cells were then treated with various concentrations of NAC (0, 0.1, 0.2, 0.5, 1, 5 and 10 mM) for 24 h. At the end of incubation, a 25-µl of 5 mg/ml of MTT reagent in culture medium was added to each well (equal to 1 mg/ml of MTT in a total volume of medium in each well of 125 µl) and the cells were incubated for 3 h at 37 °C. The resulted formazan was dissolved with 100 µl of DMSO. Optical density was measured at 570 nm using a PowerWave XS2 microplate reader. All experiments were performed in 3-independent experiments.

1.2.4 Cytotoxicity of β-sitosterol in HepG2 cells

Cytotoxicity of β-sitosterol in HepG2 cells were determined using MTT assay. Briefly, HepG2 cells were seeded in 96-well culture plate (1×10^4 cells/well) and incubated overnight. The cells were then treated with various concentrations of β-sitosterol (0, 0.05, 0.5, 1, 10, 20, 50, 100 and 200 µM) for 24 h. At the end of incubation, a 25-µl of 5 mg/ml of MTT reagent in culture medium was added to each well (equal to 1 mg/ml of MTT in a total volume of medium in each well of 125 µl) and the cells were incubated for 3 h at 37 °C. The resulted formazan was dissolved with 100 µl of DMSO. Optical density was measured at 570 nm using a PowerWave XS2 microplate reader. All experiments were performed in 3-independent experiments.

1.3 Generation of intracellular ROS by PN extract and inhibition of H_2O_2-induced intracellular ROS by NAC, PN extract and β-sitosterol

1.3.1 Generation of intracellular ROS by PN extract

Generation of intracellular ROS by PN was determined using the DCFH-DA fluorescent probe. Briefly, HepG2 cells were seeded in 96-well black plates (1×10^4 cells/well). Cells were treated with various concentrations of the PN extract (0, 0.0001, 0.001, 0.01, 0.1 and 1 mg/mL). After 3 h of incubation, 50 μM DCFH-DA was added to each well and incubated for 40 min at 37 °C. The fluorescent intensity of DCF was detected using a PowerWave XS2 microplate reader at an excitation and emission wavelength of 485 and 528 nm, respectively. H_2O_2 at the concentration of 500 μM was used as a positive control. All experiments were performed in 3-independent experiments.

1.3.2 Inhibition of H_2O_2-induced intracellular ROS by NAC

HepG2 cells were seeded in 96-well black plates (1×10^4 cells/well). Cells were treated with various concentrations of NAC (0, 0.1, 0.2, 0.5, 1, 5 and 10 mM) for 1, 2 and 6 h. After the treatment, cells were treated with 500 μM H_2O_2 for 3 h, thereafter, intracellular ROS levels were measured by the DCFH-DA assay according to the protocol as described above. All experiments were performed in 3-independent experiments.

1.3.3 Inhibition of H_2O_2-induced intracellular ROS by PN extract and β-sitosterol

HepG2 cells were seeded in 96-well black plates (1 x 10^4 cells/well). Cells in the NAC-pretreated group was treated with 5 mM of NAC for 1 h and cells in the PN or β-sitosterol pretreated groups were treated with various concentrations of the PN extract (0, 0.0001, 0.001, 0.01 and 0.1 mg/mL) or β-sitosterol (0, 0.1, 1, 2.5, 5, 10 and 20 µM) for 3, 6 and 12 h. After the treatment, cells were treated with 500 µM H_2O_2 for 3 h, thereafter, intracellular ROS levels were measured by the DCFH-DA assay according to the protocol as described above. All experiments were performed in 3-independent experiments.

1.4 Protective effects of PN extract and β-sitosterol against H_2O_2-induced cytotoxicity

MTT assay was used to determine whether PN or β-sitosterol could protect cells from H_2O_2-induced cell cytotoxicity. HepG2 cells were seeded in 96-well plates (1 x 10^4 cells/well). After 24 h, the culture medium was replaced with serum-free medium containing various concentrations of the PN extract (0, 0.0001, 0.001, 0.01 and 0.1 mg/mL) or β-sitosterol (0, 0.1, 1, 2.5, 5, 10 and 20 µM) or 5 mM of NAC (used as a positive control). After the treatment (3 h for PN and β-sitosterol, 1 h for NAC), the culture medium was discarded, and the cells were subsequently treated for 24 h with 500 µM H_2O_2 to induce oxidative stress. Cell viability was then evaluated using the MTT assay. All experiments were performed in 3-independent experiments.

1.5 Effect of PN extract and β-sitosterol on GSH levels and the activities of antioxidant enzymes (CAT, GPx, GR and SOD)

The levels of GSH and the activities of CAT, GPx, GR and SOD upon H_2O_2 and PN extract treatment were measured. HepG2 cells were seeded in petri-dishes (1 x 10^6 cells each) and incubated for 24 h at 37 $^\circ$C in a CO_2 incubator. Then, the cells were divided into 2 groups as following;

1. The cells were treated with different concentrations of PN (0, 0.01 and 0.1 mg/mL) or β-sitosterol (10 and 20 μM) for 3, 6 or 12 h and harvested at the end of treatment.

2. The cells were treated with different concentrations of PN (0, 0.01 and 0.1 mg/mL) or β-sitosterol (10 and 20 μM) for 3, 6 or 12 h then the cells subsequently incubated with culture medium for 24 h before harvested.

1.6 Effect of PN extract and β-sitosterol to restore GSH levels and the activities of antioxidant enzymes (CAT, GPx, GR and SOD) in H_2O_2 induced oxidative stress in HepG2 cells.

To evaluated the effect of PN extract and β-sitosterol to restore GSH levels and the activities of antioxidant enzymes (CAT, GPx, GR and SOD), the cells were treated with different concentrations of PN (0, 0.01 and 0.1 mg/mL) or β-sitosterol (10 and 20 μM) for 3 h prior to incubate with 500 μM of H_2O_2 for 24 h before harvested.

After the treatment, the cells were in 1.5 and 1.6 mentioned above harvested using a rubber policeman and ultrasonically homogenized on ice with specific lysis buffer [50 mM potassium phosphate buffer containing 1 mM EDTA (pH 7.0) for CAT and GR; 50 mM Tris-HCl containing 5 mM EDTA and 1 mM DTT (pH 7.5) for GPx and

HEPES buffer containing 1 mM EGTA, 210 mM mannitol and 70 mM sucrose (pH 7.2) for SOD] using cell disrupter. Activities of CAT, GPx, GR, SOD and the GSH levels in HepG2 cells were determined using commercially available assay kits following the manufacturer's protocols.

Determination of glutathione (GSH)

The assay is based on the GSH recycling system by DTNB and GR. 5,5'-Dithiobis-2-nitrobenzoic acid or DTNB is reacted with GSH in cell lysate to generate 2-nitro-5-thiobenzoic acid which has yellow colour. GSH concentration can be determined by measuring the absorbance of the product at 414 nm. Oxidized glutathione (GSSG) generated from the reaction can be reduced back to GSH by GR, and GSH reacts with DTNB again to produce more 2-nitro-5-thiobenzoic acid. Therefore, the recycling system dramatically improves the sensitivity of total GSH detection. GR catalyse the reduction of GSSG in the presence of NADPH which is oxdised to $NADP^+$ resulting in the decrease of an absorbance at 340 nm of NADPH (Figure 9). Glutathione assay kit was used in this study. The assay was initiated by adding 50 μl of each sample into each well of 96 well plate followed by 150 μl/well of freshly prepared Assay Cocktail (MES buffer; cofactor mixture comprising lyophilized powder of $NADP^+$ and glucose-6-phosphate; enzyme mixture comprising glutathione reductase and glucose-6-phosphate dehydrogenase, DTNB and water). The plate was covered with a plate cover and incubated on an orbital shaker in the dark room and the absorbance was measured at 414 nm using microplate reader at 5 minute intervals for 30 minutes for a total of 6 measurements. Because of the use of GR in this assay kit, both GSH and GSSG were measured and the assay reflects total GSH. Thus, the assay of GSH the reaction was performed as mentioned above except

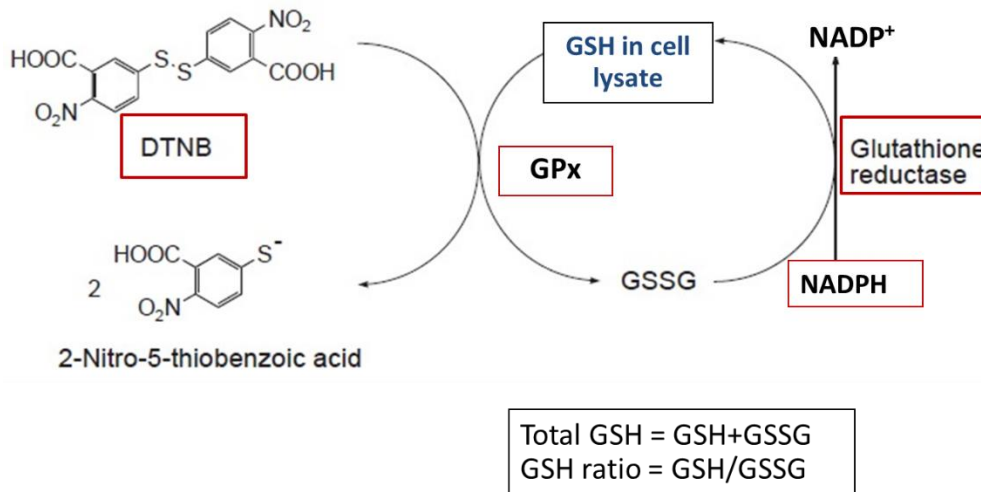the addition of GR and GSSG level was calculated by subtracting GSH from the total amount of glutathione.

Figure 9. Schematic illustration of principle of total GSH, GSH, GSSG assay [70]

Determination of catalase (CAT) activity

CAT is one of an important enzyme that protects cells from oxidative damage by reactive oxygen species. It catalyzes the decomposition of H_2O_2 to molecules of water and oxygen. To determine effect of PN on CAT activity, catalase assay kit was used. The assay is based on the reaction of CAT in cell lysate with methanol in the presence of an optimal concentration of H_2O_2. Formaldehyde produced from the reaction is measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald). Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colourless to a purple colour (Figure 10). The assay was started by adding 100 μl of diluted assay buffer, 30 μl of methanol and 20 μl of each samples into 96 well plate. The reaction was initiated by adding 20 μl of diluted H_2O_2 to all wells. The plate was covered with the plate cover and incubated on a shaker for 20 minutes at room temperature. Then, 30 μl of

diluted potassium hydroxide was added to each well to terminate the reaction followed by an addition of 30 µl of Purpald. The plate was covered and incubated for 10 minutes at room temperature on the shaker. Ten microlitres of catalase potassium periodate was added to each well and the plate was covered and incubated for 5 minutes at room temperature on a shaker then the absorbance was measured at 540 nm using a microplate reader. CAT activity of the sample were calculated using the following equation. One unit is defined as the amount of enzyme that will cause the formation 1 nmoL of formaldehyde per minute at 25 ° C.

$$\text{CAT activity (nmoL/min/mL)} = \frac{\underline{\text{µM of sample}}}{20 \text{ min}} \times \text{sample dilution}$$
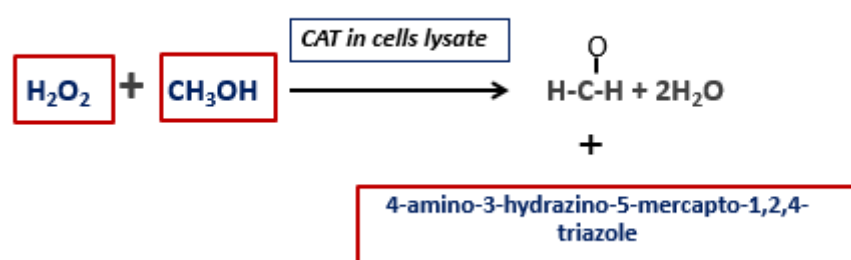

Figure 10. Principle of CAT assay [71]

Determination of glutathione peroxidase (GPx) activity

GPx reduces the peroxide group to a relatively un-reactive alcohol group, using glutathione as the reducing agent, and thus protects the cell from oxidative damage. To determine the effect of PN on the GPx activity, glutathione peroxidase assay kit was used. This assay directly measures NADPH consumption in the enzyme coupled reactions. The measured decrease in optical density at 340 nm is directly proportional to the enzyme activity in the sample (Figure 11). The assay was started by adding 100 µl of an assay buffer, 50 µl of co-substrate mixture (comprised NADPH, GSH and GR) and 20 µl of samples to the wells. The reaction was initiated by an

adding of 20 µl of cumene hydroperoxide to all wells. The plate was carefully shaked for a few seconds to mix and the absorbance was read once every minute at 340 nm using a microplate reader to obtain the absorbance at least 5 time points. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00373 μM^{-1}. One unit is defined as the amount of enzyme that will cause the oxidation of 1 nmoL of NADPH to $NADP^{+}$ per minute at at 25 °C. Activity of GPx of the sample was calculated from the following equation.

$$\text{GPx activity (nmoL/min/mL)} = \frac{\Delta 340/\text{min}}{0.00373\ \mu M^{-1}} \times \frac{0.19\ \text{mL}}{0.02\ \text{mL}} \times \text{sample dilution}$$
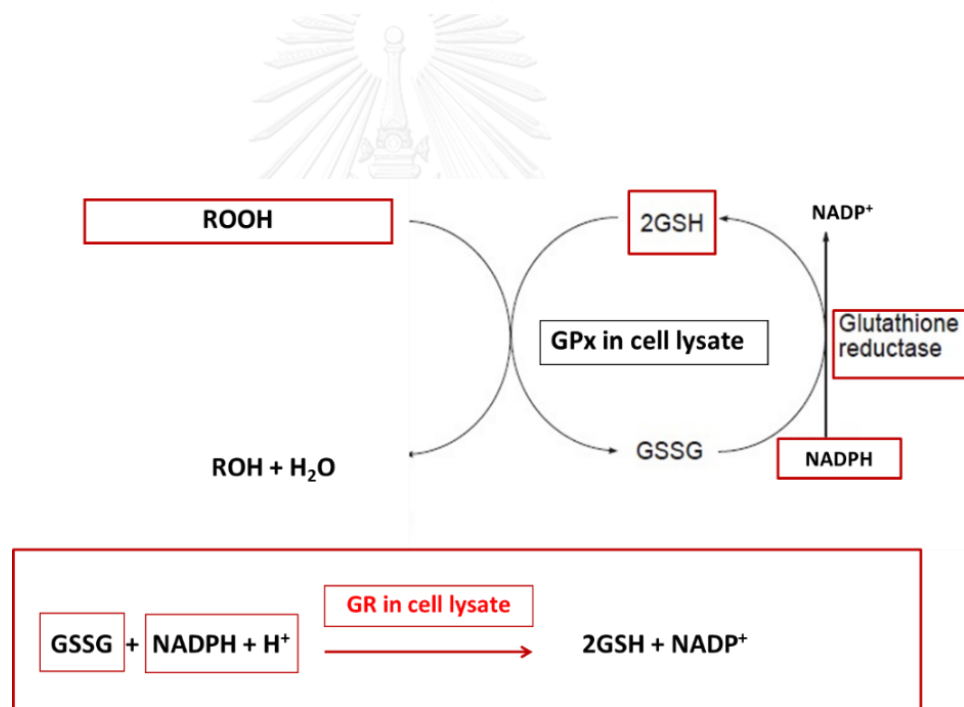

Figure 11. Principle of GPx and GR assay [67]

Determination of glutathione reductase (GR) activity

GR catalyzes the NADPH-dependent reduction of GSSG to GSH. This enzyme is essential for the GSH redox cycle which maintains adequate levels of cellular reduced GSH. A high GSH/GSSG ratio is a good indicator of oxidative stress status and DNA damage thus cell injury. The assay measures GR activity by measuring the rate of NADPH oxidation. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Since GR is present at rate limiting concentrations, the rate of decrease in the absorbance is directly proportional to the GR activity in the cell lysate (Figure 11). The effect of PN on GR activity was determined using commercially available kit. The assay was started by adding 100 µl of an assay buffer, 20 µl of GSSG and 20 µl of each samples to wells. The reaction was initiated by an adding of 50 µl of NADPH to all the wells. The plate was carefully shaked for a few seconds to mix and the absorbance was read once every minute at 340 nm using a microplate reader to obtain at least 5 time points. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of $0.00373 \ \mu M^{-1}$. One unit is defined as the amount of enzyme that will cause the oxidation of 1 nmoL of NADPH to $NADP^+$ per minute at 25 ° C. The activity of GR of sample was calculated from the following equation.

$$GR \ activity \ (nmoL/min/mL) = \frac{\Delta 340/min}{0.00373 \ \mu M^{-1}} \times \frac{0.19 \ mL}{0.02 \ mL} \times sample \ dilution$$

Determination of superoxide dismutase (SOD) activity

SOD catalyzes the dismutation of the superoxide anion to molecules of H_2O_2 and oxygen. To determine the effect of PN on SOD activity, superoxide dismutase assay kit was used. The assay uses tetrazolium that produces a water-soluble formazan dye upon reduction with superoxide anion. The rate of reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Therefore, the inhibitory activity of SOD can be determined by a colorimetric method (Figure 12). The assay was started by adding 200 µl of the diluted Radical detector (Tetrazolium salt which was diluted with assay buffer containing xanthine) and 10 µl of the sample into each well of 96 well plate and the reaction was initiated by adding 20 µl of diluted xanthine oxidase to all wells. The plate was carefully shaked for a few second to mix the solution. The plate was cover and incubated on a shaker for 20 minutes at room temperature. The absorbance was measured at 460 nm using a microplate reader. SOD activity in the cell lysate were calculated using equation from the linear regression of the standard curve substituting the linearized rate (LR) for each sample. One unit is define as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

SOD (U/mL) = [(sample LR- y-intercept) x 0.23 mL] x sample dilution

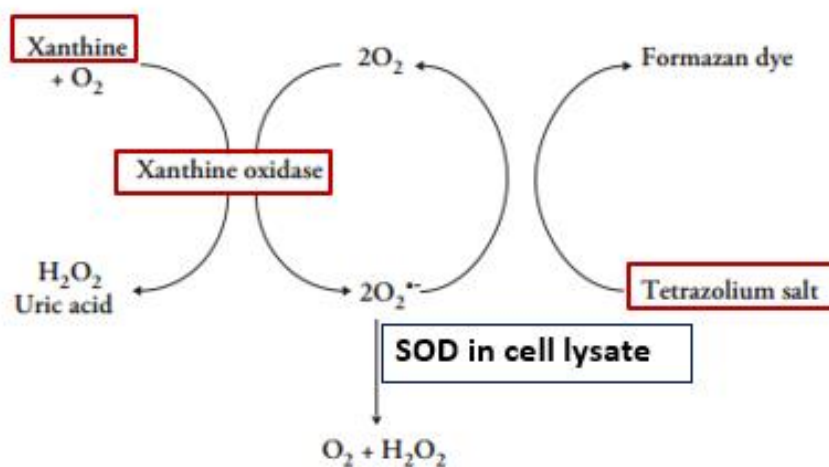Slope   0.01 mL

Figure 12. Principle of SOD assay [67]

1.7 Effect of PN extract and β-sitosterol on Nrf2 and HO-1 mRNA expression

HepG2 cells were seeded in 12-well plates, incubated overnight and treated with 0.1 mg/mL or 20 µM of β-sitosterol. Total RNA was then isolated from the cells after 0, 3, 6 and 12 h after exposure to the compounds using a RNAiso plus total RNA extraction reagent following the manufacturer's instructions. The RNA concentration was determined by measuring the absorbance at 260 nm with a Nanodrop 2000 C, and using by Beer's law with an assumed molar extinction coefficient of 40 for RNA. The cDNA was then generated with a random hexamer primer using a ReverTra Ace[®] qPCR RT kit. The expression levels of Nrf2 and HO-1 mRNA were measured by quantitative real-time RT-PCR (qRT-PCR) on Mx3000 real-time PCR system with Power SYBR green and primer pairs specific to each gene (Table 4). Each PCR reaction mixture, at a final volume of 10 µL, contained 1x Power SYBR green mastermix, 0.4 µM each of the forward and reverse primer (Table 4) and 1 ng cDNA template. PCR reactions were performed at 95 °C for 30 sec, 95 °C for 5 sec and 60 °C for 30 sec

total 40 cycles followed by of 95 °C for 60 sec, 55 °C and 95 °C for 30 sec. The

expression level of each target gene was normalized to the expression level of

glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a house-keeping gene. The

primers were designed using the Universal ProbeLibrary Assay Design Center

(https://lifescience.roche.com/webapp) and confirmed for likely target gene

specificity by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/)

searching of the NCBI GenBank database.

Table 4. The sequences of PCR primers used in the gene expression analysis

Gene name	Primer sequences (5' –3')	Accession number
NFE2L2 (Nrf2)	F: ACACGGTCCACAGCTCATC	NM_006164.3
	R: TGCCTCCAAAGTTCAATCA	
HO-1 (HMOX1)	F: GCATGCCCCAGGATTTGTCA	NM_002133.2
	R: GCTCTCCTTGTTGCGCTCAA	
GAPDH	F: CCAGCGCTGCTTTAACTC	NM_001256799.1
	R: GCTCCCCCCTGCAAATGA	

ᵃ Location is the position of the gene (5'–3') on the indicated GenBank (accession

code) sequence for NFE2L2 (NM_006164.3), HO-1 (NM_002133.2) and GAPDH

(NM_001256799.1)

1.8 Effect of PN extract and β-sitosterol on Nrf2 and HO-1 protein expression

SDS-PAGE Western blot analysis was performed to assess effects of PN extract and β-sitosterol on the expression of Nrf2 and HO-1 proteins. HepG2 cells were seeded into petri-dishes (1×10^6 cells each) and incubated for 24 h. Then, the cells were treated with different concentrations of PN (0, 0.01 and 0.1 mg/mL) or β-sitosterol (0, 10 and 20 µM) for 3 h prior to incubate with 500 µM of H_2O_2 or culture media without H_2O_2 for 24 h. To assess the time-dependent effect of PN extract or β-sitosterol on Nrf2 and HO-1, the cells were treated with 0.1 mg/mL of PN or 20 µM of β-sitosterol for 3, 6 and 12 h in the other separated experiments. After the indicated incubation times, cells were further incubated with culture medium for 24 h prior to harvesting. At the end of treatment, the cells were harvested using a rubber policeman and washed twice with ice cold PBS. Cytosolic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's protocol. Protein concentrations of the cytosolic and nuclear fractions were assessed using a Pierce® BCA protein assay kit. The fractions (20 µg protein/lane) were separated by 7.5% SDS-PAGE. The proteins were transferred onto PVDF membrane using a semi-dry transfer system, blocked overnight with 5% skimmed milk in PBS-T, and then probed with specific antibodies including rabbit polyclonal anti-Nrf2 and anti-HO-1. The membrane was then incubated with HRP-conjugated secondary antibodies. In each experiment, GAPDH house-keeping protein was also detecting for a loading control. The band intensity was measured with an Amersham imager 600 (GE Healthcare, Freiburg, Germany).

2. Inhibition and induction effect of PN extract

2.1 Inhibition of CYP enzyme activities by PN extract

Inhibition of CYP enzyme activities by PN extract was investigated using pooled human liver microsomes from 50 donors, as described previously [72]. Phenacetin, *S*-warfarin, *S*-mephenytoin, bufuralol, aniline and midazolam were used as probe substrates of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, respectively. The incubation mixture comprised potassium phosphate buffer pH 7.4 (100 mM), β-NADPH (1 mM), human liver microsomes, and the particular substrate. Incubations were carried out in the presence of PN extract at various concentrations over an appropriate time. Metabolites were extracted and quantified by HPLC (Table 5) for comparison between reactions with and without PN extract. Procedures were verified by determining IC_{50} values of known inhibitors of each CYP isoform: furafylline, sulfaphenazole, ticlopidine, quinidine, diethyldithiocarbamate and ketoconazole for CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, respectively. All experiments were performed in 3-independent experiments.

2.2 Kinetics of enzyme inhibition by PN extract

To identify mechanisms of inhibition and the apparent inhibitor constant (*Ki*) of PN, enzyme kinetic reactions were performed by varying the concentrations of PN and each substrate. The type of inhibition was estimated from Lineweaver-Burk plots and Ki was determined by a secondary plot of the slopes of the Lineweaver-Burk plots versus the concentrations of PN. All experiments were performed in 3-independent experiments.

Table 5. Experimental conditions for investigating CYP activities

CYP isoform	Selective oxidative reactions, Selective substrate concentration	Microsomal concentration, incubation time, Terminating solvent	HPLC conditions
1A2	Phenacetin-*O*-deethylation, 25 µM	0.4 mg/ml, 20 min, Ice cold acetonitrile	HPLC model L-2400 UV detector; 245 nm (Hitachi, Tokyo, Japan), Mightysil 18 RP-column (250 x 4.6 mm; Kanto chemical, Tokyo, Japan) Mobile phase: 6% Acetonitrile, 50 mM KH_2PO_4
2C9	*S*-Warfarin-7-hydroxylation, 5 µM	0.5 mg/ml, 30 min, 60% Ice cold perchloric acid	HPLC Model L-2400 FL detector; Excitation 320 nm, Emission 415 nm (Hitachi, Toyko, Japan) Mightysil 18 RP-column (250 x 4.6 mm; Kanto chemical, Tokyo, Japan) Mobile phase: 40% Acetonitrile, 0.04% H_3PO_4
2C19	S-Mephenytoin 4-hydroxylation, 200 µM	0.4 mg/ml, 60 min, Dichloromethane	HPLC model L-2400 UV detector; 204 nm (Hitachi, Tokyo, Japan), Capcell Pak C18 UG. 120 (250 x 4.6 mm; Shiseido, Tokyo, Japan) Mobile phase: 20% Acetonitrile, 50 mM Sodium phosphate buffer (pH 4.0)
2D6	Bufuralol-1-hydroxylation, 1 µM	0.2 mg/ml, 5 min, 60% Ice cold perchloric acid	HPLC Model L-2480 detector; FL Exitation 252 nm, Emission 302 nm (Hitachi, Toyko, Japan) Capcell-Pak C18 UG. 120 (250 x 4.6 mm; Shiseido, Tokyo, Japan) Mobile phase: 18% Acetonitrile, 1 mM $HClO_4$
2E1	Aniline-4-hydroxylation, 50 µM	0.5 mg/ml, 15 min, 10% Trichloro acetic acid	Microplate reader model POWERSCAN 4 microplate reader (DS Pharma Biomedical, Osaka, Japan); absorbance 640 nm
3A4	Midazolam-1-hydroxylation, 5 µM	0.1 mg/ml, 15 min, Ice cold MeOH	HPLC Model L-2400 UV detector; 220 nm (Hitachi, Toyko, Japan), Capcell-Pak C18 UG. 120 (250 x 4.6 mm; Shiseido, Tokyo, Japan), Mobile phase: 30% Methanol/20% Acetonitrile/10 mM KH_2PO_4/10 mM K_2HPO_4

2.3 Effect of PN extract on mechanism based inhibition of CYPs

Time dependent inhibition (TDI) effect of PN on human CYP1A2, CYP2C9, CYP2D6 and CYP3A4 were further investigated as previous described [73]. The pre-incubation mixture comprised potassium phosphate buffer pH 7.4 (100 mM), human liver microsomes (2-5 mg/mL), β-NADPH (1mM) and various concentration of PN set around the IC_{50} of each CYP isoform. The 10 µL of the incubated mixture were withdrawn after 30 min preincubation and were diluted 10 fold with a pre-incubated at 37 °C containing saturated concentration of each substrate, potassium phosphate buffer pH 7.4 and β-NADPH. The second reaction was terminated with organic solvents after the indicated incubation time as shown in Table 5, metabolites were extracted and quantified by HPLC (Table 5) for comparison between reactions with and without PN extract. The IC_{50} value shift of the ratio of IC_{50} values between without and with β-NADPH of a 30 min pre-incubation of PN was calculated. All experiments were performed in 3-independent experiments.

2.4 Cytotoxicity of PN extract in primary human hepatocytes

Cytotoxicity of PN in cryopreserved primary human hepatocytes was determined by MTT assay. The hepatocytes were seeded in a 96-well collagen coated plate (1 x 10^4 cells/well) and incubated overnight. The cells were then treated with several concentrations of PN extract (0, 0.0001, 0.001, 0.01, 0.1 and 1 mg/mL) for 48 h. At the end of incubation, a 25-µl of 5 mg/ml of MTT reagent in culture medium was added to each well (equal to 1mg/ml of MTT in a total volume of medium in each well of 125 µl) and the cells were incubated for 3 h at 37°C. The resulting formazan was dissolved in 100 µl of DMSO. Optical density was measured at 570 nm using a SpectraMax M5 microplate reader. All experiments were performed in 3-independent experiments.

2.5 Induction of CYP enzyme activities by PN extract

Cryopreserved primary human hepatocytes were seeded on a 96-well collagen coated plate (3 x 10^4 cells/well). After 24 h, the medium was replaced with various concentrations of PN and the cells were incubated at 37°C for 48 h. Induction of CYP activity was detected using a P450-Glo™ CYP assay according to the manufacturer's instruction. The assay consists of two components: a luminogenic CYP substrate (Luciferin-1A2, Luciferin-H, Luciferin-HEGE and Luciferin-IPA for CYP1A2, CYP2C9, CYP2C19 and CYP3A4, respectively) and Luciferin Detection Reagent.

First, the culture medium was replaced with medium containing the specific Luciferin substrate. After an appropriate time (Table 6), the culture medium of 25 µL was transferred from each well to a 96-well opaque white luminometer plate at room temperature, and 25 µl of Luciferin Detection Reagent was added to initiate a luminescent reaction. The plate was incubated at room temperature for 20 minutes, thereafter the luminescence was measured using a luminometer SpectraMax M5 microplate reader. The net signals were calculated by subtracting luminescence background values from the non-treated control. The fold inductions of CYPs activity were calculated compared with the non-treated control and the treated groups. The procedure was verified by determining the fold induction of known inducers of each CYP isoform: 100 µM omeprazole for CYP1A2, 10 µM rifampicin for CYP2C9 and CYP2C19, and 50 µM rifampicin for CYP3A4. All experiments were performed in 3-independent experiments.

Table 6. Assay conditions for CYPs induction

CYP	Substrate	Final substrate concentration (µM)	Medium volume required for 96 well plate (µL)	Incubation time (min)
1A2	Luciferin-1A2	6	50	60
2C9	Luciferin-H	100	50	180
2C19	Luciferin-HEGE	100	50	180
3A4	Luciferin-IPA	3	50	60

2.6 Effect of PN extract on CYP protein expression

SDS-PAGE Western blot analysis was performed to assess effects of PN on expression of CYP1A2, CYP2C9, CYP2C19 and CYP3A4. Primary human hepatocytes were seeded on a 6-well collagen coated plate (5.5×10^5 cells/plate) and incubated overnight. Then, cells were treated with different concentrations of PN (0, 0.001, 0.01 and 0.1 mg/mL) or positive control compounds (100 µM omeprazole for CYP1A2; 10 µM rifampicin for CYP2C9 and CYP2C19; 50 µM rifampicin for CYP3A4). After 48 h, the cells were harvested and ultrasonically homogenized at 4°C in 200 µl RIPA buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 10 mM EDTA, 0.1% SDS, 1% Tergitol® type NP-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM NaF, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors, pH 7.4. Protein concentrations were determined by BCA protein assay. Cell lysates (20 µg protein/lane) were separated by 7.5% SDS-PAGE. Proteins were transferred to a PVDF membrane using a semi-dry transfer system, blocked with 5% skimmed milk in PBS-T, and then probed with specific antibodies: mouse polyclonal anti-CYP1A2, and rabbit

polyclonal anti-CYP2C9, anti-CYP2C19 and anti-CYP3A4 antibodies. The membrane was then incubated with peroxidase-conjugated secondary antibodies and was also blotted with anti-GAPDH antibody (Santa Cruz Biotechnology) as a loading control. The band intensity was measured with a Calibrated Imaging Densitometer.

Statistical analysis

IC_{50} values were calculated using probit analysis. To determine the type of inhibition, Lineweaver-Burk plot and secondary plots were used. All results are presented as mean ± standard deviation (S.D.) of three independent experiments. Differences among means were analyzed using one way analysis of variance (ANOVA) followed by a Tukey test for multiple comparisons, with $p < 0.05$ considered as significant difference.
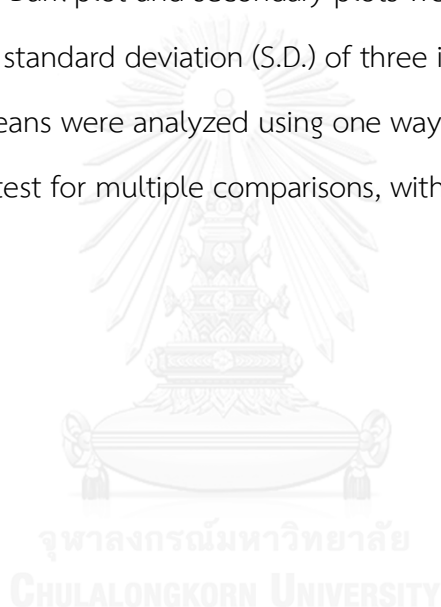
CHAPTER IV
RESULTS

1 Hepatoprotective effect of PN extract

1.1 DPPH radical scavenging activity of PN and some active constituents in the PN extract

In the DPPH radical scavenging assay, PN and some active constituents (ferulic acid, gallic acid, imperatorin, rutin, vanillic acid and β-sitosterol) were evaluated for their free radical scavenging activity using ascorbic acid as the standard control. The IC_{50} of PN extract, the compounds as well as ascorbic acid are summarized in table 7. The scavenging effect increased with the increasing concentrations of the test compounds. The IC_{50} value for PN extract and the compounds were 57, 8.48, 0.38 and 12.40 µg/mL for PN, ferulic acid, gallic acid and rutin, respectively which were comparatively lower than that of ascorbic acid except for PN. From the results of DPPH, it showed that most of the compounds are effective as antioxidant compared to ascorbic acid. Due to the limitation of the solubility of imperatorin, vanillic acid and β-sitosterol, this study could not calculate the IC_{50} of these compounds. Thus, the antioxidant effect of these compounds could not appropriately be investigated via this test model. Antioxidant effects of these compounds such as β-sitosterol via modulation of cellular antioxidants (GSH) and antioxidant enzymes were performed in the subsequent study.

Table 7. DPPH free radical scavenging activity of PN and some active constituents

Test compound	MW	IC_{50} (µM)	IC_{50} (µg/mL)
Ascorbic acid (positive control)	176.12	246.99	43.50
Ferulic acid	194.18	43.69	8.48
Gallic acid	170.12	2.25	0.38
Imperatorin	270.28	>100	> 27.08
Rutin	610.52	20.31	12.40
Vanillic acid	168.14	>100	>16.81
β-sitosterol	414.70	>100	>41.47
PN	-	-	57.00

1.2 Cytotoxicity studies

Before performing *in vitro* hepatoprotective studies, cytotoxic effect of PN, β-sitosterol, H_2O_2 and NAC were determined using MTT assay. The results showed that percent cell viability was decreased in concentration dependent manner with the % cell viability of 100 ± 6.36, 103.32 ± 8.89, 94.79 ± 8.83, 93.40 ± 5.00, 84.63 ± 5.82 and 49.69 ±13.04 % at the PN concentrations of 0.0001, 0.001, 0.01, 0.1 and 1 mg/mL respectively significant decrease of percent cell viability was shown following treatment with PN extract at the concentration of 0.1 and 1 mg/mL (Figure 13). An IC_{50} of percent cell viability following treatment with PN extract in HepG2 cells was 0.93 mg/mL. Treatment of HepG2 cells with H_2O_2 resulted in a concentration dependent decrease of cell viability. Survival of HepG2 cells after 24 h exposure to H_2O_2 at 0, 5, 10, 50, 100, 200, 500 and 1,000 µM were 100 ± 2.62, 77.46 ± 0.70, 73.75 ± 1.80, 69.44 ± 3.10, 63.74 ± 1.96, 58.05 ± 2.08, 49.28 ± 3.20 and 40.37 ± 1.64%, respectively (Figure 14) with an IC_{50} of 789.80 µM. Treatment of NAC resulted in a slight decrease of cell viability of HepG2 cells. Survival of HepG2 cells after 24 h

exposure to NAC at 0, 0.1, 0.2, 0.5, 1, 5 and 10 mM were 100 ± 0.89, 101.90 ± 3.64, 98.68 ± 1.16, 98.46 ± 2.01, 92.23 ± 1.17, 90.84 ± 0.68 and 76.55 ± 1.58 %, respectively (Figure 15). Significant decrease of cell viability was shown at NAC concentrations of 1, 5 and 10 mM. HepG2 cells incubating with β-sitosterol for 24 h resulted in a significant decrease of cell viability at 0.50 - 200 μM and the decrease of cell viability was 100 ± 5.28, 94.17 ± 2.67, 93.97 ± 5.68, 90.49 ± 3.87, 82.44 ± 5.19, 75.16 ± 2.09, 62.04 ± 2.71, 57.16 ± 1.60 and 41.95 ± 2.46% for 0, 0.05, 0.5, 1, 10, 20, 50, 100 and 200 μM of β-sitosterol, respectively (Figure 16) with an IC_{50} of 136.7 μM.
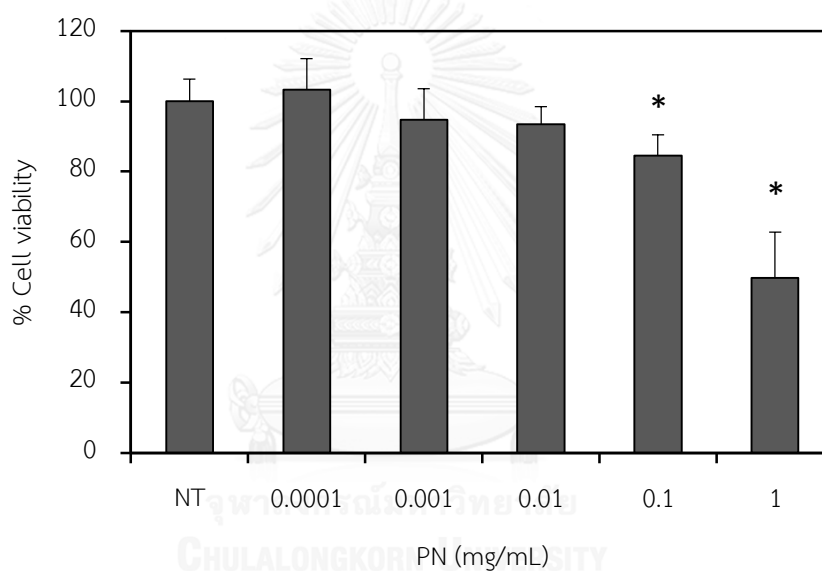
Figure 13. Viability of HepG2 cells after treatment with different concentrations of PN extract. HepG2 cells were treated for 24 h with the noted concentrations of the PN. Cell viability was determined by MTT assay. Values are shown as means ± S.D. of 3 independent experiments, * $p < 0.05$; PN vs non-treated control.
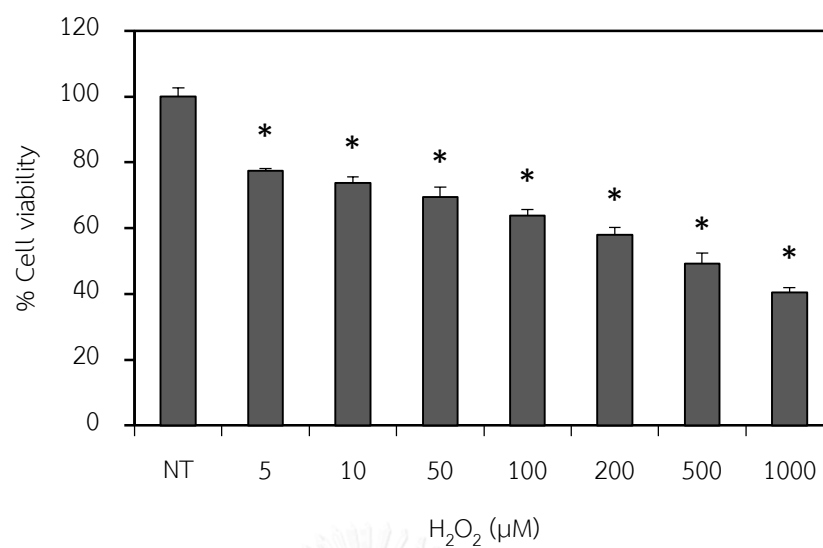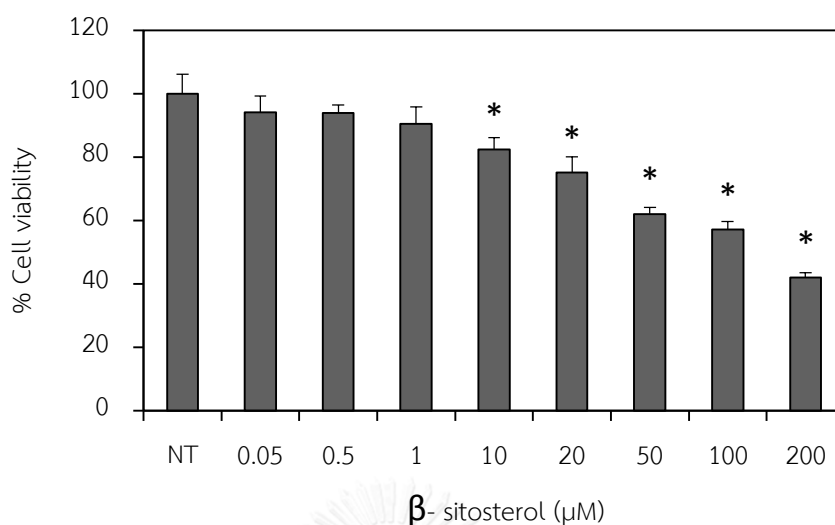
Figure 14. Viability of HepG2 cells after treatment with different concentrations of H_2O_2.
HepG2 cells were treated for 24 h with the noted concentrations of the H_2O_2.
Cell viability was determined by MTT assay. Values are shown as means ± S.D. of
3 independent experiments, * $p < 0.05$; H_2O_2 vs non-treated control.
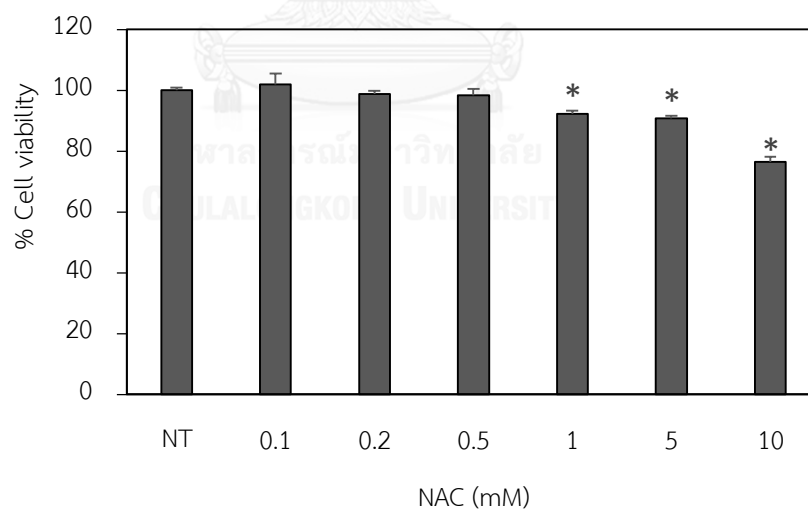
Figure 15. Viability of HepG2 cells after treatment with different concentrations of NAC.
HepG2 cells were treated for 24 h with the noted concentrations of the NAC. Cell
viability was determined by MTT assay. Values are shown as means ± S.D. of 3
independent experiments, * $p < 0.05$; NAC vs non-treated control.

Figure 16. Viability of HepG2 cells after treatment with different concentrations of β-sitosterol. HepG2 cells were treated for 24 h with the noted concentrations of the β-sitosterol. Cell viability was determined by MTT assay. Values are shown as means ± S.D. of 3 independent experiments, * $p < 0.05$; β-sitosterol vs non-treated control.

1.3 Generation of intracellular ROS by PN extract and inhibition of H_2O_2-induced intracellular ROS by NAC, PN extract and β-sitosterol

Compared to the non-treated control cells, PN exposure did not generated intracellular ROS at 0.0001, 0.001, 0.01 and 0.1 mg/mL. At the 1 mg/mL concentration, PN significantly caused intracellular ROS formation for 2.18 folds while H_2O_2 at 500 µM caused 6.26 folds induction of ROS formation as compared to the non-treated (Figure 17). Pre-treatment with NAC caused a decrease of H_2O_2-induced intracellular ROS as compared to H_2O_2 treated group. Different exposure times (1, 2, 6 h) of NAC showed the comparable ROS scavenging effects (Figure 18). Pre-treatment cells with PN (at 0.0001, 0.001, 0.01 and 0.1 mg/mL) for 3, 6 and 12 h also attenuated intracellular ROS induction by H_2O_2 as compare to H_2O_2 treated group.

The scavenging effect of PN was concentration dependent. However, the exposure times (3, 6, 12 h) to PN pre-treatment demonstrated comparable effect (Figure 19). In the same manner, pre-treatment with β-sitosterol for 3, 6 and 12 h attenuated intracellular ROS induction by H_2O_2 (Figure 20).
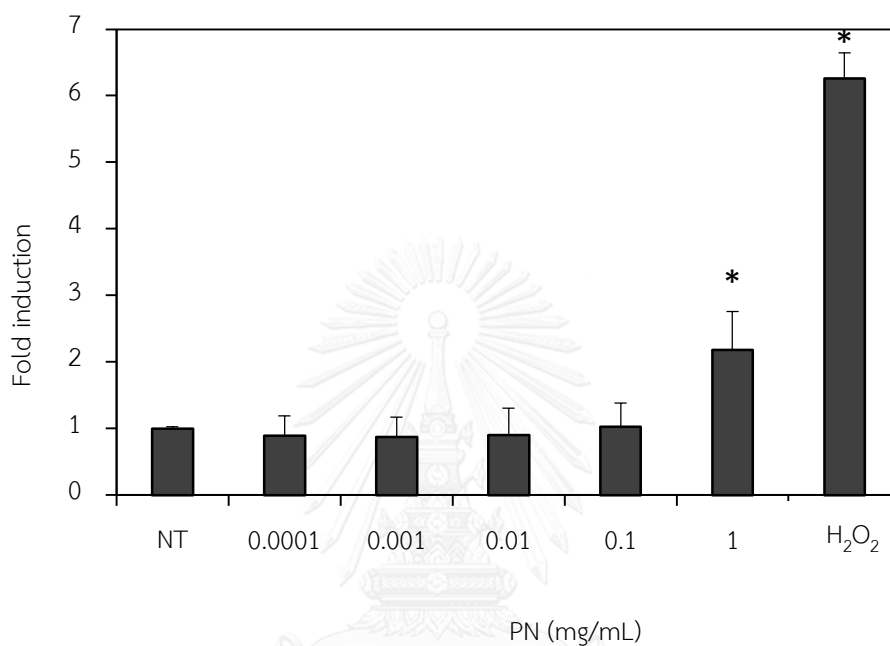

Figure 17. Generation of intracellular ROS by PN extract. HepG2 cells were treated for 3 h with the noted concentrations of the PN. Intracellular ROS were measured using DCFH-DA assay. H_2O_2 at 500 µM was used as a positive control. Values are shown as means ± S.D. of 3 independent experiments, * $p<0.05$; PN or H_2O_2 vs non-treated control.
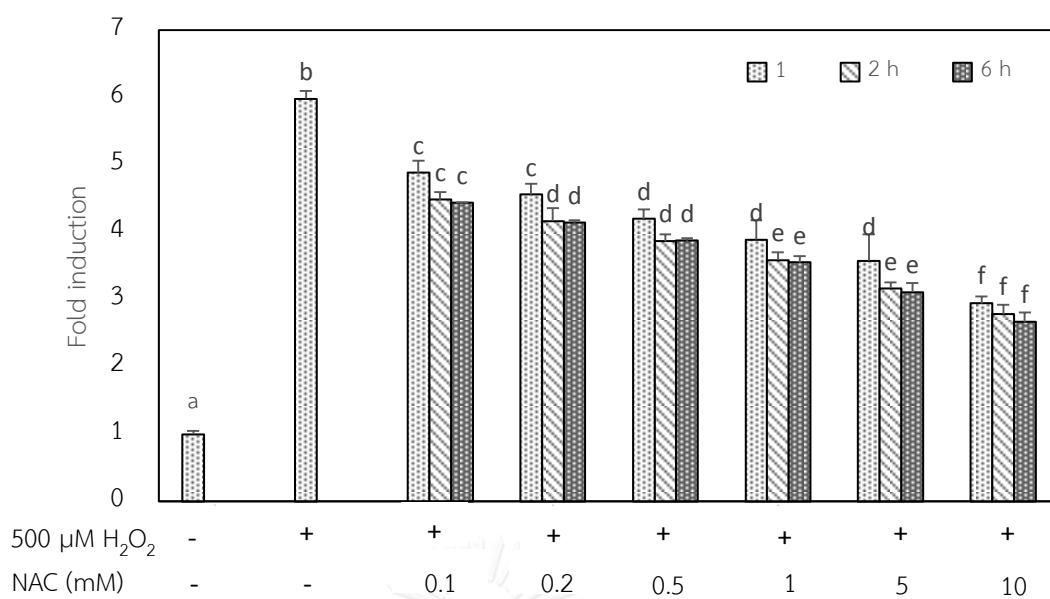
Figure 18. Effects of NAC on H$_2$O$_2$-induced intracellular ROS formation. HepG2 cells were

treated with various concentrations and exposure times of NAC, before

treatment with 500 μM of H$_2$O$_2$. Intracellular ROS levels were measured by the

DCFH-DA assay. Values are shown as means ± S.D. of 3 independent experiments.

Different letters (a, b, c, d, e, f) above bars indicate significant difference (by
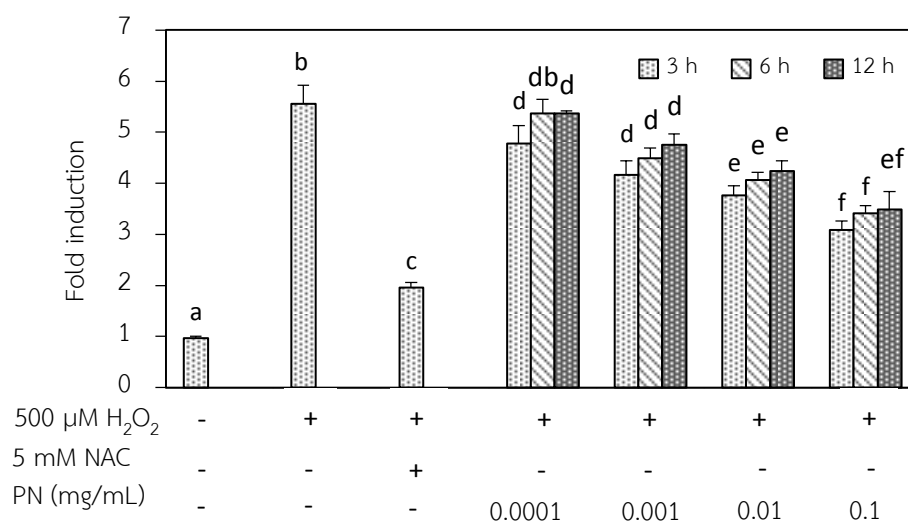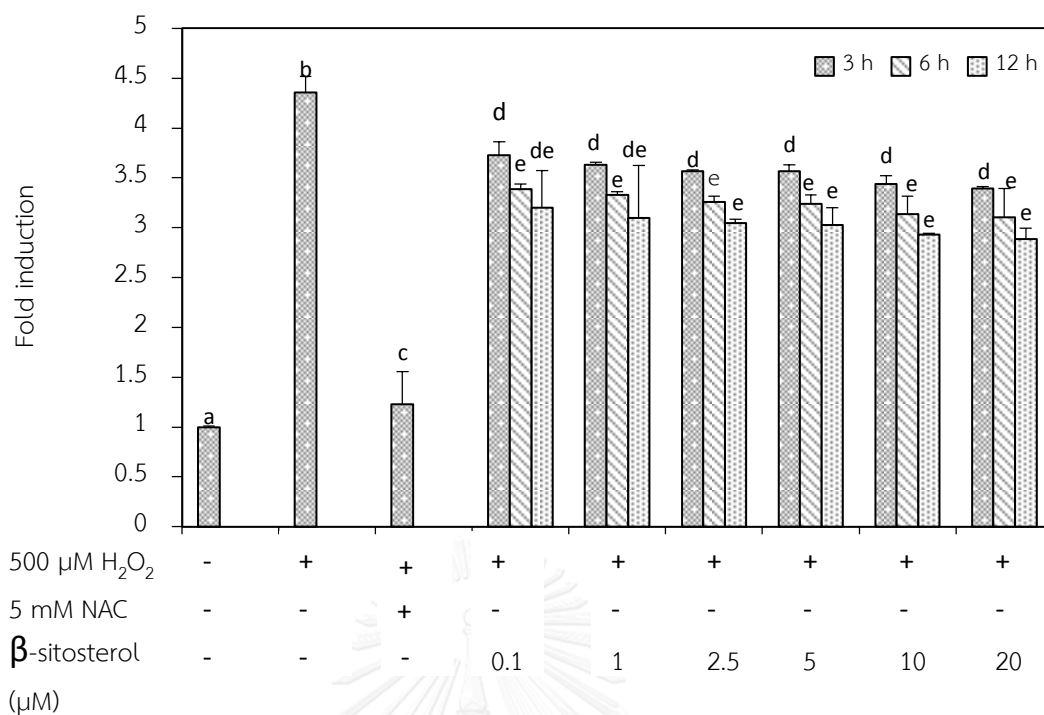
ANOVA, $p < 0.05$).

Figure 19. Effects of PN extract on H_2O_2-induced intracellular ROS formation. HepG2 cells were treated with various concentrations and exposure time of PN or 5 mM of NAC for 1 h, before treatment with 500 μM of H_2O_2. Intracellular ROS levels were measured by the DCFH-DA assay. Values are shown as means ± S.D. of 3 independent experiments. Different letters (a, b, c, d, e, f) above bars indicate significant difference (by ANOVA, $p < 0.05$).

Figure 20. Effects of β-sitosterol on H_2O_2-induced intracellular ROS formation. HepG2 cells were treated with various concentrations and exposure times of β-sitosterol or 5 mM of NAC for 1 h, before treatment with 500 μM of H_2O_2. Intracellular ROS levels were measured by the DCFH-DA assay. Values are shown as means ± S.D. of 3 independent experiments. Different letters (a, b, c, d, e) above bars indicate significant difference (by ANOVA, $p < 0.05$).

1.4 Protective effects of PN extract and β-sitosterol against H_2O_2-induced cytotoxicity

The results showed that HepG2 cells pre-treated with various concentrations of PN for 3, 6 or 12 h followed by exposure to 500 µM of H_2O_2 for 24 h showed significant attenuation of cell viability in a concentration dependent manner. Exposure time of PN pre-treatment did not significantly influence the effect (Figure 21). Pre-treatment with β-sitosterol also significant attenuated cell viability but the effects were not concentration and time dependent (Figure 22).
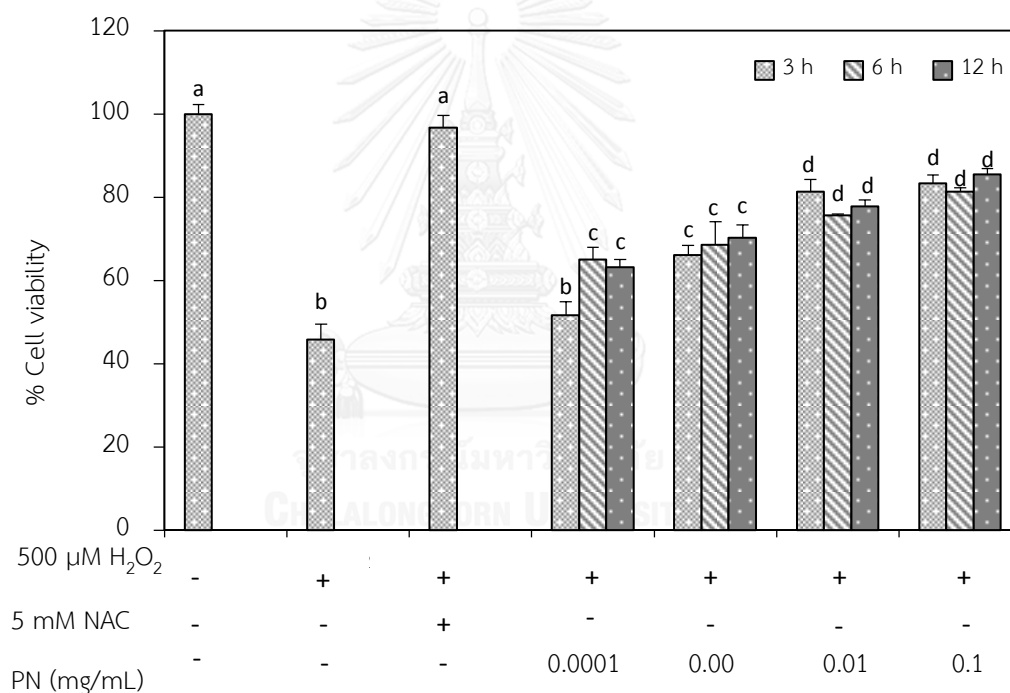
Figure 21. Protective effects of PN extract against H_2O_2-induced cytotoxicity. HepG2 cells were treated with different concentrations of PN (0–0.1 mg/mL) for 3, 6 and 12 h or 5 mM of NAC for 1 h before exposure to H_2O_2 (500 µM) for 24 h. Cell viability was determined by MTT assay. Values are shown as means ± S.D. of 3 independent experiments. Different letters (a, b, c, d) above bars indicate significant difference (by ANOVA), 5 mM NAC was used as a positive control.
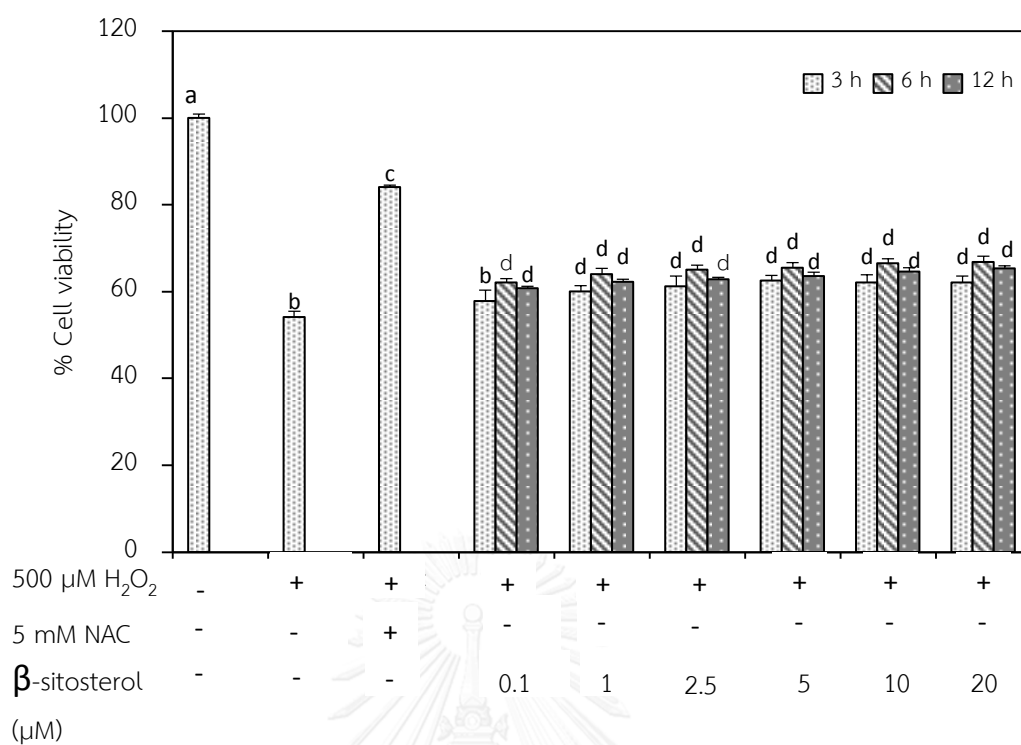
Figure 22. Protective effects of β-sitosterol against H_2O_2-induced cytotoxicity. HepG2 cells were treated with different concentrations of β-sitosterol (0–20 μM) for 3, 6 and 12 h or 5 mM of NAC for 1 h before exposure to H_2O_2 (500 μM) for 24 h. Cell viability was determined by MTT assay. Values are shown as means ± S.D. of 3 independent experiments. Different letters (a, b, c, d) above bars indicate significant difference (by ANOVA), 5 mM NAC was used as a positive control.

1.5 Effect of PN extract and β-sitosterol on GSH levels and the activities of antioxidant enzymes

Effects of PN extract and β-sitosterol on the cellular antioxidant enzyme system, including CAT, GPx, GR and SOD as well as GSH were investigated.

1.5.1 Effect of PN extract and β-sitosterol on GSH levels

The result showed that treatment HepG2 cells with PN (0.01 and 0.1 mg/mL) or β-sitosterol (10 and 20 μM) for 3, 6 and 12 h significantly increased the level of total GSH (Figure 23, 24) and GSH (Figure 25, 26) but did not affect the level of GSSG as compared to the non-treated group (Figure 27, 28). Therefore, both PN and β-sitosterol caused a significant increase of GSH/GSSG ratio (Figure 29, 30). Levels of total GSH, GSH, GSSG and GSH/GSSG ratio between the time period of treatment (3, 6 and 12 h) or between the concentrations of PN or β-sitosterol treatment were not consistently significant different. Likewise, further incubation the cells with the culture medium for 24 h before harvesting the cells, did not cause any significant differences of these parameter (Figure 23 - 30).
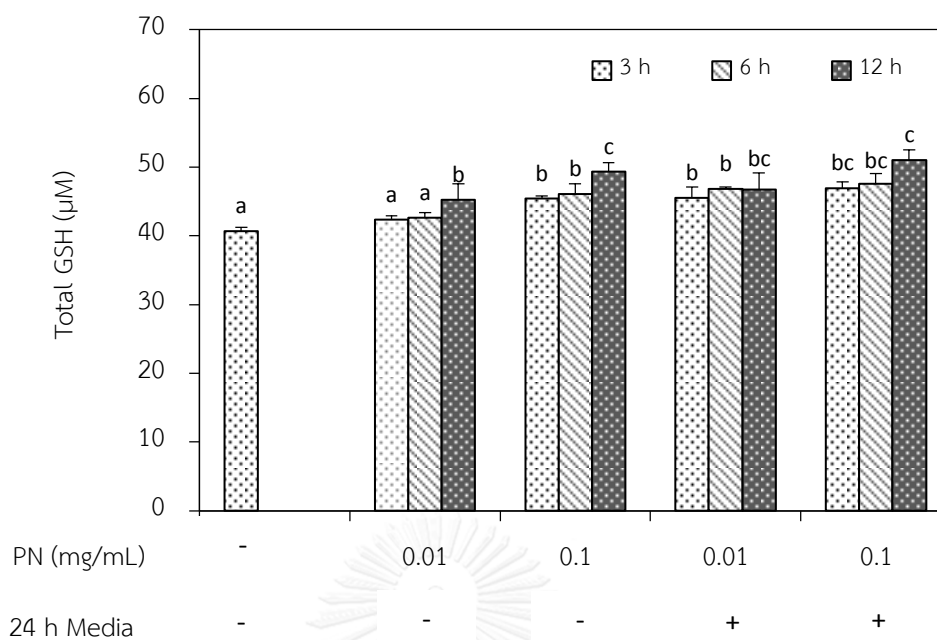
Figure 23. Effect of PN extract on total GSH level (µM). HepG2 cells were treated with different concentrations of PN (0, 0.01 and 0.1 mg/mL) and harvested at the end of treatment or subsequently incubated with culture medium for 24 h before evaluating GSH level. Values are shown as means ± S.D. of 3 independent experiments, different letters (a, b, c) above bars indicate significant difference (by ANOVA, $p < 0.05$).
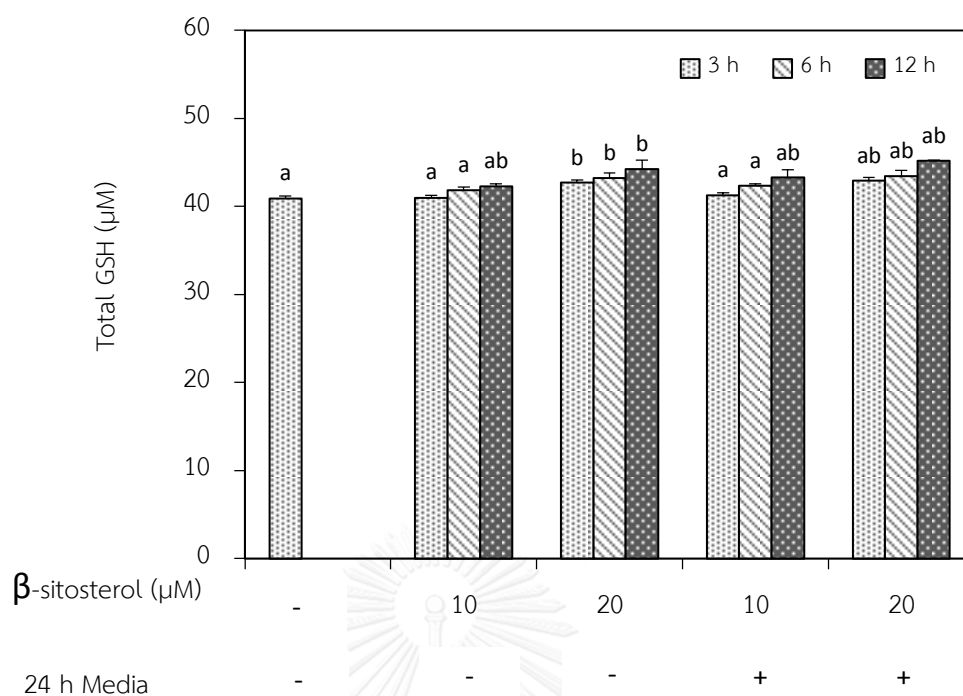
Figure 24. Effect of β-sitosterol on total GSH level (μM). HepG2 cells were treated with different concentrations of β-sitosterol (10 and 20 μM) and harvested at the end of treatment or subsequently incubated with culture medium for 24 h before evaluating total GSH level. Values are shown as means ± S.D. of 3 independent experiments, different letters (a, b) above bars indicate significant difference (by ANOVA, $p < 0.05$).
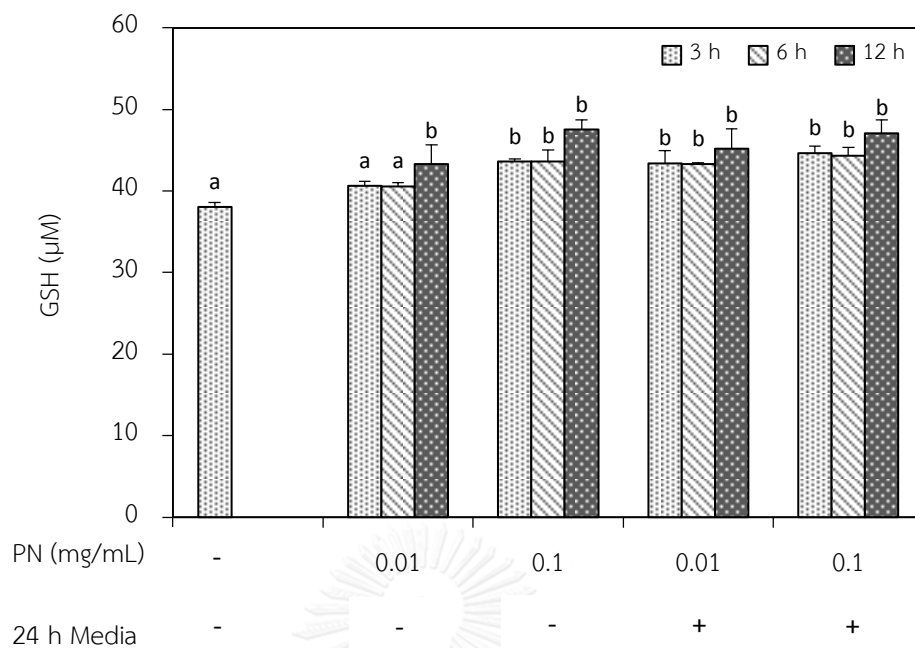
Figure 25. Effect of PN extract on GSH level (µM). HepG2 cells were treated with different concentrations of PN (0, 0.01 and 0.1 mg/mL) and harvested at the end of treatment or subsequently incubated with culture medium for 24 h before evaluating GSH level. Values are shown as means ± S.D. of 3 independent experiments, different letters (a, b) above bars indicate significant difference (by ANOVA, $p < 0.05$).
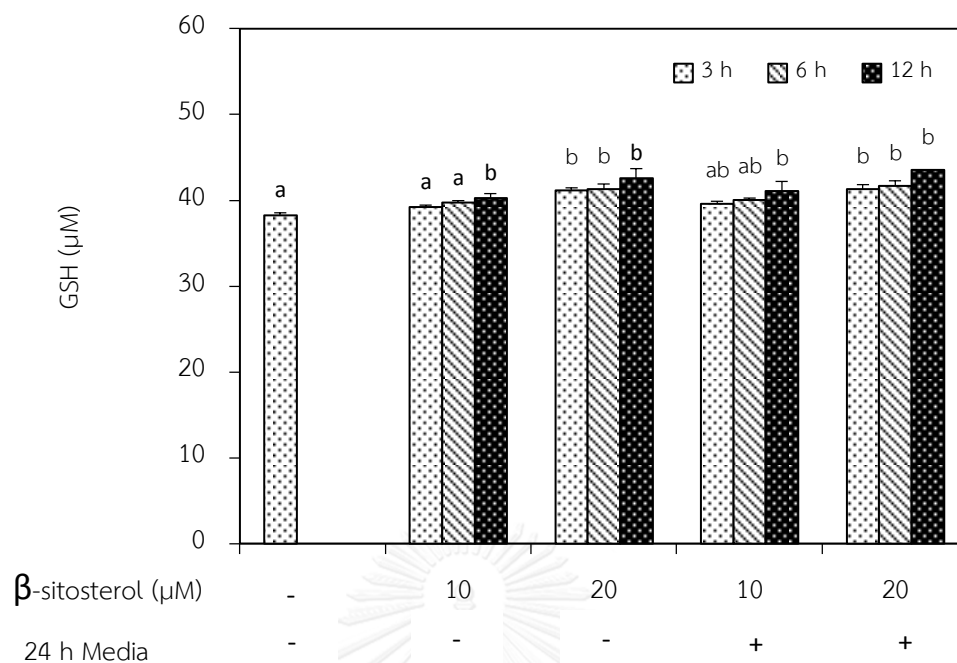
Figure 26. Effect of β-sitosterol on GSH level (μM). HepG2 cells were treated with
different concentrations of β-sitosterol (10 and 20 μM) and harvested at the
end of treatment or subsequently incubated with culture medium for 24 h
before evaluating GSH level. Values are shown as means ± S.D. of 3
independent experiments, different letters (a, b) above bars indicate significant
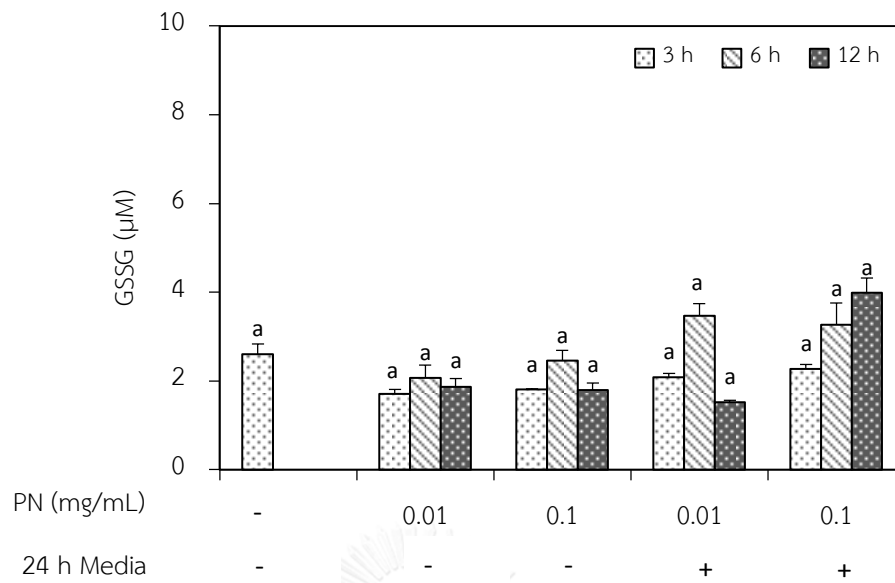difference (by ANOVA, $p < 0.05$).

Figure 27. Effect of PN extract on GSSG level (μM). HepG2 cells were treated with different concentrations of PN (0.01 or 0.1 mg/mL) and harvested at the end of treatment or subsequently incubated with culture medium for 24 h before evaluating GSSG level. Values are shown as means ± S.D. of 3 independent experiments, different letters above bars (a, b) indicate significant difference (by ANOVA, $p < 0.05$).
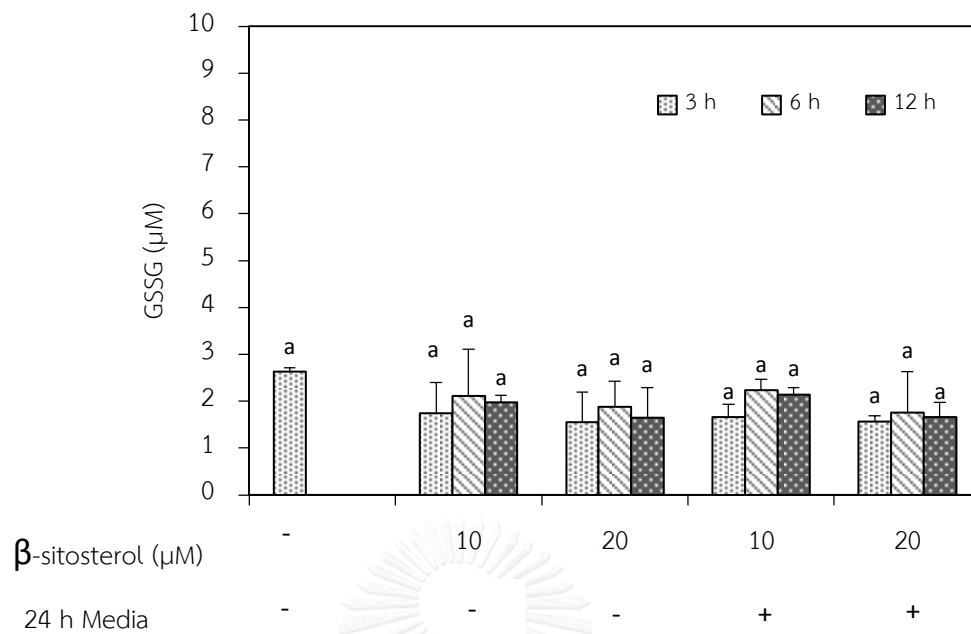
Figure 28. Effect of β-sitosterol on GSSG level (μM). HepG2 cells were treated with different

concentrations of β-sitosterol (10 and 20 μM) and harvested at the end of

treatment or subsequently incubated with culture medium for 24 h before

evaluating GSSG level. Values are shown as means ± S.D. of 3 independent

experiments, different letters above bars indicate significant difference (by
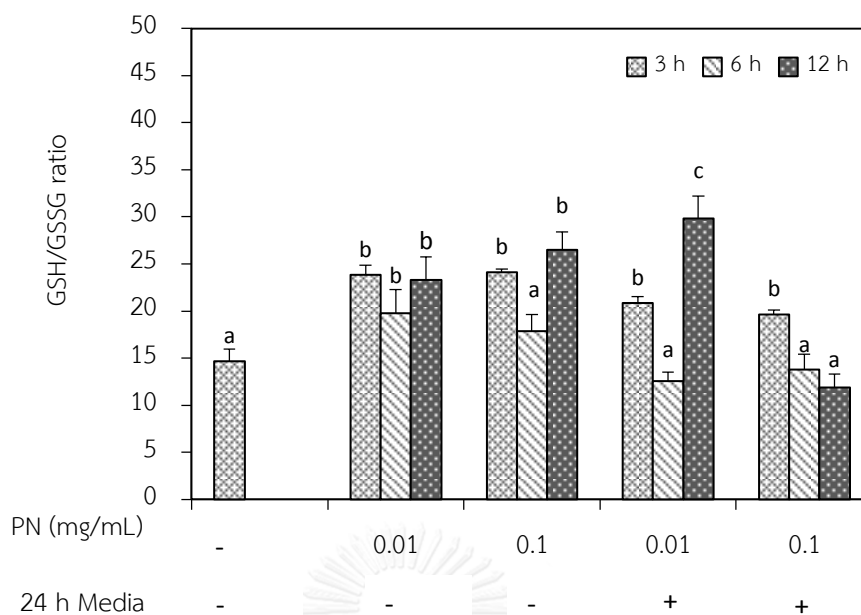
ANOVA, $p < 0.05$).

Figure 29. Effect of PN extract on GSH/GSSG ratio. HepG2 cells were treated with different

concentrations of PN (0.01 and 0.1 mg/mL) and harvested at the end of

treatment or subsequently incubated with culture medium for 24 h before

evaluating GSH and GSSG levels. GSH/GSSG ratio was calculated from the values

of GSH and GSSG level. Values are shown as means ± S.D. of 3 independent

experiments, different letters (a, b, c) above bars indicate significant difference
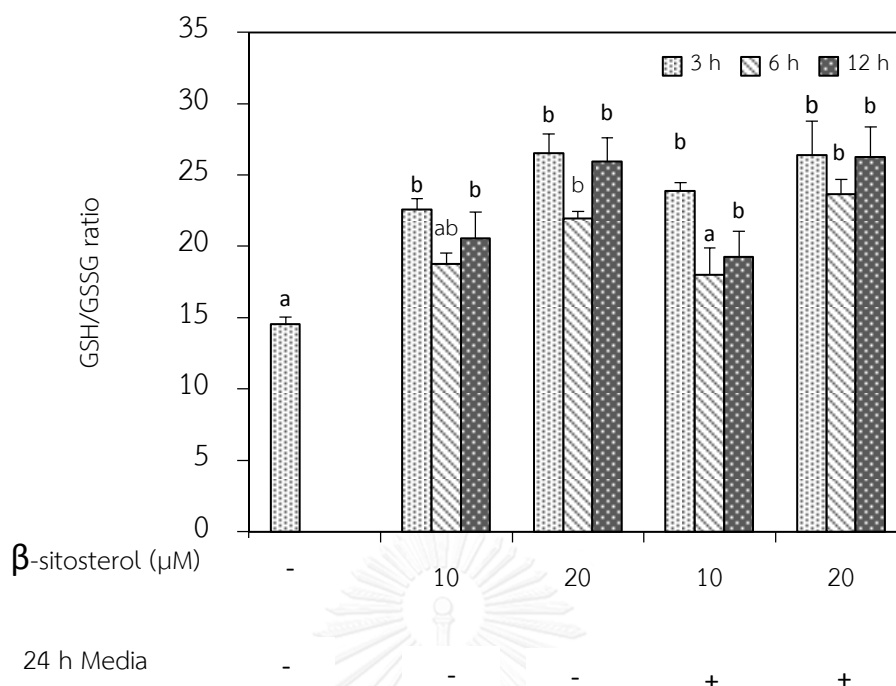
(by ANOVA, $p < 0.05$).

Figure30. Effect of β-sitosterol on GSH/GSSG ratio. HepG2 cells were treated with
different concentrations of β-sitosterol (10 and 20 μM) and harvested at the
end of treatment or subsequently incubated with culture medium for 24 h
before evaluating GSH and GSSG levels. GSH/GSSG ratio was calculated from
the values of GSH and GSSG level. Values are shown as means ± S.D. of 3
independent experiments, different letters (a, b) above bars indicate significant
difference (by ANOVA, $p < 0.05$).

1.5.2 Effects of PN extract and β-sitosterol on the activities of antioxidant enzymes (CAT, GPx, GR and SOD)

The results showed that CAT activity in HepG2 cells was significantly increased following treatment with 0.1 mg/mL of PN and the activity of this enzyme tended to be increased when the time of exposure to PN was increased (Figure 31). However, GPx, GR and SOD activities were significantly increased at the concentration of 0.01 and 0.1 mg/mL of PN and these effects tended to be shown in a concentration and time (except for SOD) dependent manner (Figure 33, 35, 37). In the same manner as PN, HepG2 cells incubating with β-sitosterol for 3 h showed a significant increased activities of enzymatic antioxidants CAT, GPx, GR and SOD as compared with non-treated control group. An increased effect was concentration dependent but no significant differences were observed between the different time period of treatment (3, 6 and 12 h) (Figure 32, 34, 36, 38). Patterns of enzyme activities in HepG2 cells (treated with PN or β-sitosterol) that were further incubated with culture medium for 24 h before harvesting and assessed the enzyme activities, were not consistently difference from the cells harvested at the noted incubation times (Figure 31-38).
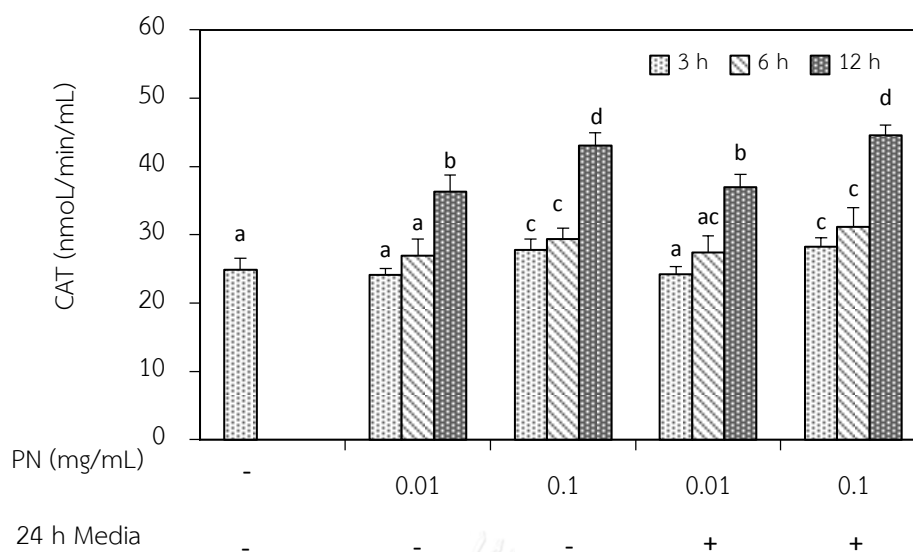
Figure 31. Antioxidant enzyme activity of CAT (nmoL/min/mL) was evaluated in HepG2

treated for 3, 6 and 12 h with PN. HepG2 cells were treated with 0.01 or 0.1

mg/mL of PN extract and harvested at the end of treatment or subsequently

incubated with culture medium for 24 h before evaluating CAT activity. Values

are shown as means ± S.D. of 3 independent experiments. Different letters (a, b,

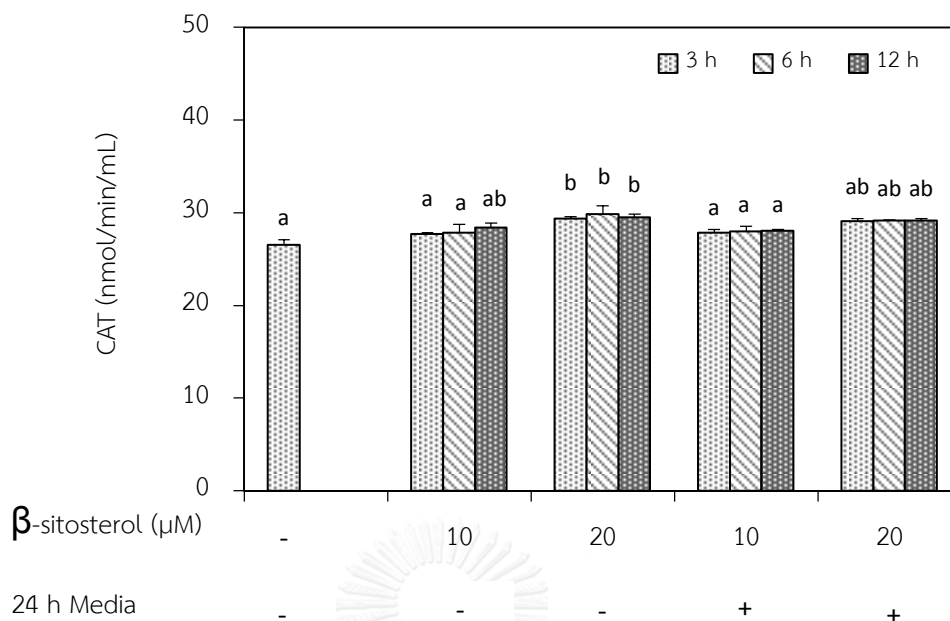c, d) above bars indicate significant difference (by ANOVA, $p < 0.05$).

Figure 32. Antioxidant enzyme activity of CAT (nmoL/min/mL) was evaluated in HepG2

treated for 3, 6 and 12 h with β-sitosterol. HepG2 cells were treated with 10 or

20 μM of β-sitosterol and harvested at the end of treatment or subsequently

incubated with culture medium for 24 h before evaluating CAT activity. Values

are shown as means ± S.D. of 3 independent experiments. Different letters (a, b)

above bars indicate significant difference (by ANOVA, $p < 0.05$).

Figure 33. Antioxidant enzyme activity of GPx (nmoL/min/mL) was evaluated in HepG2

treated for 3, 6 and 12 h with PN extract. HepG2 cells were treated with 0.01 or

0.1 mg/mL of PN and harvested at the end of treatment or subsequently

incubated with culture medium for 24 h before evaluating GPx activity. Values

are shown as means ± S.D. of 3 independent experiments. Different letters (a, b,

c, d, e) above bars indicate significant difference (by ANOVA, $p < 0.05$).

Figure 34. Antioxidant enzyme activity of GPx (nmoL/min/mL) was evaluated in HepG2

treated for 3, 6 and 12 h with β-sitosterol. HepG2 cells were treated with 10 or

20 μM of β-sitosterol and harvested at the end of treatment or subsequently

incubated with culture medium for 24 h before evaluating GPx activity. Values

are shown as means ± S.D. of 3 independent experiments. Different letters (a, b,

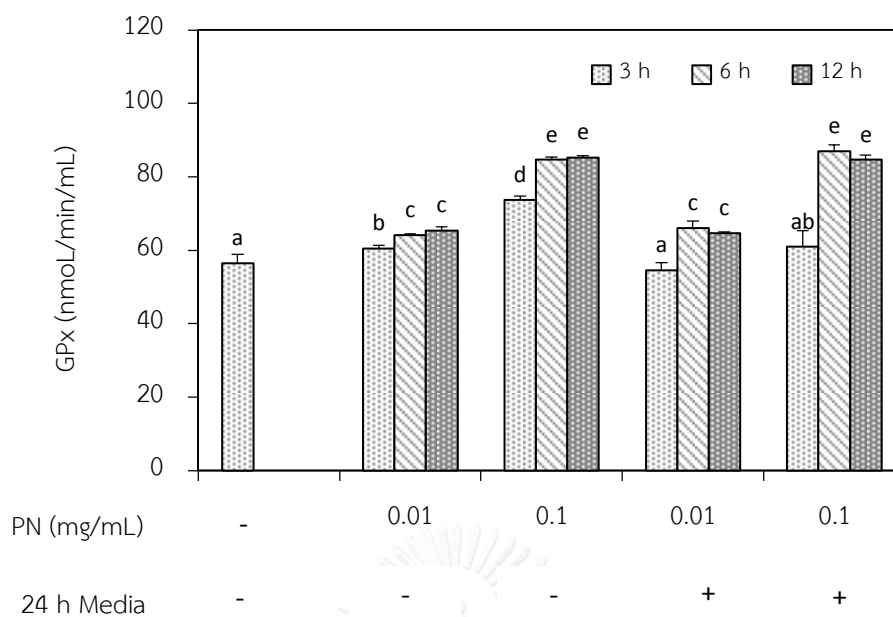c, d, e) above bars indicate significant difference (by ANOVA, p < 0.05)

Figure 35. Antioxidant enzyme activity of GR (nmoL/min/mL) was evaluated in HepG2
treated for 3, 6 and 12 h with PN extract. HepG2 cells were treated with 0.01
and 0.1 mg/mL of PN and harvested at the end of treatment or subsequently
incubated with culture medium for 24 h before evaluating GR activity. Values are
shown as means ± S.D. of 3 independent experiments. Different letters (a, b, c,
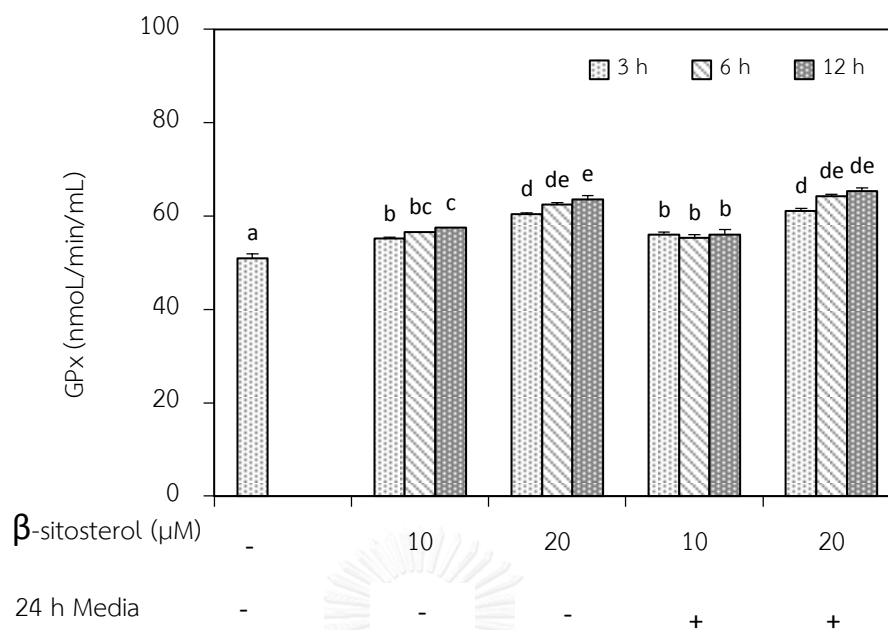d) above bars indicate significant difference (by ANOVA, $p < 0.05$).

Figure 36. Antioxidant enzyme activity of GR (nmoL/min/mL) was evaluated in HepG2 treated for 3, 6 and 12 h with β-sitosterol. HepG2 cells were treated with 10 or 20 μM of β-sitosterol and harvested at the end of treatment or subsequently incubated with culture medium for 24 h before evaluating GR activity. Values are shown as means ± S.D. of 3 independent experiments. Different letters (a, b, c) above bars indicate significant difference (by ANOVA, $p < 0.05$).
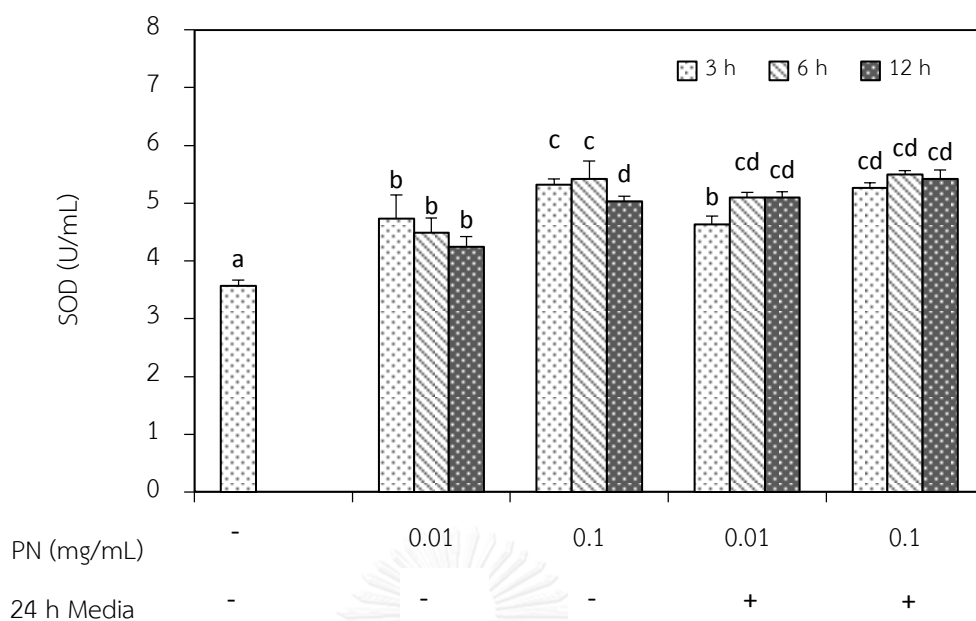
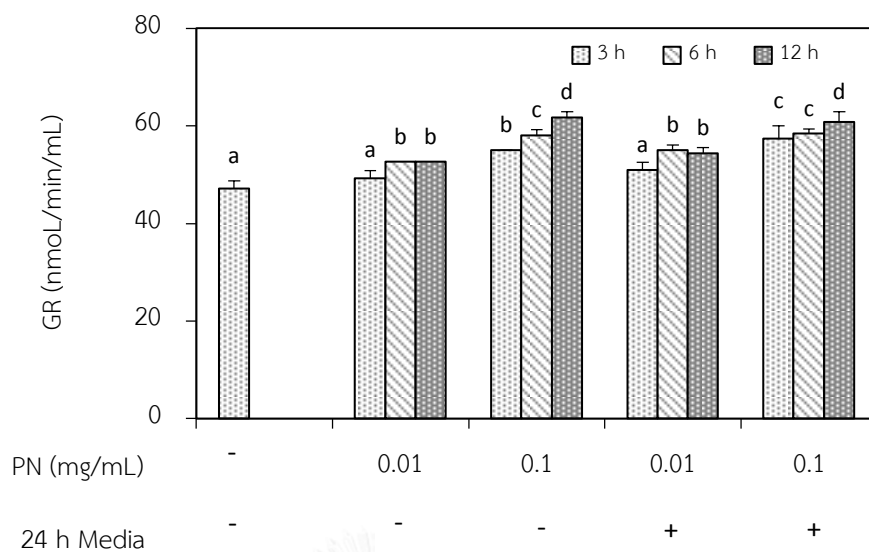Figure 37. Antioxidant enzyme activity of SOD (U/mL) was evaluated in HepG2 treated for 3, 6 and 12 h with PN extract. HepG2 cells were treated with 0.01 and 0.1 mg/mL of PN and harvested at the end of treatment or subsequently incubated with culture medium for 24 h before evaluating SOD activity Values are shown as means ± S.D. of 3 independent experiments. Different letters (a, b, c, d) above bars indicate significant difference (by ANOVA, $p < 0.05$).
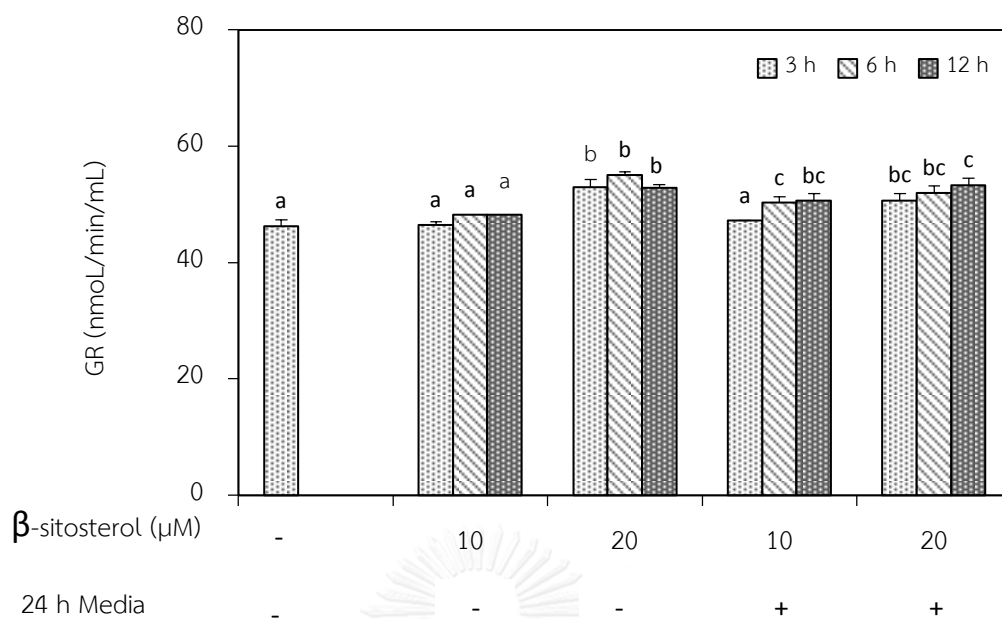
Figure 38. Antioxidant enzyme activity of SOD (U/mL) was evaluated in HepG2 treated for 3,

6 and 12 h with β-sitosterol. HepG2 cells were treated with 10 or 20 μM of β-

sitosterol and harvested at the end of treatment or subsequently incubated

with culture medium for 24 h before evaluating SOD activity. Values are shown

as means ± S.D. of 3 independent experiments. Different letters (a, b, c) above

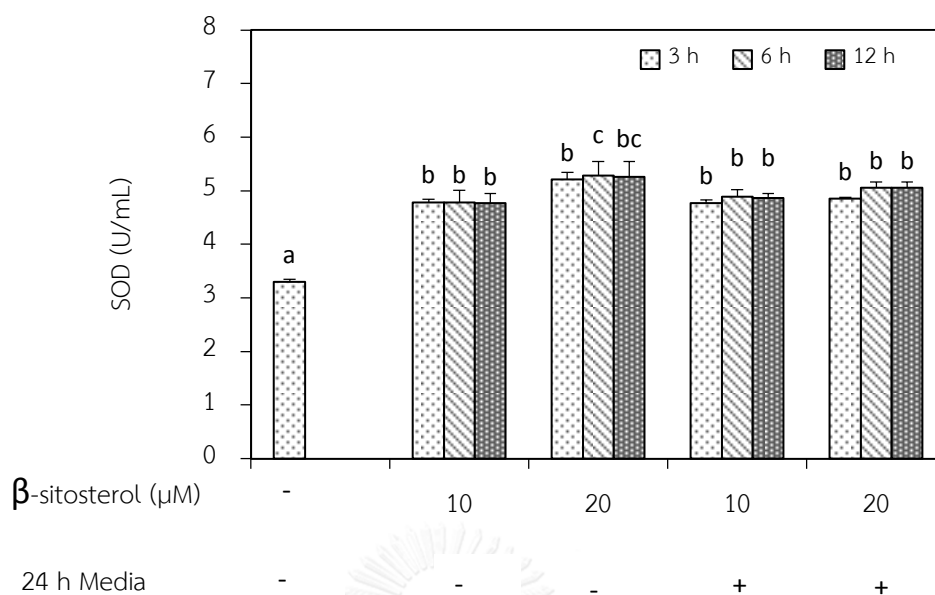bars indicate significant difference (by ANOVA, $p < 0.05$).

1.6 Effect of PN extract and β-sitosterol to restore GSH levels and the activities of antioxidant enzymes (CAT, GPx, GR and SOD) in H_2O_2-induced oxidative stress in HepG2 cells.

The result showed that H_2O_2 decreased total GSH and GSH but increased GSSG, thus decreased GSH/GSSG ratio, as compared to the non-treated group (Figure 39-42). Pre-treatment HepG2 cells with PN (0.01 and 0.1 mg/mL) significantly attenuated the depletion of total GSH (Figure 39) and GSH (Figure 40). Also, the extract attenuated an increase of GSSG caused by H_2O_2 (Figure 41), thus the ratio of GSH/GSSG ratio were increased (Figure 42) as compared to the H_2O_2 treated group. The GSH/GSSG ratio was shown to be significantly increased in a concentration dependent manner after treatment with either PN alone or PN pretreatment following with H_2O_2 as compared to the corresponding control groups (Figure 42). H_2O_2 significantly decreased the activities of CAT, GPx and GR as compared to the non-treated group. Pretreatment HepG2 cells with PN (0.01 and 0.1 mg/mL) attenuated the activities of these enzymes (Figure 43, 44, 45). Even though the activity of SOD was not affected by H_2O_2, PN pretreatment concentration-dependently increased the activity of this enzyme. PN 0.1 mg/mL increased SOD activity in the normal state of the cells while PN at both 0.01 and 0.1 mg/mL significantly increased SOD activity in the H_2O_2-induced oxidative stress cells even more than in the normal state cells (Figure 46). In the same manner as PN, pre-treatment HepG2 cells with β-sitosterol (10 and 20 μM) significantly prevented the depletion of total GSH (Figure 47) and GSH (Figure 48) but decreased the level of GSSG (Figure 49), resulted in the increase of GSH/GSSG ratio (Figure 50), as compared to the H_2O_2 treated group. Likewise, H_2O_2 significantly decreased the activities of CAT, GPx, GR and SOD as compared to the non-treated group (Figure 51, 52, 53, 54).

However, pretreatment HepG2 cells with β-sitosterol (10 and 20 μM) attenuated the activities of these enzymes (Figure 51, 52, 54) except for GR enzyme (Figure 53).

Figure 39. Effect of PN extract to restore total GSH level (μM) in H_2O_2-induced oxidative stress in HepG2 cells. HepG2 cells were treated with different concentrations of PN (0, 0.01 and 0.1 mg/mL) for 3 h prior to incubate with 500 μM of H_2O_2 for 24 h before evaluating total GSH level. Values are shown as means ± S.D. of 3 independent experiments, different letters (a, b, c) above bars indicate significant difference (by ANOVA, $p < 0.05$).

Figure 40. Effect of PN extract to restore GSH level (µM) in H_2O_2-induced oxidative stress in HepG2 cells. HepG2 cells were treated with different concentrations of PN (0, 0.01 and 0.1 mg/mL) for 3 h prior to incubate with 500 µM of H_2O_2 for 24 h before evaluating GSH level. Values are shown as means ± S.D. of 3 independent experiments, different letters (a, b, c) above bars indicate significant difference (by ANOVA, $p < 0.05$).
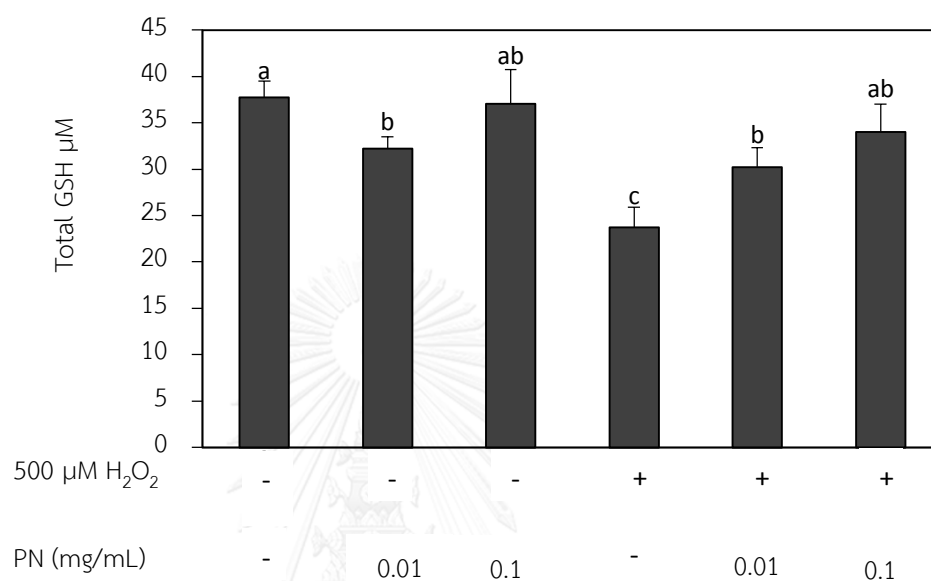
Figure 41. Effect of PN extract on the level of GSSG (μM) in H_2O_2-induced oxidative stress in

HepG2 cells. HepG2 cells were treated with different concentrations of PN (0,

0.01 and 0.1 mg/mL) for 3 h prior to incubate with 500 μM of H_2O_2 for 24 h

before evaluating GSSG level. Values are shown as means ± S.D. of 3

independent experiments, different letters (a, b) above bars indicate significant

difference (by ANOVA, $p < 0.05$).

Figure 42. Effect of PN extract on GSH/GSSG ratio in H_2O_2-induced oxidative stress in HepG2

cells. HepG2 cells were treated with different concentrations of PN (0, 0.01 and

0.1 mg/mL) for 3 h prior to incubate with 500 µM of H_2O_2 for 24 h before

evaluating GSH and GSSG levels. GSH/GSSG ratio was calculated from the values

of GSH and GSSG level. Values are shown as means ± S.D. of 3 independent

experiments, different letters (a, b, c, d, e, f) above bars indicate significant
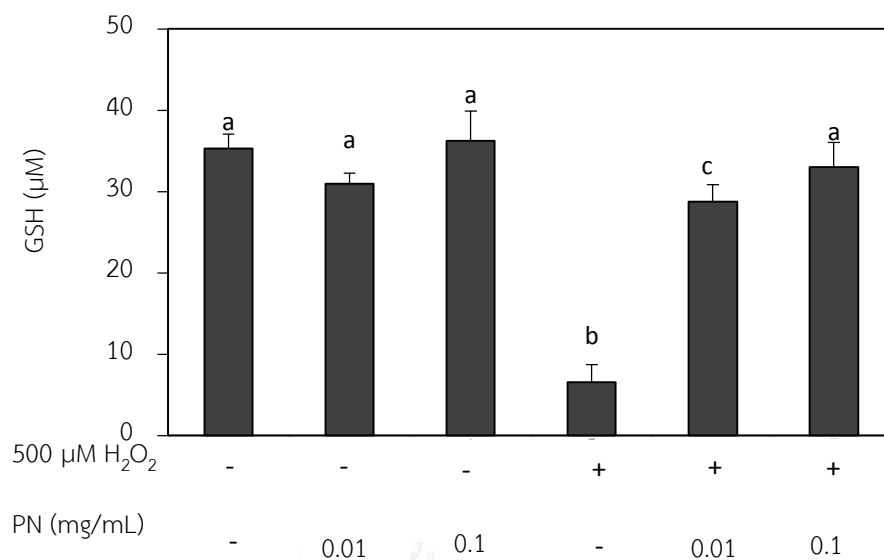
difference (by ANOVA, $p < 0.05$).

Figure 43. Effect of PN extract to restore CAT activity (nmol/min/mL) in H_2O_2-induced

oxidative stress in HepG2 cells. HepG2 cells were treated with different

concentrations of PN (0, 0.01 and 0.1 mg/mL) for 3 h prior to incubate with 500

μM of H_2O_2 for 24 h before evaluating CAT activity. Values are shown as means

± S.D. of 3 independent experiments, different letters (a, b, c) above bars
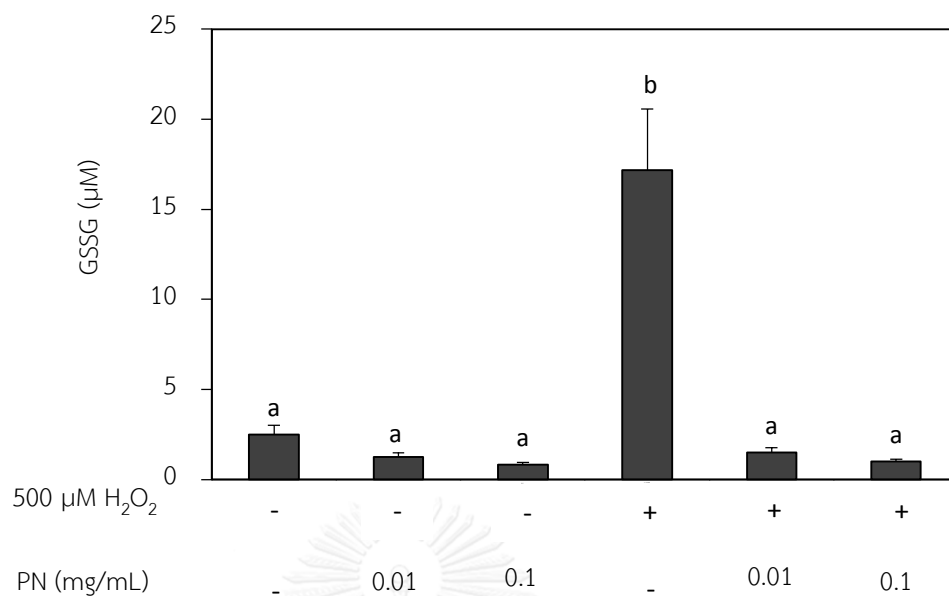
indicate significant difference (by ANOVA, $p < 0.05$).

Figure 44. Effect of PN extract to restore GPx activity (nmol/min/mL) in H_2O_2-induced

oxidative stress in HepG2 cells. HepG2 cells were treated with different

concentrations of PN (0, 0.01 and 0.1 mg/mL) for 3 h prior to incubate with 500

μM of H_2O_2 for 24 h before evaluating GPx activity. Values are shown as means ±

S.D. of 3 independent experiments, different letters (a, b, c) above bars indicate
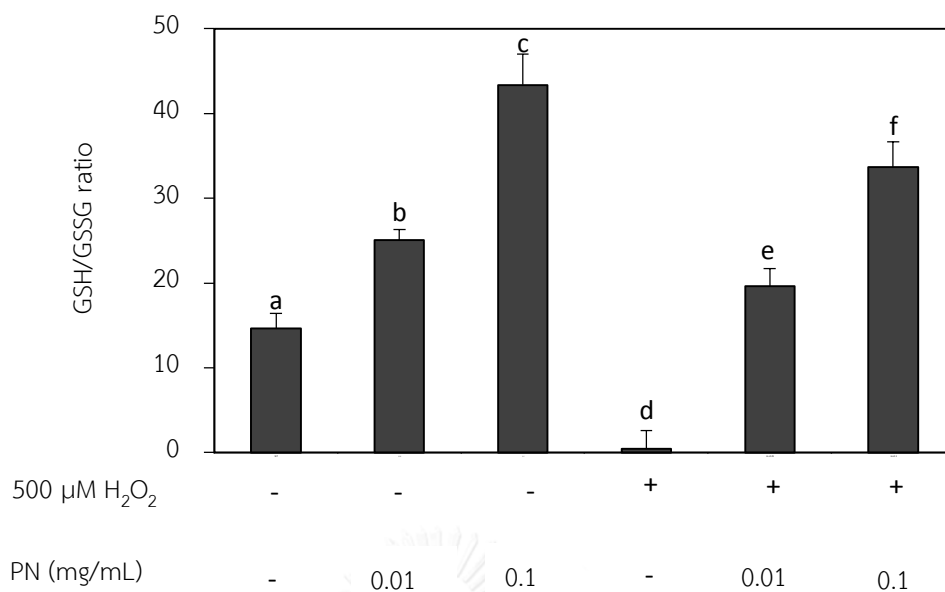
significant difference (by ANOVA, $p < 0.05$).

Figure 45. Effect of PN extract to restore GR activity (nmol/min/mL) in H_2O_2-induced

oxidative stress in HepG2 cells. HepG2 cells were treated with different

concentrations of PN (0, 0.01 and 0.1 mg/mL) for 3 h prior to incubate with 500

μM of H_2O_2 for 24 h before evaluating GR activity. Values are shown as means ±

S.D. of 3 independent experiments, different letters (a, b, c) above bars indicate

significant difference (by ANOVA, $p < 0.05$).

Figure 46. Effect of PN extract to restore SOD activity (U/mL) in H_2O_2-induced oxidative

stress in HepG2 cells. HepG2 cells were treated with different concentrations of

PN (0, 0.01 and 0.1 mg/mL) for 3 h prior to incubate with 500 µM of H_2O_2 for 24

h before evaluating SOD activity. Values are shown as means ± S.D. of 3

independent experiments, different letters (a, b, c) above bars indicate
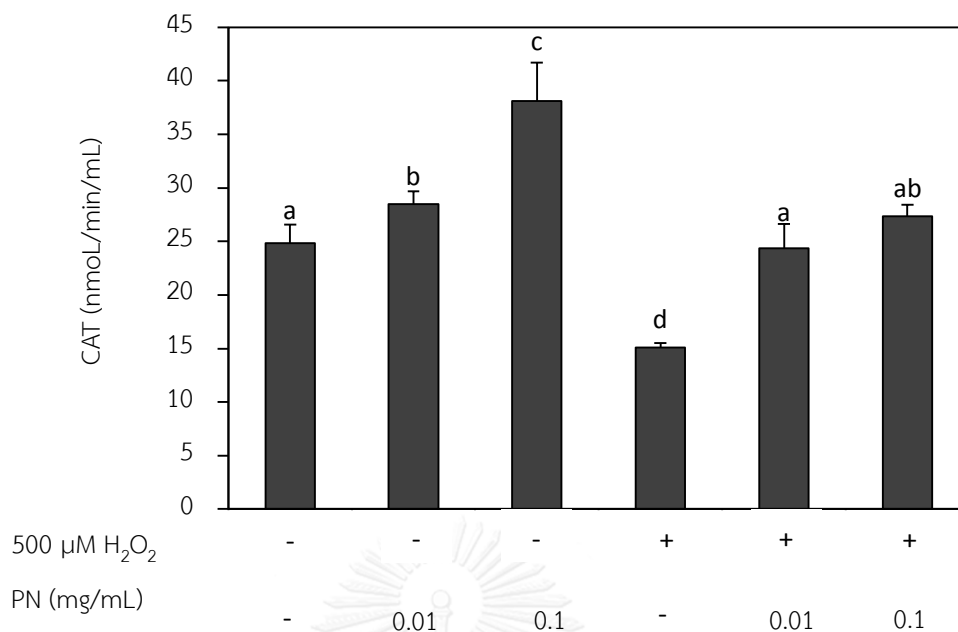
significant difference (by ANOVA, $p < 0.05$).

Figure 47. Effect of β-sitosterol to restore total GSH level (μM) in H_2O_2-induced oxidative

stress in HepG2 cells. HepG2 cells were treated with different concentrations of

β-sitosterol (0, 10 and 20 μM) for 3 h prior to incubate with 500 μM of H_2O_2 for

24 h before evaluating total GSH level. Values are shown as means ± S.D. of 3

independent experiments, different letters (a, b, c) above bars indicate
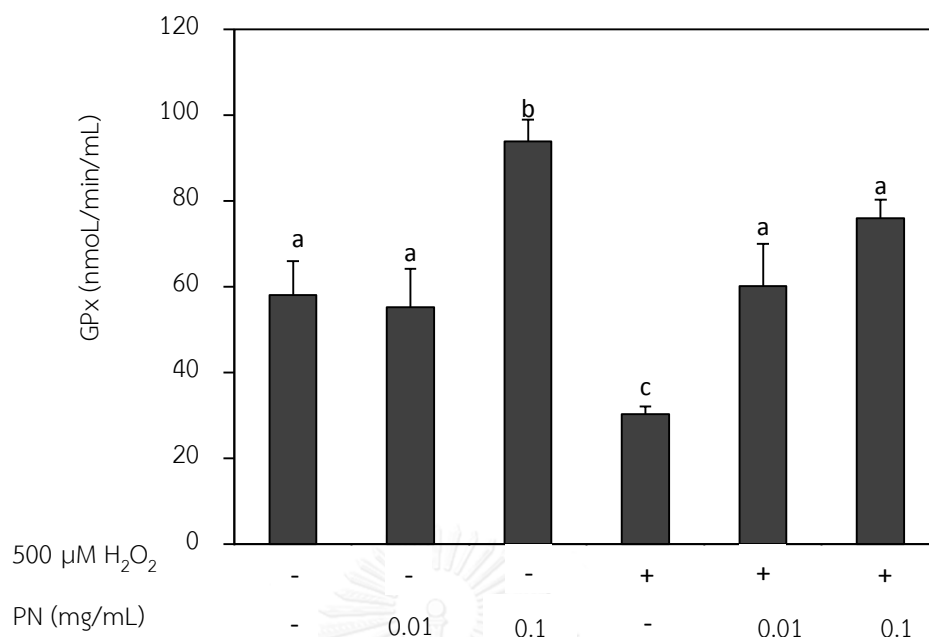
significant difference (by ANOVA, $p < 0.05$).

Figure 48. Effect of β-sitosterol to restore GSH level (µM) in H_2O_2-induced oxidative stress in HepG2 cells. HepG2 cells were treated with different concentrations of β-sitosterol (0, 10 and 20 µM) for 3 h prior to incubate with 500 µM of H_2O_2 for 24 h before evaluating GSH level. Values are shown as means ± S.D. of 3 independent experiments, different letters (a, b, c, d) above bars indicate significant difference (by ANOVA, $p < 0.05$).
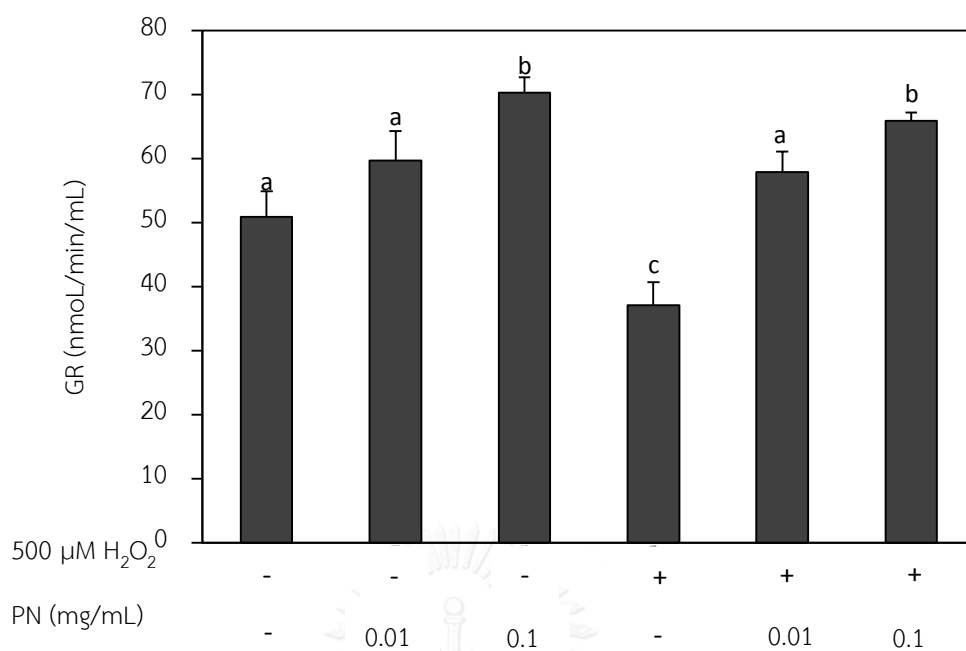
Figure 49. Effect of β-sitosterol on the level of GSSG (μM) in H_2O_2-induced oxidative stress in HepG2 cells. HepG2 cells were treated with different concentrations of β-sitosterol (0, 10 and 20 μM) for 3 h prior to incubate with 500 μM of H_2O_2 for 24 h before evaluating GSSG level. Values are shown as means ± S.D. of 3 independent experiments, different letters (a, b) above bars indicate significant difference (by ANOVA, $p < 0.05$).
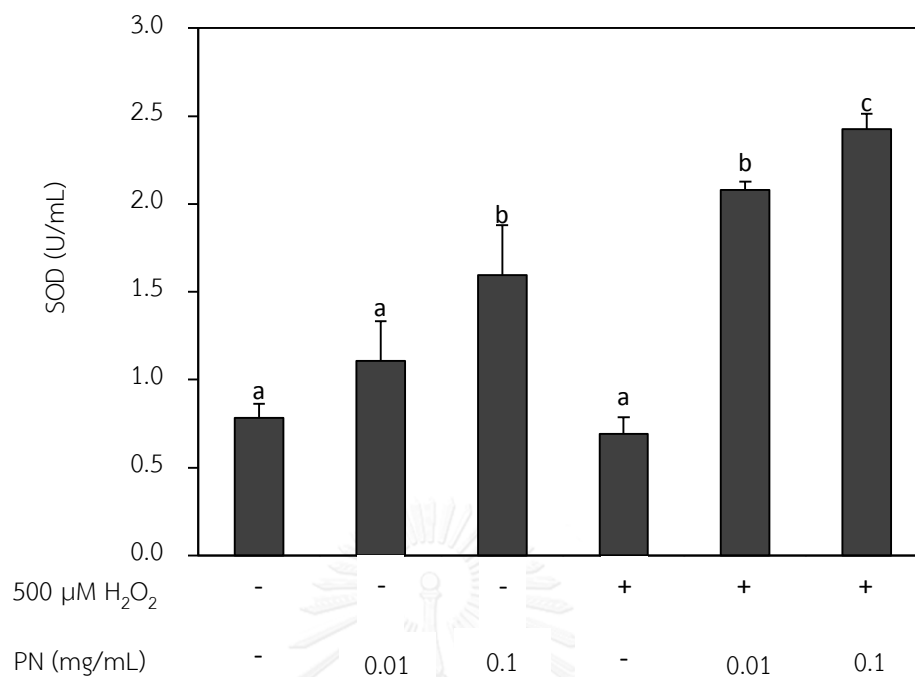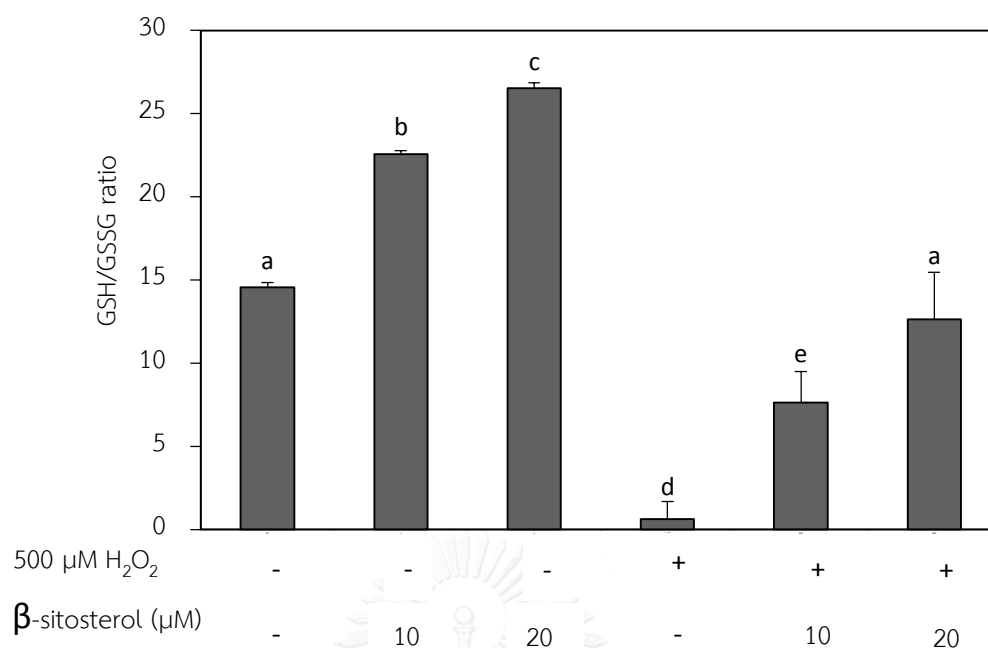
Figure 50. Effect of β-sitosterol on the GSH/GSSG ratio in H_2O_2-induced oxidative stress in HepG2 cells. HepG2 cells were treated with different concentrations of β-sitosterol (0, 10 and 20 µM) for 3 h prior to incubate with 500 µM of H_2O_2 for 24 h before evaluating GSH and GSSG levels. GSH/GSSG ratio was calculated from the values of GSH and GSSG level. Values are shown as means ± S.D. of 3 independent experiments, different letters (a, b, c, d, e) above bars indicate significant difference (by ANOVA, $p < 0.05$).
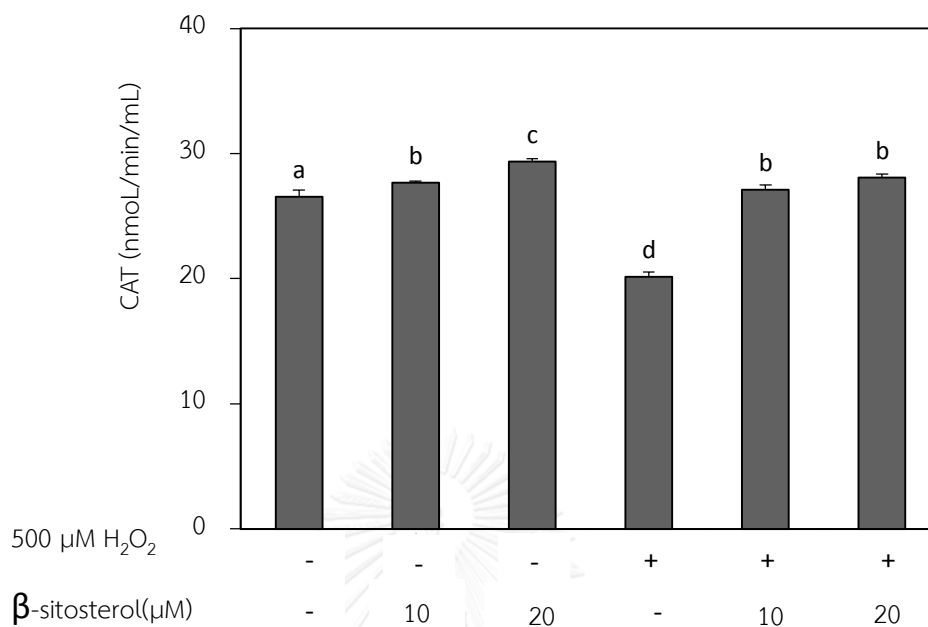
Figure 51. Effect of β-sitosterol to restore CAT activity (nmol/min/mL) in H_2O_2-induced

oxidative stress in HepG2 cells. HepG2 cells were treated with different

concentrations of β-sitosterol (0, 10 and 20 µM) for 3 h prior to incubate with

500 µM of H_2O_2 for 24 h before evaluating CAT activity. Values are shown as

means ± S.D. of 3 independent experiments, different letters (a, b, c, d) above

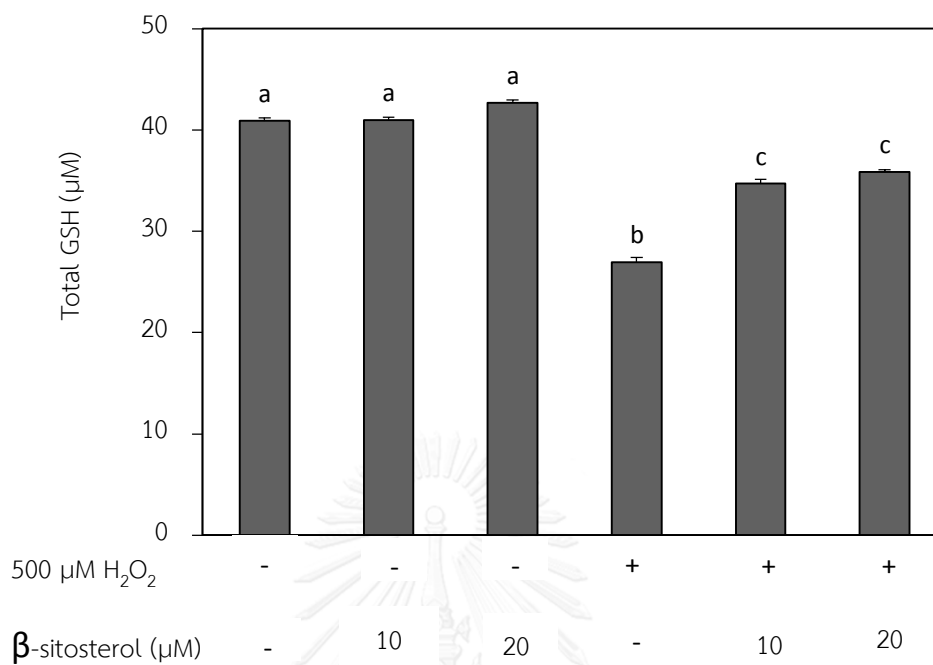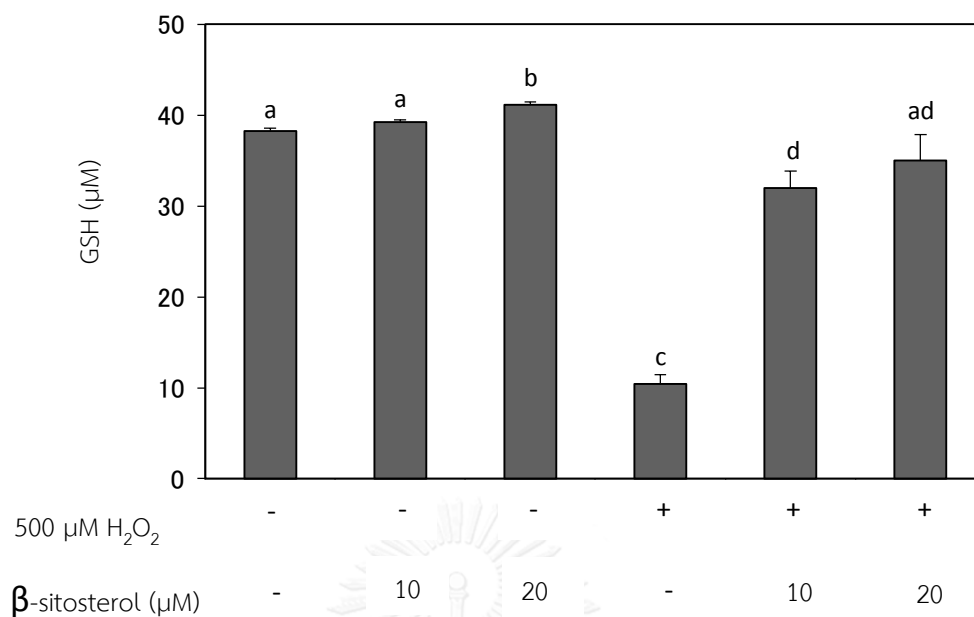bars indicate significant difference (by ANOVA, $p < 0.05$).
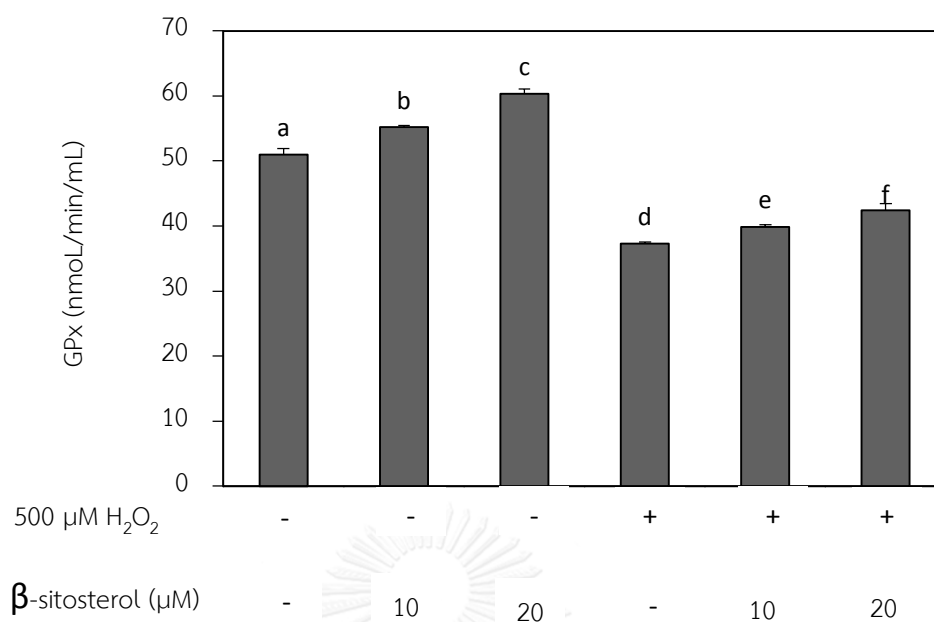
Figure 52. Effect of β-sitosterol to restore GPx activity (nmol/min/mL) in H_2O_2-induced

oxidative stress in HepG2 cells. HepG2 cells were treated with different

concentrations of β-sitosterol (0, 10 and 20 µM) for 3 h prior to incubate with

500 µM of H_2O_2 for 24 h before evaluating GPx activity. Values are shown as

means ± S.D. of 3 independent experiments, different letters (a, b, c, d, e, f)

above bars indicate significant difference (by ANOVA, $p < 0.05$).
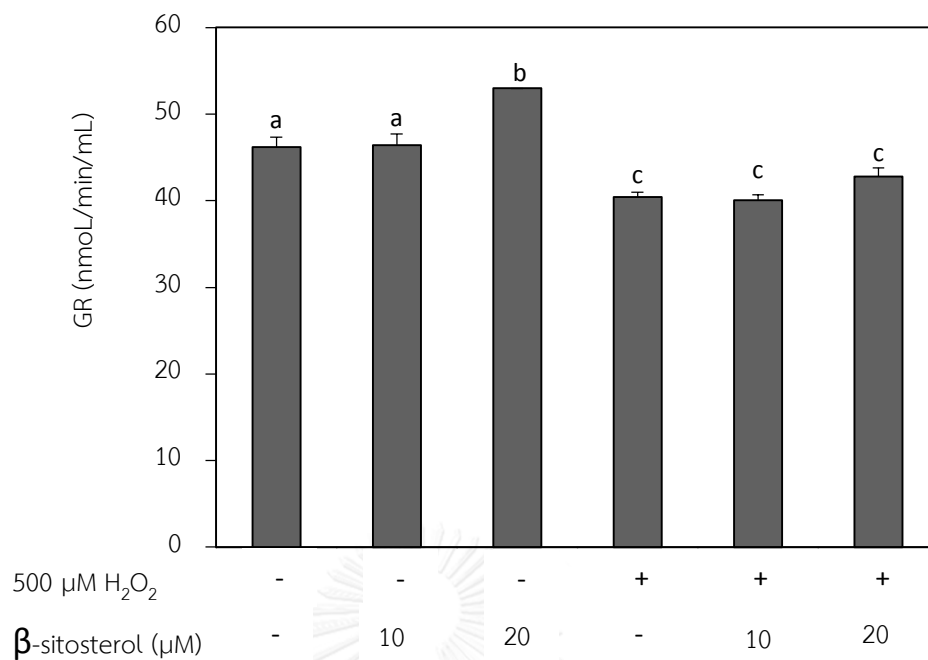
Figure 53. Effect of β-sitosterol to restore GR activity (nmol/min/mL) in H_2O_2-induced

oxidative stress in HepG2 cells. HepG2 cells were treated with different

concentrations of β-sitosterol (0, 10 and 20 μM) for 3 h prior to incubate with

500 μM of H_2O_2 for 24 h before evaluating GR activity. Values are shown as

means ± S.D. of 3 independent experiments, different letters (a, b, c) above bars

indicate significant difference (by ANOVA, $p < 0.05$).
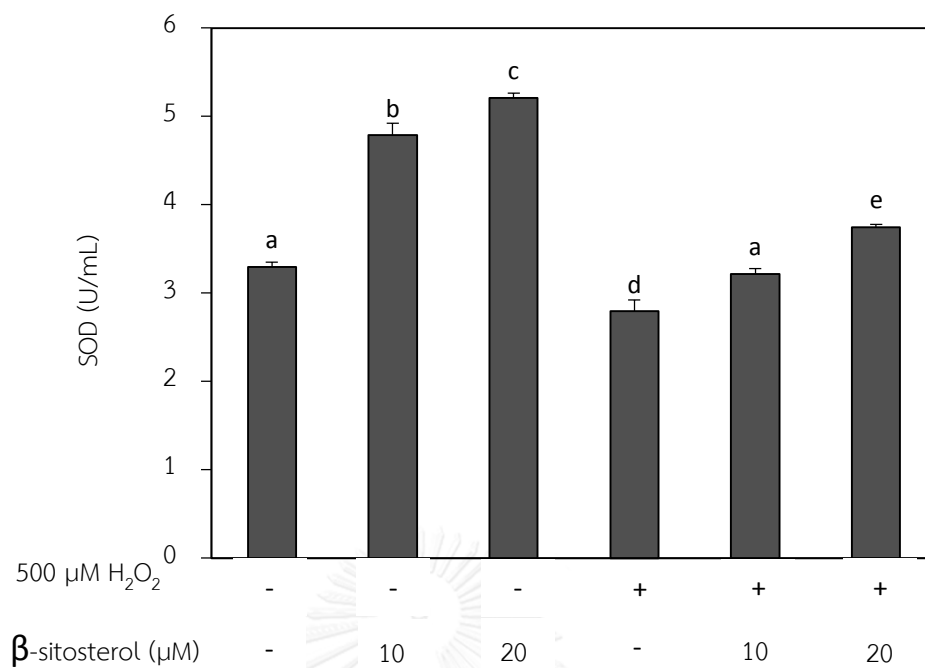
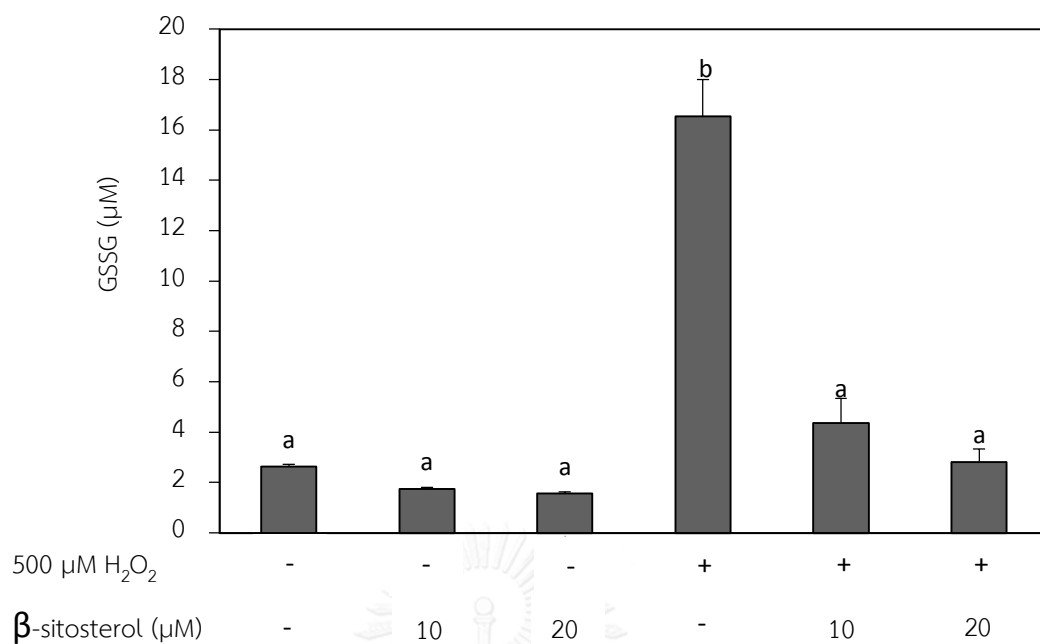Figure 54. Effect of β-sitosterol to restore SOD activity (U/mL) in H_2O_2-induced oxidative
stress in HepG2 cells. HepG2 cells were treated with different concentrations of
β-sitosterol (0, 10 and 20 μM) for 3 h prior to incubate with 500 μM of H_2O_2 for
24 h before evaluating SOD activity. Values are shown as means ± S.D. of 3
independent experiments, different letters (a, b, c, d) above bars indicate
significant difference (by ANOVA, $p < 0.05$).

1.7 Effect of PN extract and β-sitosterol on Nrf2 and HO-1 mRNA expression

The mRNA expression levels of Nrf2 and HO-1 was evaluated in HepG2 cells treated with 0.1 mg/mL of PN for 0, 3, 6 and 12 h. No induction effect on Nrf2 mRNA expression was observed at any indicated time point after 0.1 mg/mL of PN exposure (Figure 55a). In contrast, the mRNA expression level of HO-1 in HepG2 cells was significantly induced by treatment with 0.1 mg/mL of PN at 3 and 6 h of PN exposure with the fold induction of about 3 folds as compared with 0 h ($p<0.01$) and subsequently decreased at 12 h exposure (Figure 55b). Meanwhile, β-sitosterol did not have an inductive effect on the expression of both Nrf2 and HO-1 mRNA (Figure 56a, 56b).

a)   b)
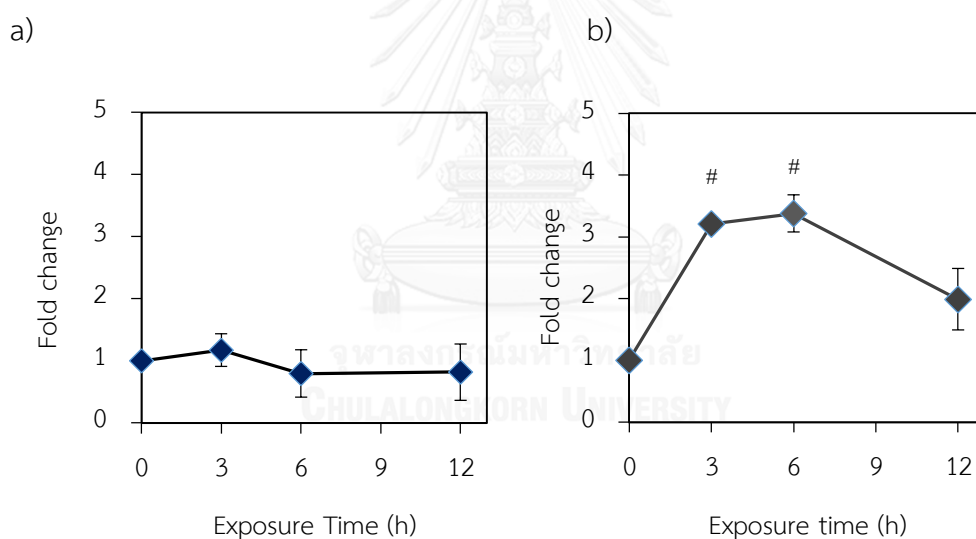
Figure 55. Effect of PN on Nrf2 (a) and HO-1 (b) mRNA expression. HepG2 cells were treated with 0.1 mg/mL of PN for 0, 3, 6 and 12 h. Nrf2 and HO-1 mRNA levels were determined by qRT-PCR and using GAPDH as an internal reference and loading control. Data are mean ± S.D. of three independent experiments. #, $p<0.01$ as compared to 0 h.

a)

b)
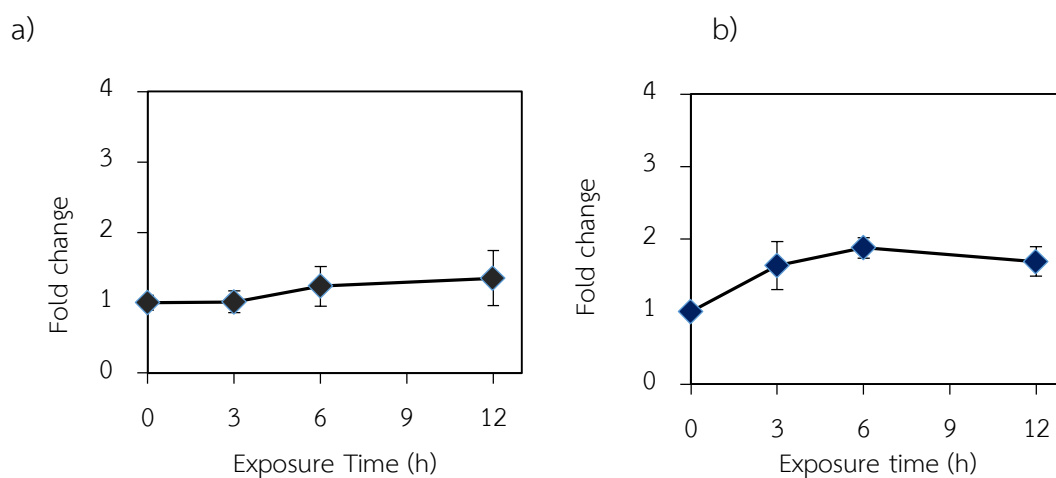
Figure 56. Effect of β-sitosterol on Nrf2 (a) and HO-1 (b) mRNA expression. HepG2 cells

were treated with 20 µM of β-sitosterol for 0, 3, 6 and 12 h. Nrf2 and HO-1

mRNA levels were determined by qRT-PCR and using GAPDH as an internal

reference and loading control. Values are shown as means ± S.D. of 3

independent experiments.

1.8 Effect of PN extract and β-sitosterol on Nrf2 and HO-1 protein expression

Accumulations of Nrf2 in cytosol and nucleus as well as HO-1 protein in the HepG2 cells treated with either PN alone or PN followed by H_2O_2 were determined. The result showed that PN itself caused significant increase of both cytosolic Nrf2 (Figure 57a, 57b) and nuclear Nrf2 proteins (Figure 57c, 57d) as compared to the non-treated HepG2 cells. No significant effect of PN alone was shown on HO-1 protein (Figure 57e, 57f). H_2O_2 caused significant decrease of both Nrf2 and HO-1 protein expression. Pretreatment of the cells with PN (0.01 and 0.1 mg/mL) prior to H_2O_2 treatment, attenuated the expression of both cytosolic Nrf2 (Figure 57a, 57b) and nuclear Nrf2 (Figure 57c, 57d) as well as HO-1 proteins (Figure 57e, 57f). Under H_2O_2 treated condition, PN at both 0.01 and 0.1 mg/mL reversed nuclear Nrf2 proteins of which the levels were significantly higher than the H_2O_2-treated control and even

significantly higher than the non-treated control (Figure 57c, 57d). However, PN at both concentrations reversed the cytosolic Nrf2 protein of which the level was significantly higher than the H_2O_2-treated control but not significantly higher than the non-treated control (Figure 57b). In addition, to know the induction time of Nrf2 and HO-1 proteins by PN, a time-course study was also undertaken. Significant induction of cytosolic Nrf2 and HO-1 proteins were observed after the exposure to 0.1 mg/mL of PN at 3, 6, 12 h of incubation (Figure 58a, 58b and 58e, 58f). In addition, Nrf2 were significantly localized to the nucleus after 12 h of the treatment with 0.1 mg/mL of PN. However, no significant differences were observed on the expression of Nrf2 and HO-1 proteins as well as the localized to the nucleus of Nrf2 among the different time of compound exposure (3, 6, 12 h) treated group.

β-sitosterol itself did not affect the levels of cytosolic Nrf2 (Figure 59a, 59b), nuclear Nrf2 (Figure 59c, 59d) and HO-1 protein (Figure 59e, 59f). Pre-treatment cells with β-sitosterol prior to H_2O_2 exposure did not significantly attenuate the expression of cytosolic Nrf2 (Figure 59a, 59b) but attenuated the HO-1 protein (Figure 59e, 59f). No significant effects of β-sitosterol cytosolic Nrf2, nuclear Nrf2 and HO-1 protein expression at 3, 6 and 12 h of exposure (Figure 60).
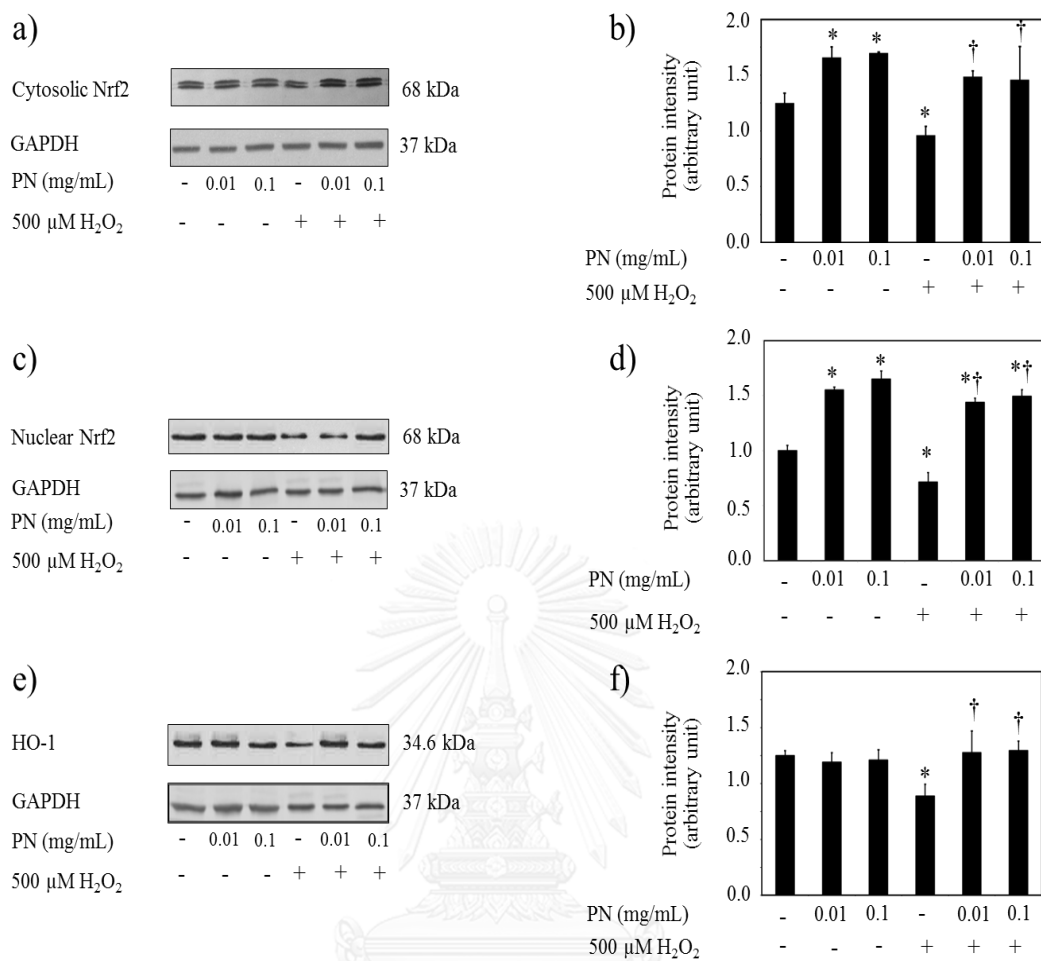
Figure 57. Effect of PN on Nrf2 (cytosolic Nrf2, a & b; nuclear Nrf2, c & d) and HO-1 (e, f)

protein expression. HepG2 cells were treated with different concentrations of PN

(0.01 and 0.1 mg/mL) for 3 h before treatment with 500 µM of H_2O_2 for 24 h.

Nrf2 and HO-1 proteins levels were determined by western blot analyses using

an enhanced chemiluminescence system. The intensity of each

chemiluminescence (protein) band was analyzed by Calibrated Imaging

Densitometer and normalized as the Nrf2 /GADPH and HO-1/GAPDH ratio. Values

are shown as means ± S.D. of 3 independent experiments. *, $p < 0.05$ as

compared to the non-treated control; †, $p < 0.05$ as compared to the H_2O_2-

treated control.
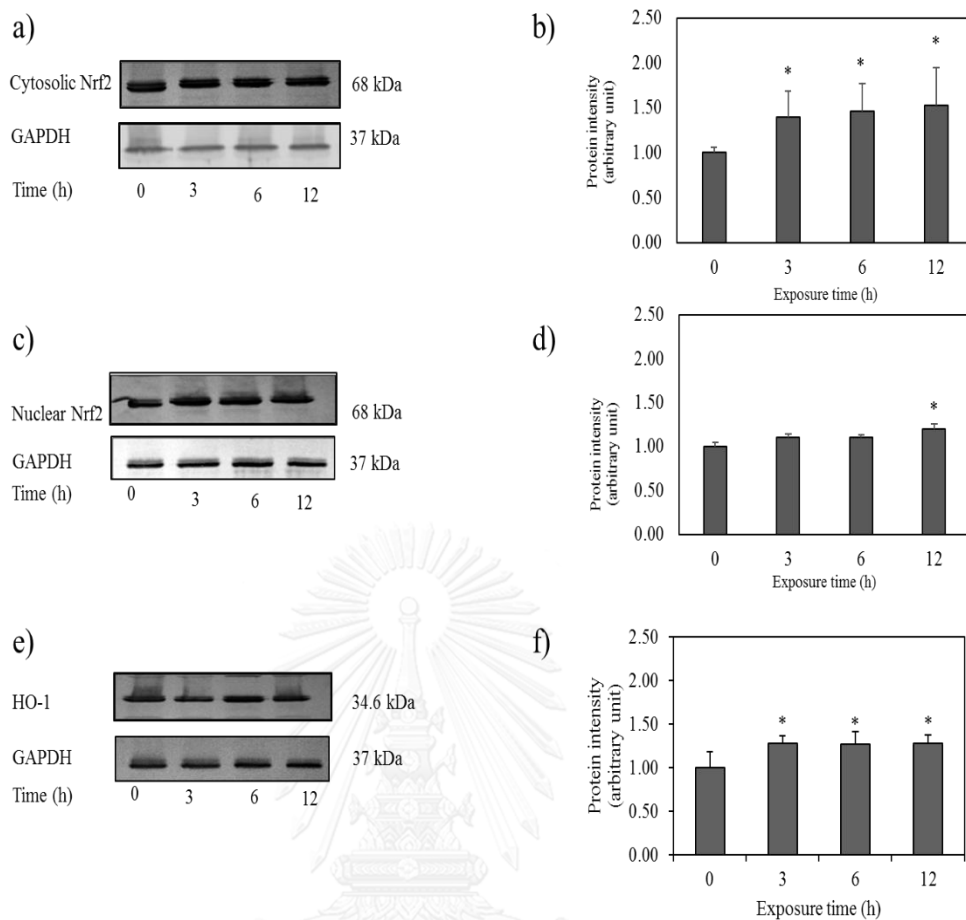
Figure 58. Time-dependent effect of PN on Nrf2 (cytosolic Nrf2, a & b; nuclear Nrf2, c & d) and HO-1 (e, f) protein expression in HepG2 cells. HepG2 cells were treated with either vehicle (NT) or 0.1 mg/mL of PN for 3, 6, 12 h. Nrf2 and HO-1 proteins levels were determined by western blot analyses using an enhanced chemiluminescence system. The intensity of each chemiluminescence (protein) band was analyzed by Calibrated Imaging Densitometer and normalized as the Nrf2 /GADPH and HO-1/GAPDH ratio. Values are shown as means ± S.D. of 3 independent experiments. *, $p<0.05$ as compared to the non-treated control.

Figure 59. Effect of β-sitosterol on Nrf2 (cytosolic Nrf2, a & b; nuclear Nrf2, c & d) and HO-1

(e, f) protein expression. HepG2 cells were treated with different concentrations

of β-sitosterol (10 and 20 μM) for 3 h before treatment with 500 μM of H_2O_2 for

24 h. Nrf2 and HO-1 proteins levels were determined by western blot analyses

using an enhanced chemiluminescence system. The intensity of each

chemiluminescence (protein) band was analyzed by Calibrated Imaging

Densitometer and normalized as the Nrf2 /GADPH and HO-1/GAPDH ratio. Values

are shown as means ± S.D. of 3 independent experiments. *, $p < 0.05$ as

compared to the non-treated control; †, $p < 0.05$ as compared to the H_2O_2-

treated control.

Figure 60. Time-dependent effect of β-sitosterol on Nrf2 (cytosolic Nrf2, a & b; nuclear Nrf2, c & d) and HO-1 (e, f) protein expression in HepG2 cells. HepG2 cells were treated with either vehicle (NT) or 20 µM of β-sitosterol for 3, 6, 12 h. Nrf2 and HO-1 proteins levels were determined by western blot analyses using an enhanced chemiluminescence system. The intensity of each chemiluminescence (protein) band was analyzed by Calibrated Imaging Densitometer and normalized as the Nrf2 /GADPH and HO-1/GAPDH ratio. Values are shown as means ± S.D. of 3 independent experiments. *, $p<0.05$ as compared to the non-treated control.

2. Inhibition and induction effect of PN extract

2.1 Inhibition of CYP enzyme activities by PN extract

The results of CYP inhibition by PN extract and known CYP inhibitors using selective substrate oxidation *in vitro* assays in human liver microsomes are shown in table 8. PN showed concentration-dependent inhibition of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 activities, with IC_{50} values of 13, 62, 67 and 88 µg/mL, respectively (Table 8). In contrast, inhibitions of CYP2C19 and CYP2E1 by PN were not observed at concentrations up to 1,000 µg/mL. The method was verified using furafylline, sulfaphenazole, ticlopidine, quinidine, diethyldithiocarbamate and ketoconazole which are selective inhibitors for CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4, respectively, gave IC_{50} values of 0.44, 0.48, 0.44, 0.059, 6.63 and 0.043 µM, respectively (Table 8).
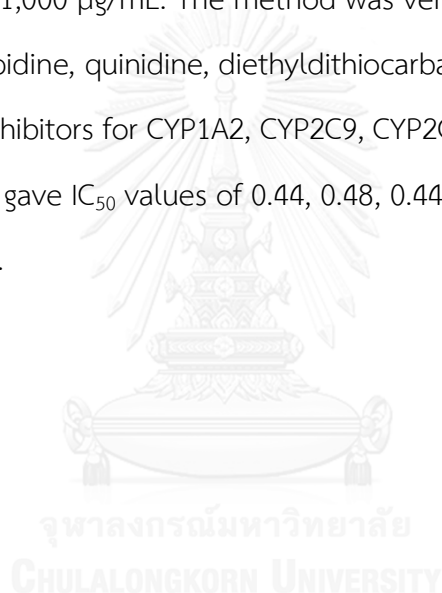
Table 8. Inhibition of CYP enzymes by PN and selective inhibitors.

CYP Isoform	Reaction	PN and Selective inhibitors	MW	IC_{50} (µM)	IC_{50} (µg/mL)	Reported IC_{50} of the selective inhibitor (µM) (references)
CYP1A2	Phenacetin *O*-deethylation	PN	-	-	13	
		Furafylline	260.25	0.44	0.115	0.48[a]
CYP2C9	*S*-warfarin 7-hydroxylation	PN	-	-	62	
		Sulfaphenazole	314.36	0.48	0.151	0.51[b]
CYP2C19	*S*-Mephenytoin 4-hydroxylation	PN	-	-	>1,000	
		Ticlopidine	263.79	0.44	0.116	0.72[c]
CYP2D6	Bufuralol 1-hydroxylation	PN	-	-	67	
		Quinidine	324.42	0.059	0.019	0.043[d]
CYP2E1	Aniline hydroxylation	PN	-	-	>1,000	
		Diethyldithiocarbamate	148.27	6.63	0.98	9.8[a]
CYP3A4	Midazolam hydroxylation	PN	-	-	88	
		Ketoconazole	531.43	0.043	0.023	0.03[e]

[a]; [74], [b]; [75], [c]; [76], [d]; [77], [e]; [78]

2.2 Kinetics of enzyme inhibition by PN extract

Enzyme kinetic reactions were performed to characterize the type of inhibition of CYPs by PN. Lineweaver-Burk and corresponding secondary plots for inhibition of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 are shown in Figure 61. The results indicated that inhibition of CYP1A2 by PN was most likely a competitive type, while the effects on CYP2C9 and CYP2D6 tended to be a mixed type. Based on the parallel pattern of Lineweaver-Burk plots for various PN concentrations, CYP3A4 inhibition was most likely an uncompetitive type. The corresponding secondary plots gave Ki values of PN with CYP1A2, CYP2C9, CYP2D6 and CYP3A4 of 34, 80, 12 and 150 µg/mL, respectively.
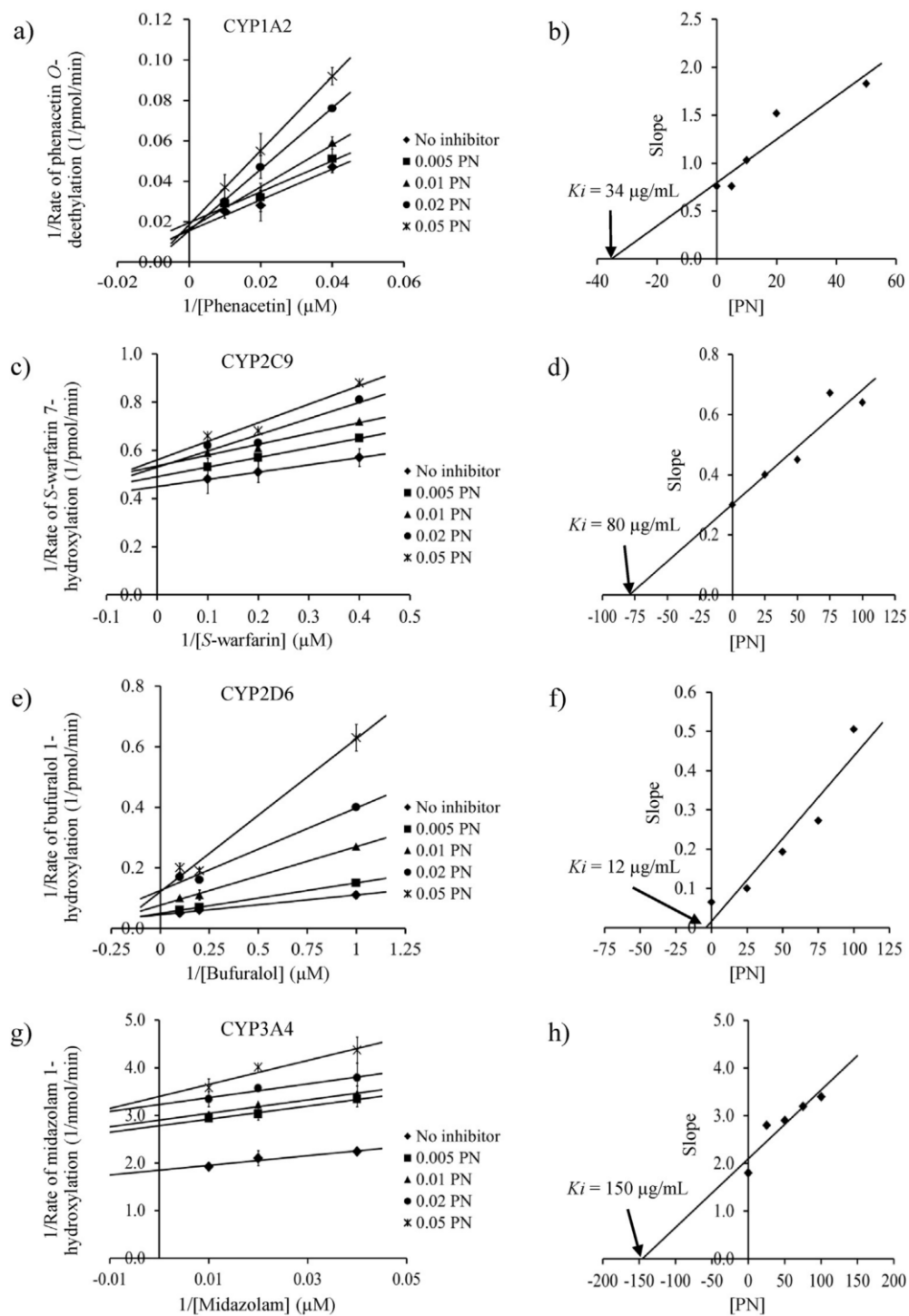
Figure 61. Lineweaver-Burk and corresponding secondary plots for inhibition of CYPs by PN extract

2.3 Effect of PN extract on mechanism based inhibition of CYPs

One of the best characterized compounds that possessed TDI is the IC_{50} value shift of the ratio of IC_{50} values between without and with β-NADPH of a 30-minute pre-incubation of a potential inhibitor; a fold shift of IC_{50} values greater than 1.5-2 would indicate the presence of TDI [73]. The result demonstrated that TDI effect of PN were not observed. The shifted IC_{50} values of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 were 14, 58, 60 and 78 µg/mL, respectively and fold shift of all CYPs were not greater than 1.5 folds (Table 9).

Table 9 IC_{50} shifted of CYPs

CYPs	IC_{50} (µg/mL)	Shifted IC_{50} (µg/mL)	Fold shift
1A2	13	14	0.92
2C9	62	58	1.06
2D6	67	60	1.11
3A4	88	78	1.12

2.4 Cytotoxicity of PN extract in primary human hepatocytes

The result demonstrated that PN at 1 mg/mL significantly decreased mitochondrial activity with cell viability of <80% compared to the non-treated group, whereas cell viability was >80% as with lower concentrations of PN (0.0001, 0.001, 0.01 and 0.1 mg/mL) (Figure 62). Therefore, PN at concentrations up to 0.1 mg/mL was subsequently used for a further study on induction of CYP enzymes.
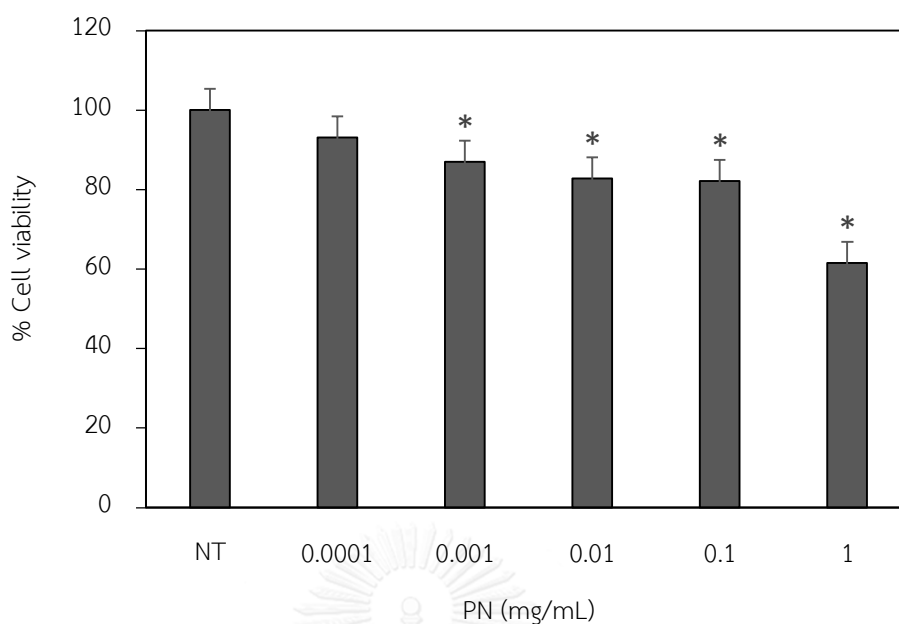
Figure 62. Cytotoxicity of PN extract in primary human hepatocytes. The hepatocytes were

treated with PN at the indicated concentrations for 48 h. Cell viability was

determined by MTT assay. NT = non-treated control. Values are shown as means

± S.D. of 3 independent experiments. * $p < 0.05$; PN vs non-treated control.

2.5 Induction of CYP enzyme activities by PN extract

Induction of CYP1A2, CYP2C9, CYP2C19 and CYP3A4 in primary human

hepatocytes was examined with PN at 0.001, 0.01 and 0.1 mg/mL. The results

showed that PN extract did not cause significant induction effect on CYPs

investigated. A selective inducer of CYP1A2, omeprazole, caused a 12-fold increase of

CYP1A2 activity as compared to the non-treated control. Similarly, rifampicin

significantly increased the activities of CYP2C9, CYP2C19 and CYP3A4, with the fold

induction of 11-, 4- and 12-fold, respectively (Figure 63).
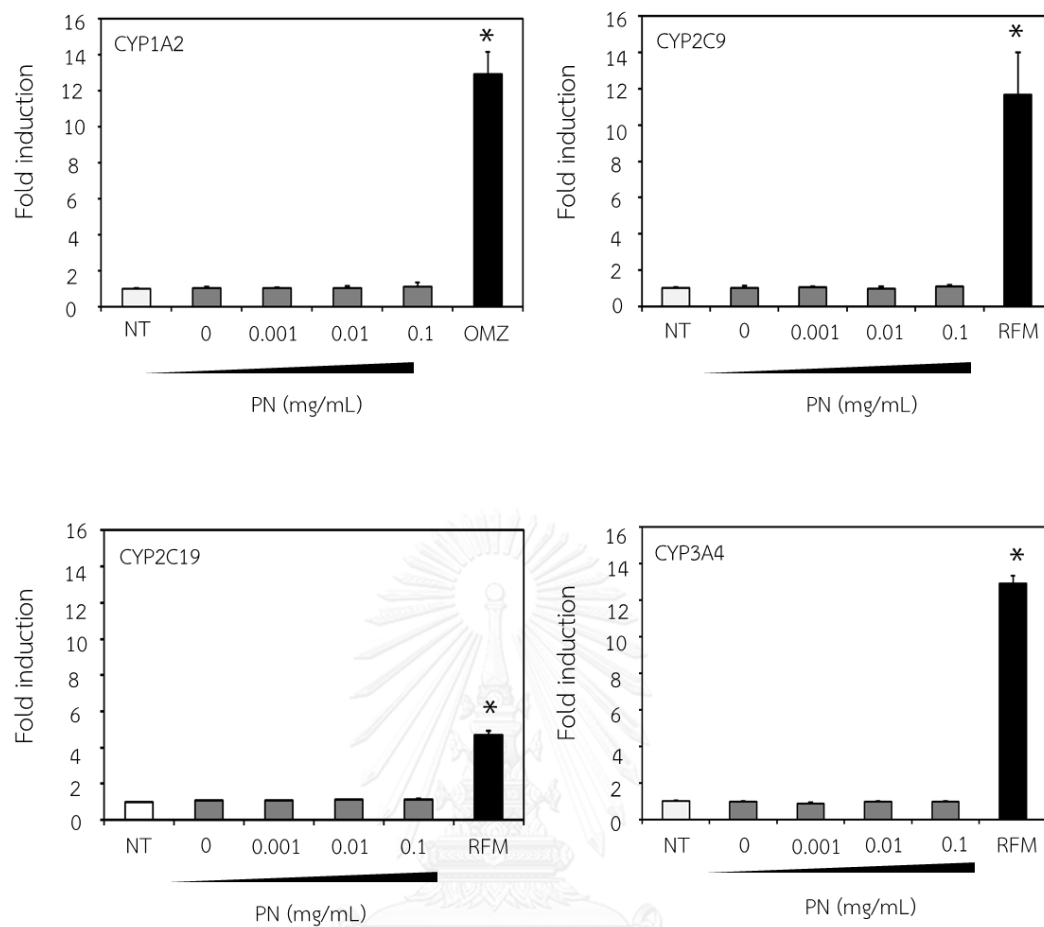
Figure 63. Induction of CYP enzymes by PN extract in primary human hepatocytes. The hepatocytes were treated with different concentrations of PN (0-0.1 mg/mL) for 48 h. CYP induction was evaluated using a P450-GloTM CYP assay. Omeprazole (OMZ) and rifampicin (RFM) were used as positive controls for CYP1A2 and CYP2C9/CYP2C19/CYP3A4, respectively. NT = non-treated control. Data are shown as mean ± S.D. (n=3). * $p < 0.05$; PN vs non-treated control.

2.6 Effect of PN extract on CYP protein expression

Western blot analysis was used to evaluate whether PN alter CYPs protein expression. PN extract at concentrations of 0.001, 0.01 and 0.1 mg/mL did not exhibit an induction effect on the expression of CYP1A2, CYP2C9, CYP2C19 and CYP3A4 proteins in primary human hepatocytes, based on western blotting (Figure 64). A selective inducer of CYP1A2, omeprazole, caused a 3-fold increase of CYP1A2 expression as compared to the non-treated control group. Similarly, rifampicin significantly increased CYP2C9, CYP2C19 and CYP3A4 protein expression by about 2- to 3-fold as compared to the non-treated control.
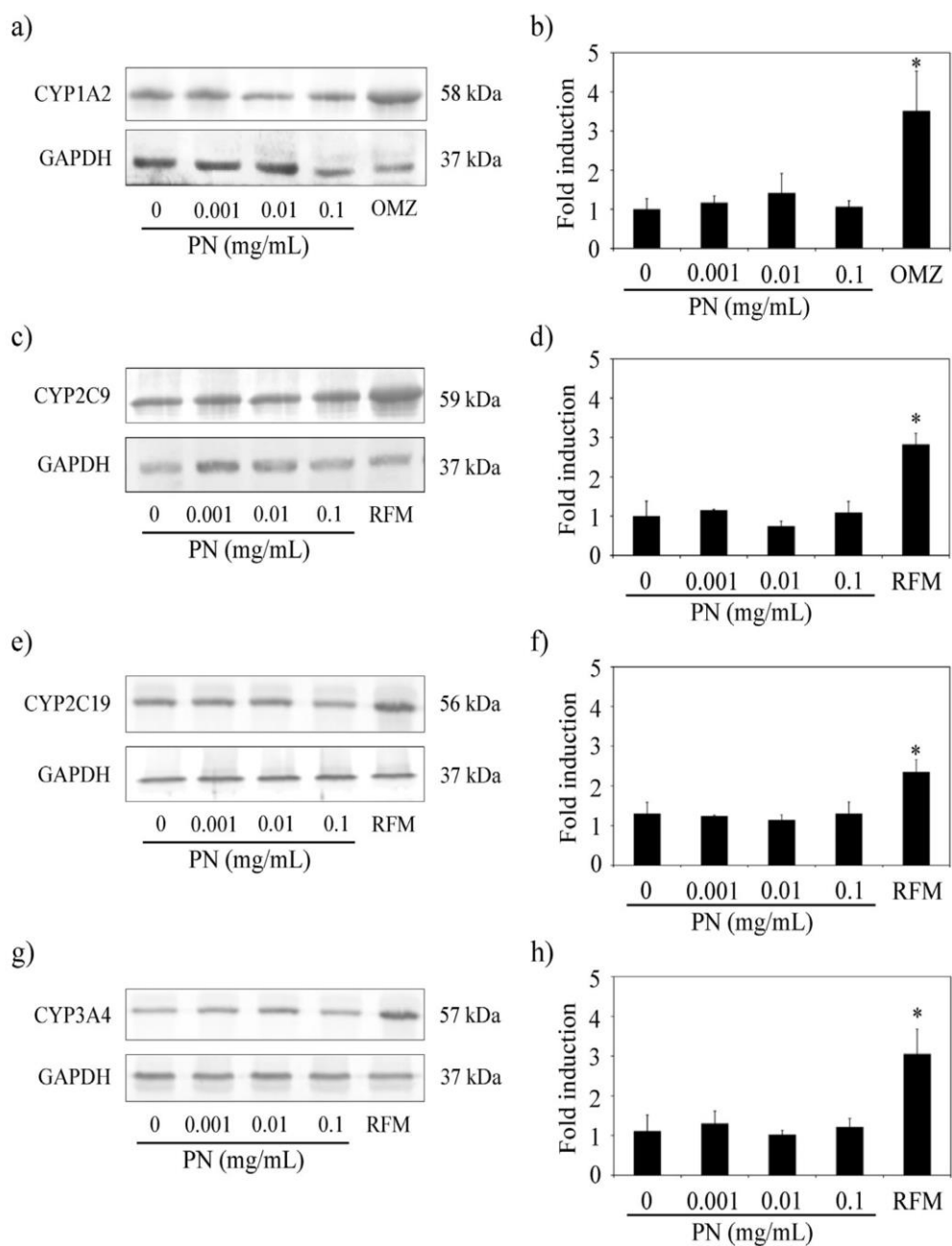
Figure 64. Effect of PN extract on CYP protein expression in primary human hepatocytes. The hepatocytes were treated with different concentrations (0-0.1 mg/mL) of PN for 48 h. CYP proteins were detected by SDS-PAGE Western blot analysis. Values are shown as means ± S.D. of 3 independent experiments. * $p < 0.05$; PN vs non-treated control. OMZ = omeprazole (100 µM), RFM = rifampicin (10 µM for CYP2C9 and CYP2C19; 50 µM for CYP3A4).

CHAPTER V

DISCUSSION AND CONCLUSION

1. **Hepatoprotective effect of PN extract**

In this study, hepatoprotective effect of PN extract against H_2O_2-induced oxidative stress was investigated using HepG2 which is a hepatocellular carcinoma cells that possess various antioxidant enzymes as in the normal cells. SOD, CAT and GPx activities as well as total GSH content in HepG2 cells are 2.8, 4.3, 2.9 and 1.4 folds higher than in Chang cells, the human normal cell line [79]. The higher GSH content in HepG2 cell is consistent to the significant higher activities of γ-glutamyl cysteine synthetase and glutathione synthetase as compared to that found in Chang cells. Two other glutathione-related enzymes, GR and GST are significant lower in HepG2 cells [75]. In addition, HepG2 cells have been widely used to assess the cytotoxic and antioxidant effects of many compounds by many groups of researchers [6], [80], [81]. In this present study, H_2O_2 at 500 µM was used to induce ROS and cause cytotoxic effect to HepG2 cells so as to investigate the hepatoprotective effect of PN. This concentration of H_2O_2 is used in other previous studies [76] and was shown in the present study that it significantly decreased cell viability to approximately 50% (Figure 14) and induced ROS about 6.26 folds of the non-treated cells (Figure 18). During investigation the hepatoprotective effect of PN extract against H_2O_2-induced oxidative stress, NAC was used as a positive control. Five millimolar of NAC was used to pretreated cells for 1 h prior to expose with H_2O_2. Even though NAC at 5 mM caused significant decreased of cell viability (with % cell viability of 90.84 ± 0.68%) as compared to the non-treated cells (Figure 15), this concentration of NAC attenuated ROS generation caused by H_2O_2 in HepG2 cells at 1, 2 and 6 h (Figure 18). Also, NAC at 5 mM and 1 h incubation has also been used in other studies [82], [83].

Thus, in this study NAC at 5 mM was used as positive control with the pretreatment time of 1 h.

To study the hepatoprotective effect of PN, various concentrations of PN (0-1 mg/mL) were firstly tested for its effect on cell viability. Even though significant decrease of cell viability was shown by PN at 0.1 (84.63 ± 5.82%) and 1 (49.69 ± 13.04% cell viability) mg/mL (Figure 13), ROS was only significantly generated by PN at 1 mg/mL but not at ≤ 0.1 mg/mL. Thus, this study chose the concentrations of PN upto 0.1 (such as 0.0001, 0.001, 0.01 and 0.1) mg/mL for the subsequent hepatoprotective study. The result showed that PN at 0.001, 0.01 and 0.1 mg/mL significantly attenuated ROS induction (Figure 19) and cell viability (Figure 21) against H_2O_2-induced cell injury in a concentration-dependent manner despite significantly less potent than NAC which was used as a positive control. However, times of PN incubation (3, 6 and 12 h) of each concentration of PN exhibited the same trend (Figure 21). Thus, PN was pretreated to cells for 3 h prior to expose cells to H_2O_2 in the subsequent study. In addition, the effects of PN on cellular antioxidant parameters (total GSH, GSH, GSSG and GSH/GSSG ratio) as well as the activities of antioxidant enzymes (CAT, GPx, GR and SOD) were proved to be comparable as the cells were further incubated with culture medium for 24 h (Figure 23, 25, 27, 29, 31, 33, 35 and 37). These results indicated that PN had already existed in the cells during the subsequent study that cells were exposed to H_2O_2 for 24 h after PN pretreatment. Hepatoprotective effects associated with ROS scavenging relating mechanism of PN were explained by our findings that in both normal and H_2O_2-induced oxidative stress conditions, PN increased the activities of various antioxidant enzymes such as CAT (Figure 43), GPx (Figure 44), GR (Figure 45) and SOD (Figure 46). Regarding the cellular antioxidant glutathione, PN extract itself did not much affect the level of total GSH (Figure 39), GSH (Figure 40) and GSSG (Figure 41) but the ratio

of GSH/GSSG ratio shown to be significant increased by PN extract (Figure 42). Under the H_2O_2-induced oxidative stress conditions, PN significantly attenuated the level of total GSH (Figure 39), GSH (Figure 40) with a decreased of GSSG (Figure 41). An increase of GSH with a decrease of GSSG resulting in an increase of GSH/GSSG ratio (Figure 42) was shown in a concentration dependent manner following PN pretreatment. As the GSH/GSSG ratio is regarded as a good indicator of oxidative stress status and DNA damage thus cell injury [84], an increase of this ratio by PN pretreatment supports our hypothesis that PN possesses hepatoprotective action associated to its antioxidant effects. These findings are consistent to the previous reports regarding effects of the extracts of herbs which are constituent in PN. The hepatoprotective effects of the extracts of Kot Sao [6], Kot Kan Prao [7], Kot Kradook [8], Kot Khamao [9] and Kot Chula-lumpa [10] were demonstrated *in vitro* (HepG2 cells, liver slice culture) or *in vivo* (mice, rats). Cytoprotective and antioxidant effects of the extracts on other tissues/cells were also investigated such as Kot Phung Pla on rat heart tissues [11], [85], Kot Hua Bua on endothelial cells [12], Kot Jatamansi on C6 glioma cell [13] and Kot Chiang in human [14]. They found that the extract increased the activities of the antioxidant enzymes such as CAT [11], GPx [10], [12], [85], GR [85], SOD [10], [12], GSH [11], vitamin C and E [11]. However, activation of Nrf2 and HO-1 proteins by these extracts of plants in PN has never been reported in the previous studies.

This study also investigated the hepatoprotective and antioxidant effects of β-sitosterol, one of the active constituents in PN extracts. This compound seemed to possess less antioxidant capacity using DPPH radical scavenging assay (Table 7). Concentrations of β-sitosterol used in the study were 10 and 20 μM, the concentrations which exhibited less cytotoxicity (Figure 16), attenuated intracellular ROS induction by H_2O_2 (Figure 20) and cell viability (Figure 22). Effects of β-sitosterol

alone or β-sitosterol pretreatment prior to H_2O_2 exposure on total GSH, GSH, GSSG and GSH/GSSG ratio (Figure 47-50) as well as the activities of antioxidant enzymes (Figure 51-54) were similar to results of PN extract.

This study further investigated the molecular mechanism of antioxidant effect of PN by assessing Nrf2 and HO-1 activation. The Nrf2 antioxidant response pathway is the primary cellular defense against the cytotoxic effects of oxidative stress. Nrf2 is localized in the cytoplasm under normal condition and bound to its inhibitor protein Kelch-like ECH-associated protein 1 (Keap1). The complex is disrupted by exposure to several stimuli, then free Nrf2 translocates to the nucleus to form heterodimers with small oncogene family proteins. This leads to the selective recognition of the antioxidant response element (ARE) on target genes, resulting in the regulation of gene expression of many cellular antioxidant enzymes such as CAT, SOD, GPx, GR [86] as well as phase II detoxifying enzymes such as HO-1, NAD(P)H dehydrogenase (quinone 1) (NQO1), glutathione S-transferase (GST) and glutamate cysteine ligase (GCL) [87]. HO-1 is an enzyme that catalyzes the degradation of heme. This produces biliverdin, iron, and carbon monoxide. Increased HO-1 activity attenuates cell apoptosis and decreases superoxide formation [88]. In this study, activation of Nrf2 and HO-1 were assessed by determine both protein levels via western blotting. H_2O_2 treated HepG2 cells showed a decrease of Nrf2 and HO-1 protein level which was reversed by pretreatment with PN (Figure 57). Increased expression of Nrf2 protein by PN explained the increased expression of various antioxidant enzymes following PN treatment found in this study. PN may also increase localization of Nrf2 from cytosol to nucleus as shown by the significant increase of nuclear Nrf2 under the H_2O_2-treated condition as compared to the non-treated control. Increased expression of HO-1, thus decreased superoxide formation as well as increased expression of various antioxidant enzymes via Nrf2 activation explain the protective effect of PN

against H_2O_2 induced oxidative stress in HepG2 cells in this study. β-sitosterol only attenuated HO-1 protein indicated the minor effect of β-sitosterol on the antioxidant effect of PN extract. No significant effect of incubation times of the PN extract (Figure 58) or β-sitosterol (Figure 60) on the expression of Nrf2 and HO-1 protein assured that the compounds were within the cells during 24 h of further incubation.

In conclusion, this study demonstrated that PN possessed hepatoprotective effect against H_2O_2-induced oxidative stress in HepG2 cells. The mechanism of this effect might be explained by an attenuation of ROS production, modulation of GSH level and activation of antioxidant enzymes including CAT, GPx, GR, HO-1 and SOD. Effects of PN on these antioxidant enzymes and the cellular antioxidant (GSH) were consistent to the findings of Nrf2 activation by PN extract.

2. Inhibition and induction effect of PN extract

This study focused on the inhibitory and inductive effects of PN on human CYPs to address the possibility of herb-drug interaction in concomitant use of PN with other drugs. In previous reports, extracts from herbs such as Kot Soa, Kot Kradook, Kot Phung Pla, Kot Chula-lumpa and Kot Hua Bua, which are constituents of PN, inhibit CYP3A in rats [52], [53]. Gallic acid, one of the main compounds in Kot Phung Pla and Kot Chula-lumpa [55], inhibits CYP3A activity in human liver microsomes [51]. In addition, Kot Soa also inhibits CYP2C and CYP2D in rats [89]. However, since there is species variation among CYPs in different animals [90], human liver microsomes and cryopreserved human hepatocytes are recommended for use in regulatory drug-drug interaction studies [74], [91]. In this study, the protocol for determining the IC_{50} was verified using a selective inhibitor of each CYP isoform. As shown in Table 5, the IC_{50} values of these compounds were consistent with those found in previous studies [70], [75], [76], [77], [78]. In human liver microsomes, PN extract inhibited the activities of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 with an IC_{50} of <100 μg/mL, but

had no or a negligible inhibitory effect on CYP2C19 and CYP2E1 at concentrations up to 1 mg/mL. In term of herb-drug interaction, inhibition of CYP enzyme activities can lead to drug toxicity or decrease of drug efficacy (in the case of prodrugs). From a safety perspective, close monitoring of the plasma concentration is required when drugs with a narrow therapeutic index, such as theophylline (metabolized via CYP1A2) and warfarin (metabolized via CYP2C9), are co-administered with compounds that are inhibitors of these CYP isoforms [92], [93]. Inhibition of CYP3A4 by PN might increase the risk of an adverse effect of rhabdomyolysis with certain statins [94]. In contrast, tamoxifen, a prodrug that undergoes biotransformation into an active form mainly by CYP2D6, might have a decreased therapeutic effect if administered with PN [95]. To characterize the type of inhibition and the *Ki* values of PN with CYP1A2, CYP2C9, CYP2D6 and CYP3A4, enzyme kinetic reactions were performed. Patterns of Lineweavere-Burk plots demonstrate the type of inhibition of PN with CYPs, while the *Ki* values indicate the potential for drug-drug interactions *in vivo* [96]. PN extract exhibited competitive (CYP1A2), mixed type (CYP2C9 and CYP2D6) and uncompetitive (CYP3A4) inhibition, with *Ki* values between 12 and 150 µg/mL. In basic models for making a decision to conduct an *in vivo* drug-drug interaction study, the degree of interaction (R) is estimated as R = 1+[I]/*Ki*. A clinical study using an appropriate probe substrate is recommended if the calculated R value is >1.1 [16]. In this equation, [I] is the mean steady-state C_{max} of total drug following administration of the highest proposed clinical dose or the concentration of inhibitor exposed to the active site of the enzyme. The *Ki* values are the apparent inhibitor constants obtained from this *in vitro* enzyme inhibition study (Figure 58 and Table 10). It is not possible to measure the blood PN concentrations because the extract comprises several compounds. Therefore, this study calculated an approximate [I] from the recommended single oral dose of PN (e.g. 100 mg) divided by the total body volume of 5 L [97], which gave a blood PN concentration of about 20 µg/mL.

As shown in Table 10, the calculated [I]/Ki resulted in a prediction that the extract is "possible" or "likely" to inhibit CYP1A2, CYP2C9, CYP2D6 and CYP3A4 *in vivo* according to the U.S. FDA Guidance for Industry (2006) [91]. The inhibitory effects of PN on CYP2C9, CYP2D6 and CYP3A4 are consistent with those in previous reports for herb extracts and phytochemicals which are the constituents of PN [52], [53], [51], [89], [98].

Generally, mechanism of CYPs inhibition can be classified as either reversible or time dependent inhibition (TDI). TDI are defined as irreversible covalent binding or quasi-irreversible noncovalent tight binding of reactive intermediate metabolite to the CYP that catalyzes its formation as a result in the loss of CYP activity. Perhaps one of the best characterized compounds that possessed TDI is the IC_{50} value shift of the ratio of IC_{50} values between with and without NADPH of a 30-minute pre-incubation of a potential inhibitor; a fold shift of IC_{50} values greater than 1.5-2 would indicate the presence of TDI [97]. In this study, an IC_{50} folds-shift of all CYPs investigated were <1.5 (Table 9), indicated that the inhibition of CYP1A2, CYP2C9, CYP2D6 and CYP3A4 found in this study was not the TDI. Further investigated on the kinetic parameters (K_I and K_{inact}) are not necessary.

Table 10. Prediction of clinical CYP inhibition by PN using the *in vitro* data

CYPs isoform	Ki (µg/mL)	[I]/Ki	Prediction of inhibition effects *in vivo*
1A2	34	0.59	Possible
2C9	80	0.25	Possible
2D6	12	1.67	Likely
3A4	150	0.13	Possible

Meanwhile, PN did not demonstrate inductive effect on CYP1A2, CYP2C9, CYP2C19 and CYP3A4 activities and on their protein expression. This negative results of *in vitro* CYP induction suggest that further *in vivo* induction study of PN on these CYP isoforms is not necessary [16]. In this study, we did not investigate the inductive effect of PN on CYP2D6 and CYP2E1. For the reasons, CYP2D6 is generally not found to be inducible, and drug interaction due to CYP2E1 induction is not often reported. In addition, the recent Guidance for industry on drug interaction studies (2012) [16] does not include these two CYP enzymes in the list of CYPs that are recommended to be investigated for the induction potential. Beside the herb-drug interaction issue, no inductive effect of PN on CYP1A2, CYP2C9, CYP2C19 and CYP3A4 is an advantageous characteristic of PN. The extract will not cause an increased risk of consumer to the toxicity, mutagenesis, and carcinogenesis from toxicants that are bioactivated by these CYP isoforms. For instant, CYP1A2 and CYP3A4 have been known to bioactivate many toxicants and procarcinogens [99]. Thus lack of inductive effect, together with the observed inhibitory effect of PN on both CYP isoforms are the favorable profile of the extract in this aspect.

In conclusion, PN extract inhibited CYP1A2, CYP2C9, CYP2D6 and CYP3A4, but not CYP2C19 and CYP2E1, in human liver microsomes, and did not induce CYP1A2, CYP2C9, CYP2C19 and CYP3A4 in primary human hepatocytes *in vitro*. Based on the *Ki* of PN for the four inhibited CYP enzymes, inhibition effects of PN *in vivo* are possible or likely. Therefore, precautions should be taken when PN is co-administered with drugs that are metabolized by CYP1A2, CYP2C9, CYP2D6 or CYP3A4.

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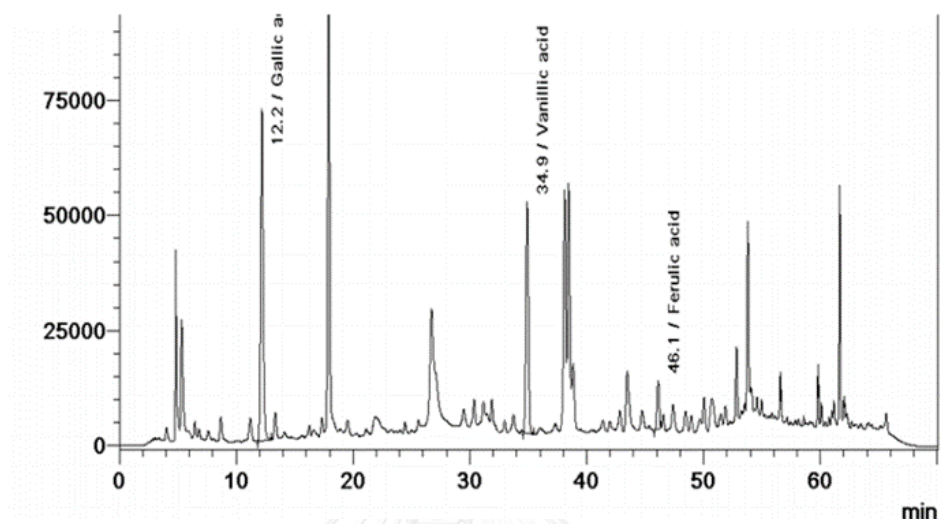
APPENDIX

Figure 65. Retention times of gallic acid, vanillic acid and ferulic acid in 50 mg of PN [68]

VITA

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