ฤทธิ์ปกป้องของฟิลแลนทินในเซลล์ตับเพาะเลี้ยงปฐมภูมิของหนูแรทที่ได้รับเอทานอล

นางสาวเหมวลา เชิดชูพันธ์เสรี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเภสัชศาสตร์ชีวภาพ
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

PROTECTIVE ACTIVITY OF PHYLLANTHIN IN ETHANOL-TREATED PRIMARY CULTURE OF RAT HEPATOCYTES

Miss Hemvala Chirdchupunseree

A Dissertation Submitted in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy Program in Biopharmaceutical Sciences

Faculty of Pharmaceutical Sciences

Chulalongkorn University

Academic year 2009

Thesis Title	PROTECTIVE ACTIVITY OF PHYLLANTHIN IN ETHANOL-TREATED PRIMARY CULTURE OF RAT HEPATOCYTES
By	Miss Hemvala Chirdchupunseree
Field of Study	Biopharmaceutical Sciences
Advisor	Associate Professor Pornpen Pramyothin, Ph.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Faculty of
(Associate Professor Pintip Pongpech, Ph.D.) Pharmaceutical Sciences

THESIS COMMITTEE

..... Chairman
(Associate Professor Nongluksna Sriubolmas, Ph.D.)

..... Advisor
(Associate Professor Pornpen Pramyothin, Ph.D.)

..... Examiner
(Associate Professor Pol. Lt. Col. Somsong Lawanprasert, Ph.D.)

..... Examiner
(Associate Professor Chaiyo Chaichantipyuth, Ph.D.)

..... Examiner
(Associate Professor Thitima Pengsuparp, Ph.D.)

..... External Examiner
(Professor Pawinee Piyachaturawat, Ph.D.)

เหมวลา เชิดชูพันธ์เสรี : ฤทธิ์ปกป้องของฟิลแลนทินในเซลล์ตับเพาะเลี้ยงปฐมภูมิของหนูแรทที่ได้รับเอทานอล (PROTECTIVE ACTIVITY OF PHYLLANTHIN IN ETHANOL-TREATED PRIMARY CULTURE OF RAT HEPATOCYTES) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.พรเพ็ญ เปรมโยธิน, 135 หน้า.

ทำการศึกษาฤทธิ์ปกป้องของฟิลแลนทิน (สารประกอบหลักของต้นลูกใต้ใบ *Phyllanthus amarus* Schum. et Thonn.) และกลไกในการปกป้องเซลล์ตับเพาะเลี้ยงปฐมภูมิของหนูแรทที่ได้รับเอทานอล เซลล์ตับเพาะเลี้ยงปฐมภูมิของหนูแรทได้รับฟิลแลนทิน (1, 2, 3, 4 ไมโครกรัม/มิลลิลิตร) 24 ชม. ก่อนให้เอทานอล (80 ไมโครลิตร/มิลลิลิตร) เป็นเวลา 2 ชม. พบว่าเอทานอลมีฤทธิ์ลด %cell viability เพิ่มการหลั่งเอนไซม์ transaminases (ALT และ AST) เพิ่มการสร้างสารอนุมูลอิสระ (ROS) ภายในเซลล์ และเพิ่มการเกิด lipid peroxidation ฟิลแลนทินแสดงฤทธิ์ปกป้องเซลล์โดยต้านผลดังกล่าวที่เกิดขึ้นจากความเป็นพิษของเอทานอล ฟิลแลนทินยังมีผลต่อความสามารถต้านออกซิเดชันภายในเซลล์ตับเพิ่มขึ้น ได้แก่ระดับ total glutathione การทำงานของเอนไซม์ superoxide dismutase (SOD) และ glutathione reductase (GR) ที่ลดลงจากการได้รับเอทานอล กลับสู่ระดับปกติ ฟิลแลนทินไม่มีผลต่อการทำงานของเอนไซม์ ADH และ ALDH ขณะที่การทำงานของ ALDH ถูกยับยั้งโดยเอทานอล การให้ฟิลแลนทินเป็นเวลา 24 ชม. มีผลเหนี่ยวนำการทำงานของ transcription factor Nrf2 ซึ่งพบว่ามีการทำงานลดลงเมื่อได้รับเอทานอลแบบเฉียบพลัน การให้ฟิลแลนทินเป็นเวลา 24 ชม. ก่อนการให้เอทานอล เพิ่มการแสดงออกของโปรตีนต้านออกซิเดชัน ได้แก่ SOD1 (Cu-ZnSOD), SOD2 (MnSOD), GR and GPx ในเซลล์ตับ จากผลการทดลองแสดงให้เห็นว่าฤทธิ์ปกป้องตับของฟิลแลนทินต่อการบาดเจ็บจากภาวะเครียดออกซิเดชันโดยเอทานอล เกิดจากฤทธิ์ในการต้านออกซิเดชันผ่านการกระตุ้นการทำงานของ transcription factor Nrf2 และการเหนี่ยวนำการแสดงออกของโปรตีนโดยเฉพาะเอนไซม์ SOD และ GR ซึ่งอยู่ภายใต้การควบคุมของ Nrf2.

สาขาวิชา เภสัชศาสตร์ชีวภาพ ลายมือชื่อนิสิต เหมวลา

ปีการศึกษา 2552 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก พรเพ็ญ

4976957133 MAJOR: BIOPHARMACEUTICAL SCIENCES

KEYWORDS : PROTECTIVE ACTIVITY / PHYLLANTHIN / ETHANOL / PRIMARY CULTURE OF RAT HEPATOCYTES

HEMVALA CHIRDCHUPUNSEREE: PROTECTIVE ACTIVITY OF PHYLLANTHIN IN ETHANOL-TREATED PRIMARY CULTURE OF RAT HEPATOCYTES. THESIS ADVISOR: ASSOC. PROF. PORNPEN PRAMYOTHIN, 135 pp.

In the present study, the protective activity of phyllanthin (a principal constituent of *Phyllanthus amarus* Schum. et Thonn.) and its possible hepatoprotective mechanisms were investigated against ethanol-induced hepatotoxicity in primary cultures of rat hepatocytes. Primary culture of rat hepatocytes (24 h culturing) were incubated with phyllanthin (1, 2, 3, 4 µg/ml) for 24 h prior to 2 h exposure with ethanol (80 µl/ml). Ethanol decreased %cell viability, increased the release of transaminases (ALT and AST) with the increase in the production of intracellular ROS and lipid peroxidation. Phyllanthin exhibited protective effect by attenuating on changes induced by ethanol. Phyllanthin also restored the antioxidant capability of rat hepatocytes including level of total glutathione and activities of superoxide dismutase (SOD) and glutathione reductase (GR) which were reduced by ethanol. Phyllanthin did not affect the activities of ethanol-metabolizing enzymes ADH and ALDH which ALDH activity was suppressed by ethanol. Twenty four hours pretreatment with phyllanthin effectively induced the activation of transcription factor Nrf2 which was reduced by acute ethanol treatment. This was concomitant with the increase in protein expression of antioxidant enzymes: SOD1 (Cu-ZnSOD), SOD2 (MnSOD), GR and GPx in hepatocytes pretreated with phyllanthin before giving ethanol. These results suggested the hepatoprotective effect of phyllathin against ethanol-induced oxidative stress causing rat liver cell damage due to its antioxidant activity through the activation of transcription factor Nrf2 and induction of protein expression especially antioxidant enzymes SOD and GR under the control of Nrf2.

Field of Study: Biopharmaceutical Sciences Student's Signature Hemvala.

Academic Year: 2009 Advisor's Signature

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude and sincere appreciation to my advisor, Associate Professor Pornpen Pramyothin, Ph.D. for her guidance, advice, kindness, encouragement and support throughout my study.

I sincerely thank Associate Professor Chaiyo Chaichantipyuth, Ph.D. for his guidance, suggestions and excellent help in plant identification and isolation of phyllanthin.

I wish to thank all staff members at many places as follows; Pharmacological Action of Natural Products Research Unit, Department of Pharmacology and Physiology; Department of Biochemistry and Microbiology; Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University for their kind assistances.

I would like to thank the Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for supporting the instrument.

This research work is supported by Grant to Support High Potential Research Unit, Ratchadaphiseksomphot Endowment Fund and the Graduate Research Fund from the Graduate school, Chulalongkorn University.

My special thanks also extend to all my friends and laboratory members for their help, sincerity, kindness and friendship.

Finally, I wish to express my deepest appreciation and infinite gratitude to my parents for their love, unconditional encouragement, understand and support given to me that they are deserved to be mentioned as a part of my success.

CONTENTS

LIST OF TABLES

page

LIST OF FIGURES

page

page

page

page

page

LIST OF ABBREVIATIONS

ADH	=	Alcohol dehydrogenase
ALD	=	Alcoholic liver disease
ALDH	=	Aldehyde dehydrogenase
ALT	=	Alanine aminotransferase
ARE	=	Antioxidant response element
AST	=	Aspartate aminotransferase
ATP	=	Adenosine triphosphate
CAT	=	Catalase
CCl_4	=	Carbon tetrachloride
Cu-ZnSOD	=	Copper-zinc superoxide dismutase
DMSO	=	Dimethyl sulfoxide
EtOH	=	Ethanol
FT-IR	=	Fourier Transform Infrared Spectrometer
GPx	=	Glutathione peroxidase
GR	=	Glutathione reductase
GSH	=	Reduced glutathione
GSSH	=	Oxidized glutathione/glutathione disulfide
GST	=	Glutathione *S*-transferase
HPLC	=	High performance liquid chromatography
Keap1	=	Kelch-like-ECH-associated protein 1
MAPK	=	Mitogen activated protein kinase
MnSOD	=	Manganese superoxide dismutase
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD^+	=	Nicotinamide adenine dinucleotide
NADPH	=	Reduced nicotinamide adenine dinucleotide phosphate
NF-κB	=	Nuclear factor-kappa B
Nrf2	=	Nuclear factor-erythroid 2-related factor 2
PBS	=	Phosphate buffered saline

ROS	=	Reactive oxygen species
SDS-PAGE	=	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SOD	=	Superoxide dismutase
TBARS	=	Thiobarbituric acid reactive substances
TBST	=	Tris buffered saline-Tween 20
TLC	=	Thin layer chromatography
g	=	Gram
U/L	=	Unit per liter
^{o}C	=	Degree Celsius
µl	=	Microliter
µg	=	Microgram

CHAPTER I

INTRODUCTION

Background and Rationale

Ethanol, a widely consumed organic solvent, is the most common cause of chronic liver disease worldwide. In the liver, the oxidation of ethanol by cytosolic alcohol dehydrogenase (ADH) gives rise to acetaldehyde, which is further oxidized to acetate by mitochondrial aldehyde dehydrogenase (ALDH).

Ethanol can initiate cell injury by variety of mechanisms. The steps are likely to differ in acute and chronic exposures to ethanol (Zima and Kalousova, 2005). The production of acetaldehyde, a highly reactive intermediate, is acutely toxic. It binds covalently to proteins and other macromolecules (Paradis et al., 1996; Niemela, 1999; Barry, 2006). Moreover, oxidation of ethanol causes the alteration of the redox status (decrease in the NAD^+/NADH ratio) and damage to mitochondria resulted in the reduction of ATP production. Ethanol also affects the immune systems and alters cytokine production (e.g. interleukine-8, TNF-α and leukotriene B4) with the increase in bacterial derived endotoxin which subsequently activates the Kupffer cells (Wheeler, 2003; Rao, Seth, and Sheth, 2004).

One deleterious factor in ethanol metabolism is the potential for generating excess amounts of reactive oxygen species (ROS) which triggers liver injury by a variety of different pathways. Recently, there are evidences supported that acute and chronic ethanol exposure affects cells and tissues injury by mechanisms that involve oxidative stress, a well recognized key step in pathogenesis of ethanol-induced liver injury which plays a central role in the development of alcoholic liver disease (Sergent et al., 2001; Zima et al., 2001; Hoek and Pastorino, 2002; Wu and Cederbaum, 2003).

ROS can highly react with and damage biomolecules such as fats, proteins, and DNA. These results may have profound effects on cell viability and function leading to cell death and tissue damage. In addition, ethanol also reduces the levels of antioxidants that can eliminate ROS and also interferes with the regulation of cellular stress response machinery. Oxidative stress usually occurred from the imbalance

between pro-oxidants and endogenous antioxidants (Koch et al., 2004; Das and Vasudevan, 2007).

Signal transduction mechanisms involving ethanol and its metabolites are still largely unknown. However, it has been reported that the development of alcoholic liver disease is related to the activation of mitogen activated protein kinase (MAPK) signaling and the activation of transcription factors such as nuclear factor-κB (NF-κB), activating protein 1 (AP-1) and nuclear factor-erythroid 2-related factor 2 (Nrf2). These responses are cell-type specific and depend on the dose of ethanol (Roma´n et al., 1999; Jokelainen, Reinke, and Nanji, 2001; Zima and Kalousova, 2005; Lamlé et al., 2008).

Nrf2, the basic leucine zipper (bZip) transcription factor, regulates the expression of important cytoprotective enzymes in response to oxidative stress. Nrf2 may play a role in protecting liver, as evidenced by increased mortality associated with liver failure in ethanol-fed *Nrf2*$^{-/-}$ mice (Lamlé et al., 2008). Some studies demonstrated the key role of Nrf2 in the adaptive response against oxidative stress involving CYP2E1 in livers or hepatocytes of chronic alcohol-fed or pyrazole-treated rats and mice (Cederbaum, 2006, 2009). HepG2 cells expressing CYP2E1 (E47 cells) increased Nrf2 mRNA and protein expression (when compared with control HepG2 C34 cells) by the increase in the up-regulation of Nrf2-regultated genes, glutamate cysteine ligase catalytic subunit (GCLC) and heme oxygenase 1 (HO-1) (Gong and Cederbaum, 2006; Cederbaum, 2009).

Phyllanthin, a lignan compound, was isolated from *Phyllanthus amarus* Schum. et Thonn. (family: Euphorbiaceae; In Thai “Luk Tai Bai”) This herbal medicine traditionally applied in the treatment of many liver diseases (Figure 1).

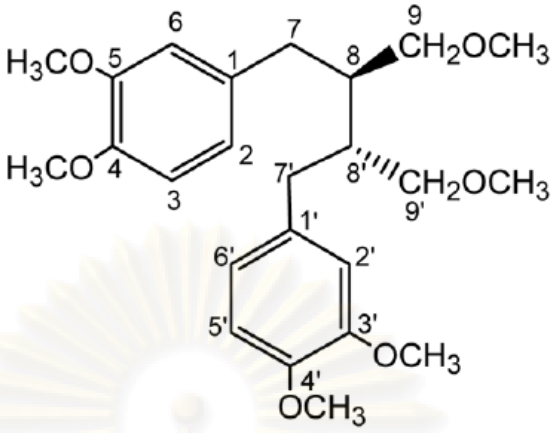


Figure 1: Chemical structure of phyllanthin (MW 418.53).

Several pharmacological actions of *Phyllanthus amarus* have been reported including anti-viral, anti-inflammatory and hepatoprotective activities (Kiemer et al., 2003; Notka, Meier, and Wagner, 2003; Raphael and Kuttan, 2003; Notka, Meier, and Wagner, 2004; Chattopadhyay, Agrawal, and Garg, 2006; Harish and Shivanandappa, 2006; Kassuya et al., 2006; Naaz, Javed, and Abdin, 2007; Pramyothin et al., 2007; Sarkar and Sil, 2007; Faremi et al., 2008; Sabir and Rocha, 2008; Harikumar, Kuttan, and Kuttan, 2009a; Krithika and Verma, 2009). On contrary, there were few studies examining the pharmacological activity of phyllanthin, a hepatoprotective marker of *Phyllanthus amarus*. Phyllanthin showed the protection against carbon tetrachloride (CCl_4) and galactosamine induced cytotoxicity in primary culture of rat hepatocytes (Syamasundar et al., 1985). In the *in vitro* study, phyllanthin and hypophyllanthin exhibited their hepatoprotection through a mechanism independent of β-glucuronidase inhibition (Joshi and Priya, 2007).

The antioxidative ability of crude extracts from plants belonging to *Phyllanthus* species and phyllanthin was reported to be strong to moderate as measured by the ability to quench DPPH free radical (Harish and Shivanandappa, 2006; Fang, Rao, and Tzeng, 2008). Twenty four hours pretreatment with phyllanthin in smaller concentrations showed higher antioxidative and hepatoprotective properties than the extract of *Phyllanthus amarus* in CCl_4-induced HepG2 cell injury (Krithika et al., 2009).

Ethanol metabolism occurs predominantly in the liver which is the most frequently and intensively studied organ. The deleterious effect of ethanol exposure depends essentially on animal models which is applied to disclose and define the roles of genetic and environment factors (Siegmund, Haas, and Singer, 2005). In the present study, acute ethanol treatment was performed in primary culture of rat hepatocytes. This selected model render to evaluate direct effects of test compound on ethanol treated cells to clarify the molecular mechanisms of cytotoxicity and cytoprotection.

Since the molecular mechanism involving antioxidant activity of phyllanthin against ethanol induced hepatotoxicity has not yet been done. This study aimed to investigate the protective activities of phyllanthin and its possible mechanisms by using primary culture of rat hepatocytes as the model and ethanol as the hepatotoxin.

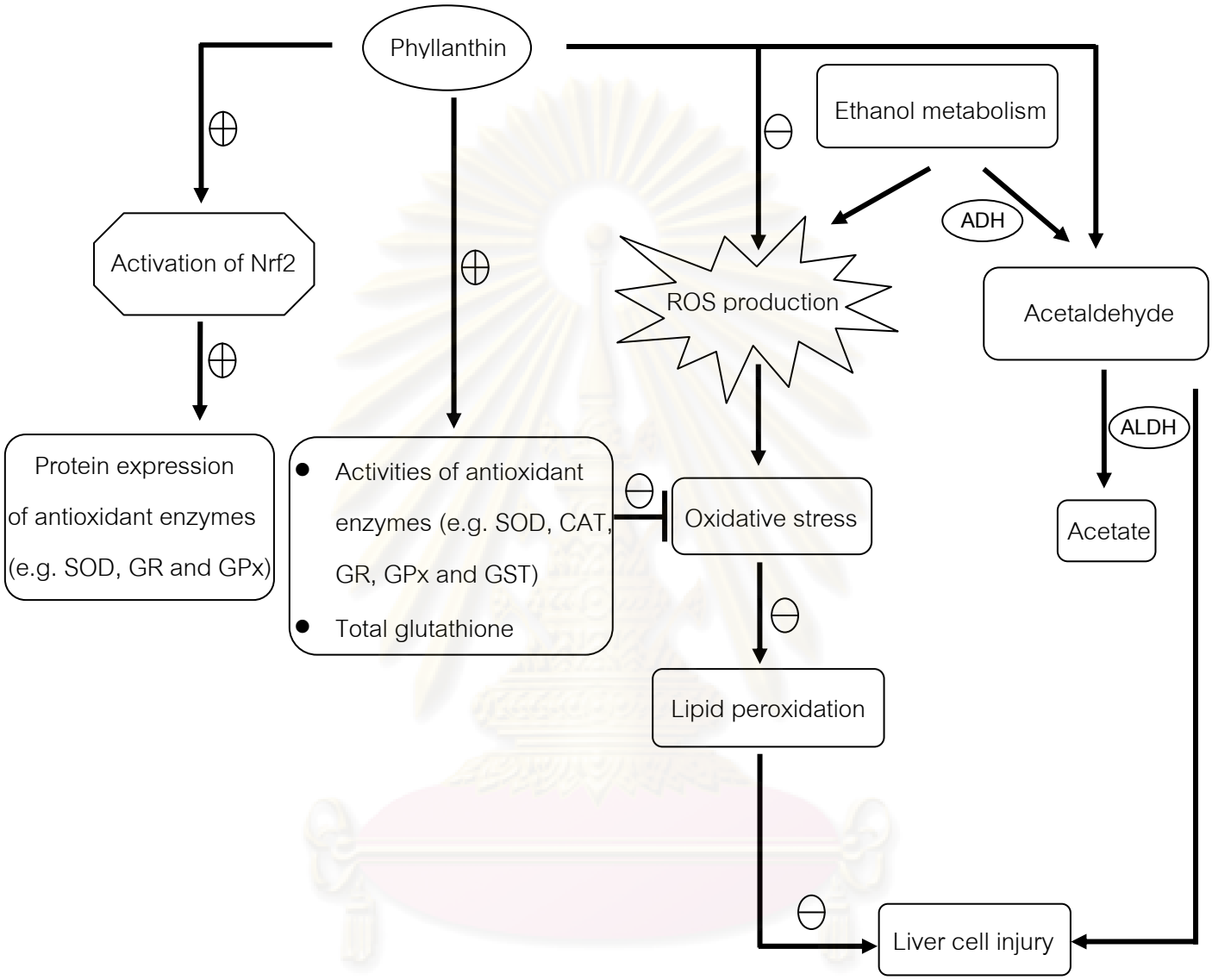
Hypothesis

1. Phyllanthin has cytoprotective activity in ethanol-treated primary culture of rat hepatocytes.
2. Phyllanthin has effect on the activities of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx)), glutathione *S*-transferase (GST) and ethanol-metabolizing enzymes (alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)) in ethanol-treated primary culture of rat hepatocytes.
3. Phyllanthin has antioxidant activities via the activation of transcription factor Nrf2 and the expression of cytoprotective proteins in ethanol-treated primary culture of rat hepatocytes.

Objectives

1. To investigate cytoprotective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes.
2. To investigate the possible cytoprotective mechanism of phyllanthin. The effect of phyllanthin on the activities of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx)), glutathione *S*-transferase (GST) and ethanol-metabolizing enzymes (alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH)) in ethanol-treated primary culture of rat hepatocytes were examined.
3. To investigate the molecular mechanism of antioxidant activities of phyllanthin involving the activation of transcription factor Nrf2 and the expression of cytoprotective proteins in ethanol-treated primary culture of rat hepatocytes.

Conceptual framework



Ethical problem

The protocols of experiment in animals in this project were approved by the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Animal Care and Use Committee (IACUC) (Animal Use Protocol No. 09-33-015 and Approval No. 09-33-015). The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.

Expected benefit from the study

Be able to explain and provide more insight information of the hepatoprotective activity of phyllanthin against ethanol-induced liver cell injury with its possible mechanisms.

This important information may be of therapeutic strategies in attempts to ameliorate or prevent the toxic effect of ethanol.

CHAPTER II

LITERATURE REVIEWS

Phyllanthus amarus

Phyllanthus amarus Schum. et Thonn. (Euphorbiaceae) grows widely in the tropical and subtropical parts of all countries. It is an annual, glabrous herb grows up to 15-60 cm. high with erect stem, naked below and slender and spreading leaf branches. Leaves are numerous, sub-sessile, pale green often distichously imbricate, glaucous below, elliptic to oblong, obtuse and subulate. Flowers arise in leaf axis, very numerous, males 1-3 and females solitary. Sepals of male are orbicular and obovate to oblong in females (Figure 2).

Figure 2: *Phyllanthus amarus* (http://plantes-rizieres guyane.cirad.fr/dicotyledones/euphorbiaceae/phyllanthus_amarus).

Leaves and other aerial portions of the plant are used in the traditional medicine for a variety of ailments including flu, dropsy, diabetes, jaundice, asthma, bronchial infections and diuretic and to lower blood pressure. In addition, it is considered one of the best herbs for treating liver disorders.

Several compounds are inherent in *Phyllanthus amarus* including phyllanthin, hypophyllanthin, phyltetralin, nirtetralin, niranthin, trimethyl-3,4-dehydrochebulate, methylgallate, gallic acid, ellagic acid, rhamnocitrin, methyl brevifolincarboxylate, β-sitosterol-3-*O*-β-D-glucopyranoside, quercitrin, rutin, amariin, 1-galloyl-2,3-dehydrohexahydroxydiphenyl(DHHDP)-glucose, repandusinic acid, geraniin, corilagin, phyllanthusiin D and quercetin-3-O-glucoside (Dhalwal, Biradar, and Rajani, 2006; Leite et al., 2006; Fang, Rao, and Tzeng, 2008; Londhe et al., 2008, 2009).

Phyllanthin is the bioactive lignan and major phytoconstituent presented in whole plant of *Phyllanthus amarus* and also found in plants of other *Phyllanthus* species.

Pharmacological studies of *Phyllanthus amarus*

Several pharmacological activities of *Phyllanthus amarus* were reported as follows:

Antiviral activity

Phyllanthus amarus has the ability to suppress virally induced cancers. It showed inhibitory activity on HIV replication both *in vivo* and *in vitro* studies. The water/alcohol extract of *Phyllanthus amarus* blocked HIV-1 attachment and the HIV-1 enzymes integrase, reverse transcriptase and protease to different degrees. Moreover, a gallotannin containing fraction and the isolated ellagitannins, geraniin and corilagin were shown to be the most potent mediators for these antiviral activities. Administration of *Phyllanthus amarus* to volunteers could reduce HIV replication by more than 30% (Notka, Meier, and Wagner, 2003, 2004).

Oral administration of the extract of *Phyllanthus amarus* decreased the progression of FMuLv-induced erythroleukemia. Treatment with plant induced the expression of p53 and p45NFE2 and decreased the expression of Bcl-2 in the spleen of infected mice (Harikumar, Kuttan, and Kuttan, 2009a).

Antimicrobial activity

The methanolic extract of *Phyllanthus amarus* showed significant concentration-dependent antibacterial activity particularly against gram-negative microbes (Mazumder, Mahato, and Mazumder, 2006). Its ethanolic extract showed antibacterial

properties by inhibiting all strains tested with minimum inhibitory concentrations of 0.25 to 16 mg/ml (Kloucek et al., 2005).

Antifungal activity

The antifungal activity of various solvent extracts (ether, chloroform, ethyl acetate and ethyl alcohol) of *Phyllanthus amarus* was investigated and found that chloroform fraction of the aerial part showed significant inhibitory effect against dermatophytic fungi (*Microsporum gypseum*) (Agrawal et al., 2004).

Antinephrotoxic activity

Aqueous extract of *Phyllanthus amarus* was reported to have the protective effect on acetaminophen- and gentamicin-induced nephrotoxic rats. Pretreatment with 100-400 mg/kg/day of aqueous extract attenuated the elevations of serum creatinine, blood urea nitrogen and tubulonephrosis caused by acetaminophen (Adeneye and Benebo, 2008).

Radioprotective activity

In vitro and *in vivo* studies indicated that *Phyllanthus amarus* has potential protective activity against radiation-induced lethality, lipid peroxidation and DNA damage (Jagetia, 2007). Its extract (250 and 750 mg/kg) inhibited the myelosuppression and elevated the levels of antioxidant enzymes in the blood and liver in mice (Harikumar and Kuttan, 2007).

Antiinflammatory activity

The methanolic extract of *Phyllanthus amarus* (50, 200, and 1000 mg/kg) significantly inhibited gastric lesions induced by intragastric administration of absolute ethanol (8 ml/kg) (Raphael and Kuttan, 2003).

EtOH/H_2O and hexane extracts showed an inhibition of LPS-induced production of NO and PGE_2 in KC and in RAW264.7. Both extracts reduced expression of iNOS and COX-2 and inhibited activation of NF-kappaB, but not AP-1.

Phyllanthus amarus inhibited induction of interleukin(IL)-1beta, IL-10, and interferon-gamma in human whole blood and reduced TNF-alpha production *in vivo* (Kiemer et al., 2003).

In mice model, the hexane extract (HE), the lignan-rich fraction (LRF), or the isolated lignans (phyltetralin, nirtetralin, niranthin) exhibited marked anti-inflammatory properties by inhibiting carrageenan-induced paw edema and neutrophil influx (Kassuya et al., 2005). Hexane extract (100 µg/ml) and niranthin (30 µM) (but not nirtetralin or phyltetralin) decreased the specific binding of [(3)H]-PAF in mouse cerebral cortex membranes. Moreover, niranthin exhibited anti-inflammation and anti-allodynia which probably mediated through its direct antagonistic action on the platelet activating factor (PAF) binding sites (Kassuya et al., 2006).

Hypoglycemic, hypocholesterolemic and anti-hypertensive activities

The methanolic extract of *Phyllanthus amarus* at 200 and 1000 mg/kg reduced blood sugar in alloxan diabetic rats by 6% and 18.7%, respectively (Raphael, Sabu, and Kuttan, 2002). The aqueous leaf and seed extracts (150, 300 and 600 mg/kg, p.o.) decreased the fasting plasma glucose and cholesterol and reduced body weight in mice (Adeneye, Amole, and Adeneye, 2006). Aqueous leaf extract (5 mg to 80 mg/kg, i.v.) showed blood pressure lowering effect in normotensive male rabbits probably by the combined effects of myocardial depression, muscarinic receptor mediated vascular smooth muscle relaxation and calcium channel ion blockade in vascular smooth muscle (Amaechina and Omogbai, 2007).

Anticarcinogenic and antimutagenic activities

Phyllanthus amarus extracts have anticarcinogenic and antimutagenic activities both *in vivo* and *in vitro*. Methanolic extract was able to inhibit the activation and mutagenicity of 2-acetaminofluorene (2-AAF), aflatoxin B_1 and direct acting mutagens including sodium azide (NaN(3)), N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and 4-nitro-*0*-phenylenediamine (NPD) (Raphael et al., 2002). In addition, oral administration of *Phyllanthus amarus* extract significantly inhibited urinary mutagenicity produced in rats by benzo[a] pyrene (Raphael et al., 2002).

Aqueous extract treatment exhibited potent anticarcinogenic activity against 20-methylcholanthrene (20-MC) induced sarcoma development and increased the survival of tumor harboring mice (Rajeshkumar et al., 2002). Aqueous extract of the entire plant showed antimutagenic effect against the induction causing by 2-aminofluorene (AF2), 2-aminoanthracene (2AA) and 4-nitroquinolone-1-oxide (4-NQO) in *Salmonella typhimurium* strains TA98 and TA100, and in *Escherichia coli* WP2 u vrA/pKM101 (Sripanidkulchai et al., 2002).

The 75% methanolic extract (250 and 750 mg/kg, p.o.) reduced the toxic side effects of cyclophosphamide (CTX) in mice by inhibiting the myelosuppression and improving WBC count, bone marrow cellularity as well as the number of maturing monocytes (Kumar and Kuttan, 2005).

The hexane extract, the lignans-rich fraction and the isolated lignans (nirtetralin, niranthin or phyllanthin) showed cytotoxic effect and multidrug resistance (MDR) reversing properties in human leukaemia cell lines, K-562 and its vincristine-resistant counterpart Lucena-1 (Pgp-overexpressing subline) (Leite et al., 2006). Administration of *Phyllanthus amarus* extract with N-methyl-N-nitro-N-nitrosoguanidine (MNNG) reduced stomach cancer in Wistar rats (Raphael et al., 2006).

The apoptotic effect of *Phyllanthus amarus* was evaluated. At doses 100 and 200 µg/ml showed the induction of caspase-3 and inhibition of Bcl-2 expression in DLA cells culture (Harikumar, Kuttan, and Kuttan, 2009b).

Hepatoprotective activity

Ethanolic extract of *Phyllanthus amarus* (0.3 g/kg/day, p.o.) showed the protective effect on aflatoxin B_1-induced liver damage in mice. It exhibited strong hepatoprotective capability in reducing the intracellular level of reactive oxygen species by enhancing both enzymatic and non-enzymatic antioxidant levels, it enhances liver regeneration against alcohol induced liver injury in partial hepatectomised Albino rats (Chattopadhyay, Agrawal, and Garg, 2006; Naaz, Javed, and Abdin, 2007). Administration of aqueous extract of *Phyllanthus amarus* protected against carbon tetrachloride (CCl_4)-induced liver damage in female mice (Krithika and Verma, 2009).

Aqueous extract of *Phyllanthus amarus* protected rat liver cell injury from acute and chronic ethanol induced toxicity by bringing the levels of AST, ALT, HTG and TNF-α back to normal similar to silymarin the reference hepatoprotective agent (Pramyothin et al., 2007). In the *in vitro* study, aqueous extract (1-4 mg/ml) increased %MTT reduction assay and decreased the release of transaminases (AST and ALT) in primary cultures of rat hepatocytes being treated with ethanol (Pramyothin et al., 2007).

Both *in vivo* and *in vitro* studies, an alcoholic extract inhibited activities of aniline hydroxylase (an indicator of CYP 2E1 activity, IC_{50} 50 µg/ml) and aminopyrine demethylase (an indicator of CYP 1A, 2A 2B, 2D and 3A activities, IC_{50} >1000 µg /ml) (Harikumar and Kuttan, 2006).

Hepatoprotective effect of aqueous, ethanolic and methanolic extracts was due to their antioxidant activities (Harish and Shivanandappa, 2006; Pramyothin et al., 2007; Sarkar and Sil, 2007; Faremi et al., 2008; Sabir and Rocha, 2008).

The methanolic leaf extract (250 and 500 mg/kg/day) may protect the liver against ethanol-induced oxidative damage by possibly reducing the rate of lipid peroxidation and increasing the antioxidant defence mechanism in rats (Faremi et al., 2008).

Rat liver mitochondria and pBR322 plasmid DNA were used as *in vitro* models to study radioprotective activity of pure compounds isolated from *Phyllanthus amarus.* The result showed that the radioprotective activity of ellagitannins (amariin, 1-galloyl-2,3-dehydrohexahydroxydiphenyl(DHHDP)-glucose, repandusinic acid, geraniin, corilagin phyllanthusiin D) and flavonoids (rutin, quercetin-3-O-glucoside) may due to their ability to scavenge different radicals more or less efficiently resulted in the relieve of oxidative stress (Londhe et al., 2009). Their ability to scavenge free radicals including 2,2-diphenyl-2-picrylhydrazyl (DPPH), 2,2-azobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) / ferrylmyoglobin, ferric reducing antioxidant power (FRAP) and pulse radiolysis was also evaluated. Amariin, repandusinic acid and phyllanthusiin D showed stronger antioxidant activity among the ellagitannins and were comparable to the flavonoids (rutin and quercetin 3-O-glucoside) (Londhe et al., 2008).

The hepatoprotective and antioxidative property of *Phyllanthus amarus* extract and phyllanthin were compared in CCl_4 induced toxicity in HepG2 cell line. After 24 hours pretreatment with phyllanthin and extract, phyllanthin showed higher activity with smaller concentration (Krithika et al., 2009).

Liver

The liver is the largest glandular organ of the body. It receives a dual blood supply consisting of the hepatic portal vein and hepatic arteries. Supplying approximately 75% of the liver's blood supply, the hepatic portal vein carries venous blood drained from the spleen, gastrointestinal tract, and its associated organs. The hepatic arteries supply arterial blood to the liver, accounting for the remainder of its blood flow. Blood flows through the sinusoids and empties into the central vein of each lobule. The central veins coalesce into hepatic veins, which leave the liver and empty into the inferior vena cava.

Liver plays a major role in metabolism and has a number of functions in the body, including glycogen storage, decomposition of red blood cells, plasma protein synthesis, hormone production, and detoxification. It is particularly frequent target organs of toxicity that are often injured or selective injured by xenobiotics, both experimentally and clinically.

Ethanol metabolism

Three metabolic pathways of ethanol involve the following enzymes: alcohol dehydrogenase (ADH), microsomal ethanol oxidation system (MEOS) and catalase.

When consumed in moderate amounts, the major part of ethanol is metabolized by alcohol dehydrogenase in the liver.

$$CH_3CH_2OH + NAD^+ \longrightarrow CH_3CHO + NADH + H^+$$

In this reaction a hydride ion is transferred from ethanol to NAD^+ (Cunningham and Bailey, 2001).

The microsomal electron transport system also participates in ethanol oxidation via catalysis by the cytochrome P450 isoenzymes (Lieber and DeCarli, 1970). The enzymes in this family include 2E1, 1A2 and 3A4 isoforms (Salmela et al., 1998), which vary in their capacity to oxidize ethanol. They catalyze the following reaction sequence:

$$CH_3CH_2OH + NADPH + H^+ + O_2 \longrightarrow CH_3CHO + NADP^+ + 2H_2O$$

The cytochrome CYP2E1 isoform is induced by chronic ethanol consumption.

Peroxisomal activity also contributes to ethanol oxidation in the liver, as is seen in the following reactions:

$$RCH_2CH_2COSCoA + O_2 \xrightarrow{\text{AcylCoA oxidase}} RCH = CHCOSCoA + H_2O_2$$

$$CH_3CH_2OH + H_2O_2 \xrightarrow{\text{Catalase}} CH_3CHO + 2H_2O$$

Ethanol oxidation gives rise to acetaldehyde, which is further oxidized by hepatic aldehyde dehydrogenase (ALDH).

$$CH_3CHO + NAD^+ \longrightarrow CH_3COOH + NADH + H^+$$

The mitochondrial form of aldehyde dehydrogenase plays a prominent role in maintaining a low concentration of acetaldehyde. The acetate is then activated by acetyl CoA synthase to acetyl CoA (Cunningham and Bailey, 2001).

Mechanism of ethanol (alcohol) induced tissue injury

Ethanol acts through numerous pathways to affect the liver and other organs, leading to the development of alcoholic liver disease (ALD) (Wu and Cederbaum, 2003; Day, 2006) (Figure 3).

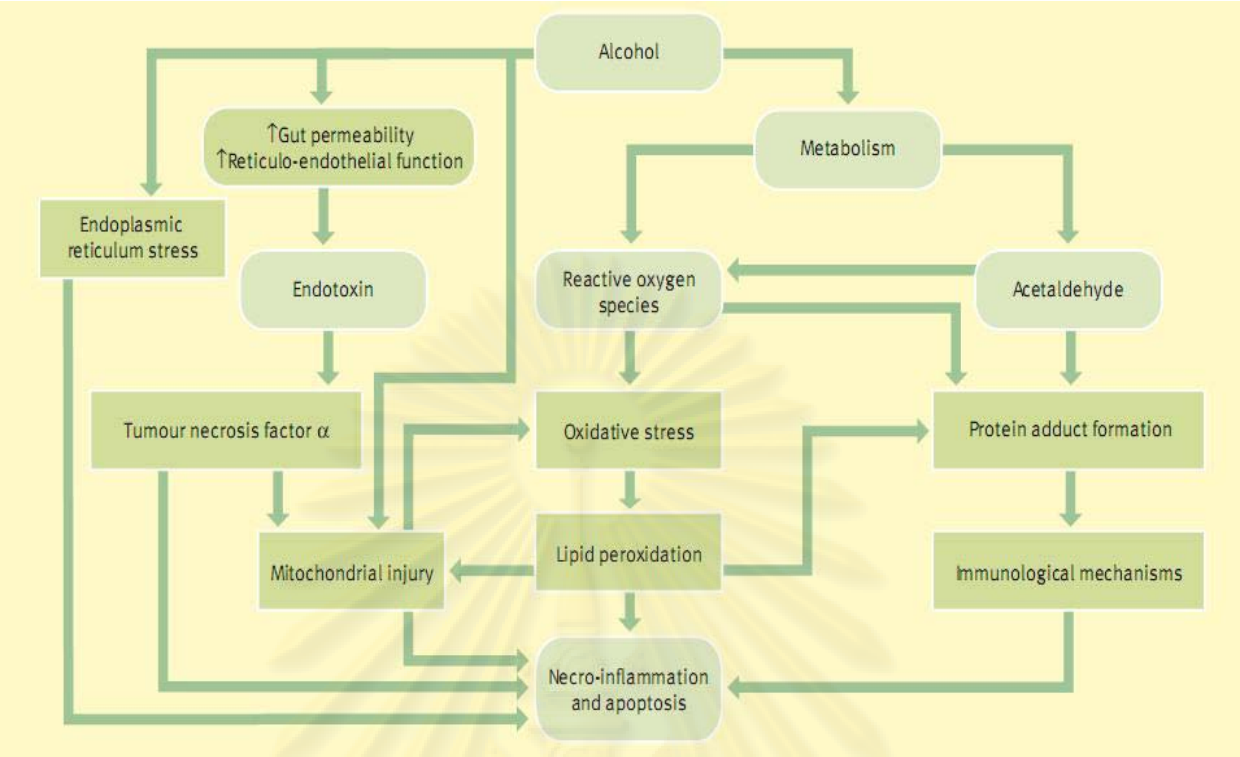


Figure 3: Possible mechanisms of ethanol (alcohol) induced tissue injury (Day, 2006).

The excessive free radicals production is one factor that has been reported as a key role in many pathways of alcohol-induced liver injury and has been focused in many studies. Reactive oxygen species (a class of oxygen-containing free radicals) are very important molecules in damaging or causing complete degradation of cellular essential complex molecules such as lipids, proteins and DNA. Several studies shown that both acute and chronic ethanol exposures can increase ROS generation and enhance peroxidation of lipids, proteins and DNA.

Many factors and processes are involved in causing ethanol-induced oxidative stress, including

- Ethanol metabolism by alcohol dehydrogenase and aldehyde dehydrogenase leads to formation of one molecule of NADH. These changes in the NAD^+/NADH ratio, provide more starting material and raise activity of the respiratory chain reaction including heightened oxygen use and ROS production.

- Ethanol metabolism by alcohol dehydrogenase increases the production of acetaldehyde (reactive metabolite) which interacts with cellular proteins and lipids and leads to radical formation results in cell damage.
- Ethanol induced oxidative stress through the impairment of cellular antioxidant defense mechanisms including antioxidant enzymes and glutathione (GSH).
- Induction of CYP2E1 in chronic ethanol administration.
- Activation of Kupffer cells.

Oxidative stress

Oxidative stress occurs in the presence of an excess of reactive molecules called free radicals and/or impairing of antioxidant molecules which can eliminate them.

Xenobiotic-induced oxidative stress not only can cause direct oxidative cell injury, due to the production of strong oxidant, but is also involved in signal transduction and the regulation of gene expression via redox-sensitive mechanisms.

Free radicals and cell damage

Free radicals contain unpaired electrons and therefore seek to obtain other electrons to produce a stable pair in its own molecules. They are highly reactive molecules that interact with other cellular structures.

Reactive oxygen species (ROS) are small, highly reactive oxygen-containing molecules. Small amounts of reactive oxygen species are constantly generated in aerobic organisms in response to both external and internal stimuli. ROS produce in cells include hydroxyl radicals ($HO^{\bullet}$), superoxide anions ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). The hydroxyl radical is particularly unstable and will react rapidly and non-specifically with most biological molecules. This species is produced from hydrogen peroxide in metal-catalyzed redox reactions such as the Fenton reaction.

These oxidants can damage cells by starting chemical chain reactions such as lipid peroxidation, or by oxidizing DNA or proteins. Damage to DNA can cause mutations and possibly cancer, if not reverse by DNA repair mechanisms, while damage to proteins can cause enzyme inhibition, protein denaturation and degradation.

Lipid peroxidation

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. Two important features characterize lipid peroxidation and distinguish it from the oxidation of other cellular compartments including

- The original radical-induced damage at a particular site in a membrane lipid is readily amplified and propagated in a chain-reaction-like fashion, thus spreading and damaging across the membrane.
- The products arising from lipid peroxidation (e.g. alkoxy radicals and toxic aldehydes) may be equally reactive as the original ROS themselves and damage cells by additional mechanism.

Mechanism of lipid peroxidation

As with any radical reaction the reaction consists of three major steps: initiation, propagation and termination (Figure 4).

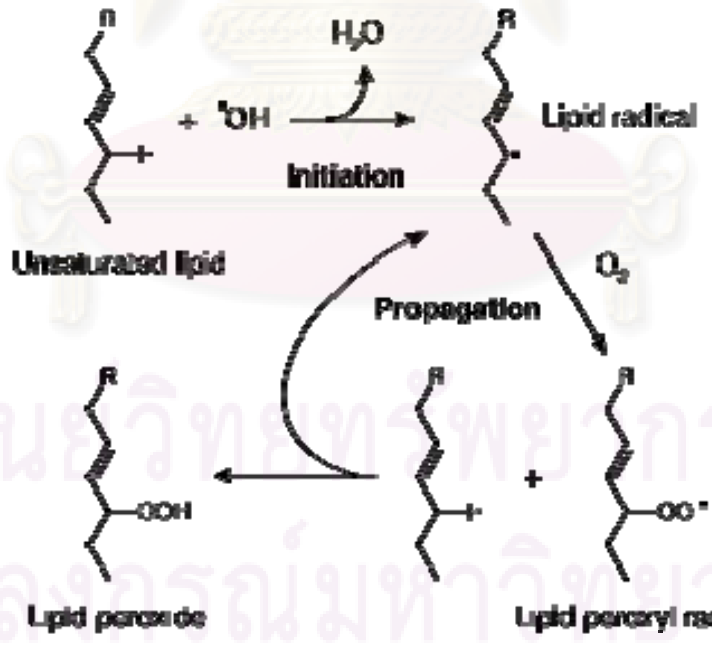


Figure 4: Mechanism of lipid peroxidation.

(http://en.wikipedia.org/wiki/Lipid_peroxidation)

Initiation

Initiation is the step whereby a fatty acid radical or lipid radical is produced. The initiators in living cells are most notably reactive oxygen species (ROS), such as $OH^{\bullet}$, which combines with a hydrogen atom from fatty acid to make water and lipid radical.

Propagation

The lipid radical is not a very stable molecule, so it reacts readily with molecular oxygen, thereby creating a lipid peroxyl radical. This is an unstable species that reacts with another free fatty acid producing a different fatty acid radical and a hydrogen peroxide or lipid peroxide if it had reacted with itself. This cycle continues as the new fatty acid radical reacts in the same way.

Termination

When a radical reacts it always produces another radical, which is why the process is called a "chain reaction mechanism". The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough for there to be a high probability of two radicals actually colliding. Living organisms have evolved different molecules to catch free radicals and protect the cell membrane. One important antioxidant is alpha-tocopherol, also known as vitamin E. Other anti-oxidants made within the body include the enzymes superoxide dismutase, catalase, and peroxidase.

Antioxidant defense mechanisms

All aerobic living forms have defenses against ROS. An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols or polyphenols.

Although oxidation reactions are crucial for life, they can also be damaging so plants and animals maintain complex systems of multiple types of antioxidants, such as

glutathione, vitamin C and vitamin E as well as enzymes such as catalase, superoxide dismutase and various peroxidases. Low levels of antioxidants, or inhibition of antioxidant enzymes, cause oxidative stress and may damage or kill cells.

Three important intracellular enzymes constitute antioxidant defense include superoxide dismutase (SOD), catalase, and GSH peroxidase/GSSG reductase system.

Superoxide dismutases

Superoxide dismutases (SODs) are metalloenzymes which catalyze the dismutation of superoxide ($O_2^{-\bullet}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2) at a rate 10^4 times as fast as spontaneous dismutation at neutral pH. SODs serve an important antioxidant defense in nearly on cells exposed to oxygen. They have been shown to play a key role in protecting cells and tissues against oxidative stress.

Three different forms of superoxide dismutase have been characterized in eukaryotes:

- A copper- and zinc-containing form (Cu-ZnSOD or SOD1) is a 32 kDa dimeric protein that was initially described as being restricted to the cytoplasm. However, recently it has also been detected in lysosomes, peroxisomes, nuclei and the mitochondrial intermembrane space.
- A manganese-containing form (MnSOD or SOD2) is widely distributed in bacteria, yeast, plants and animals. It is a 40 kDa enzyme with four subunits in mammals that contains a Mn^{3+} ion in the catalytic site. MnSOD is insensitive to cyanide and is located in the mitochondria. The activity of MnSOD in mammals is about 1 to 10% of the total SOD activity (Cu-ZnSOD plus MnSOD). The enzyme plays a pivotal role in safeguarding mitochondria from oxidative stress as well as in regulating intramitochondrial $O_2^{-\bullet}$ concentration (and consequently H_2O_2 concentration).
- A copper- and zinc-containing form in the extracellular matrix (EC-SOD or SOD3) is a tetrameric glycoprotein with molecular mass of 135 kDa. There are several isoforms of EC-SOD, and they seem to be bound to cell surface, especially in the lung and blood vessels. The biological function of EC-SOD

appears to be connected with the extracellular control of $O_2^{-\bullet}$ interaction with $^{\bullet}NO$ (which forms $ONOO^-$).

The major form is SOD1 which accounts for nearly 90% while the mitochondrial enzyme, SOD2, is approximately 10%.

Catalase

Catalase (CAT) decomposes H_2O_2 to water and oxygen. Mammalian catalase is a homotetrameric protein of about 240 kDa that contain one heme group and one NADPH molecule per subunit. The enzyme is localized primarily in peroxisomes, but smaller amounts are found in mammalian heart mitochondria; both of these organelles are responsible for intense H_2O_2 production. Catalase is also present in most organisms, including vertebrates, invertibrates, plants, fungi and bacteria. In vertibrates, catalase is present in all tissues with particularly high activity in erythrocytes, liver, kidney and adipose tissue. The Michaelis-Menten constant (K_m) of catalase is higher than the K_m of glutathione peroxidase (GPx), which suggests that catalase scavenges H_2O_2 efficiently at high H_2O_2 concentrations.

Glutathione redox cycle

The glutathione redox cycle is a mechanism which scavenges lipid peroxides and H_2O_2. The enzymes in the cycle include glutathione peroxidase (GPx) and glutathione reductase (GR) (Figure 5).

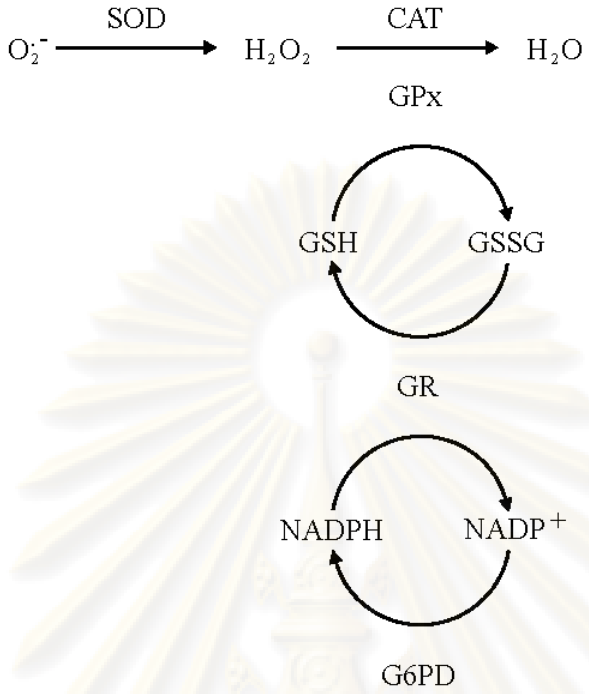


Figure 5: Glutathione redox cycle; GPx = Glutathione peroxidase, GSH = Glutathione, GSSG = Oxidized glutathione, GR = Glutathione reductase, NADP = Nicotinamide adenine dinucleotide phosphate, NADPH = Reduced nicotinamide adenine dinucleotide phosphate, G6PD = Glucose 6-phophate dehydrogenase (http://herkules.oulu.fi/isbn9514266625/html/x494.html).

In this reaction glutathione (GSH) is used as a cosubstrate to metabolize H_2O_2 to H_2O and oxidized glutathione (GSSG). Oxidized glutathione (GSSG) is reduced back to GSH by glutathione reductase (GR). This reaction requires NADPH regenerated by glucose 6-phosphate dehydrogenase (G6PD). The capacity to recycle GSH makes the GSH cycle a pivotal antioxidant defence mechanism for cells to prevent the depletion of cellular thiols.

Glutathione peroxidase

Glutathione peroxidases (GPx) are four different selenoenzymes that have similar function. These enzymes are typical in animal kingdom, some of them have found in plants and yeast. GPx is a homotetramer with one selenium atom per subunit and ranges in size from 76 to 105 kDa depending on animal species. GPx catalyses the

decomposition of H_2O_2, some organic hydroperoxides (such as fatty acid hydroperoxide, LOOH), and artificial substrates (cumene hydroperoxide and *tert*-butyl hydroperoxide) using GSH as a cosubstrate.

GPx localizes in the cytosol and mitochondria. Organs with high metabolic rate such as liver, lung and kidney produce more H_2O_2 by their mitochondria and have higher activities of GPx than do other tissue. The glutathione cycle is complementary to catalase in scavenging H_2O_2. The K_m value of GPx for H_2O_2 is lower than that for catalase and in contrast to catalase, GPx also reduces lipid peroxides. Several studies have suggested that GPx is not merely complementary to catalase but has a critical role in the inhibition/prevention of cellular oxidative challenge.

Glutathione reductase

Glutathione reductase (GR) is a flavoprotein that catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH), which can again act as antioxidants scavenging reactive oxygen species in the cell. This enzyme is an important cellular antioxidant. It is essential for the GSH redox cycle which maintains adequate levels of cellular GSH. The activity of glutathione reductase is used as indicator for oxidative stress. The regeneration of glutathione through GR requires NADPH, which in turn is regenerated through glucose-6-phosphate dehydrogenase (G6PD).

Glutathione

Glutathione (GSH) is a tripeptide found in most forms of aerobic life. It contains an unusual peptide linkage between the amine group of cysteine and the carboxyl group of the glutamate side chain. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced.

In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems as well as reacting directly with oxidants. Due to its high concentration and its central role in maintaining the cell's redox state, glutathione plays important roles in critical

physiological functions and protection against oxidative damage. It functions by detoxifying electrophiles and scavenging free radicals. Its homeostasis is maintained by a balance between the rate of synthesis and the combined rate of utilization and loss through efflux. The biosynthesis of GSH is first catalyzed by □-glutamylcysteine synthetase (□-GCS) and then by glutathione synthetase (GSS) (Figure 6).

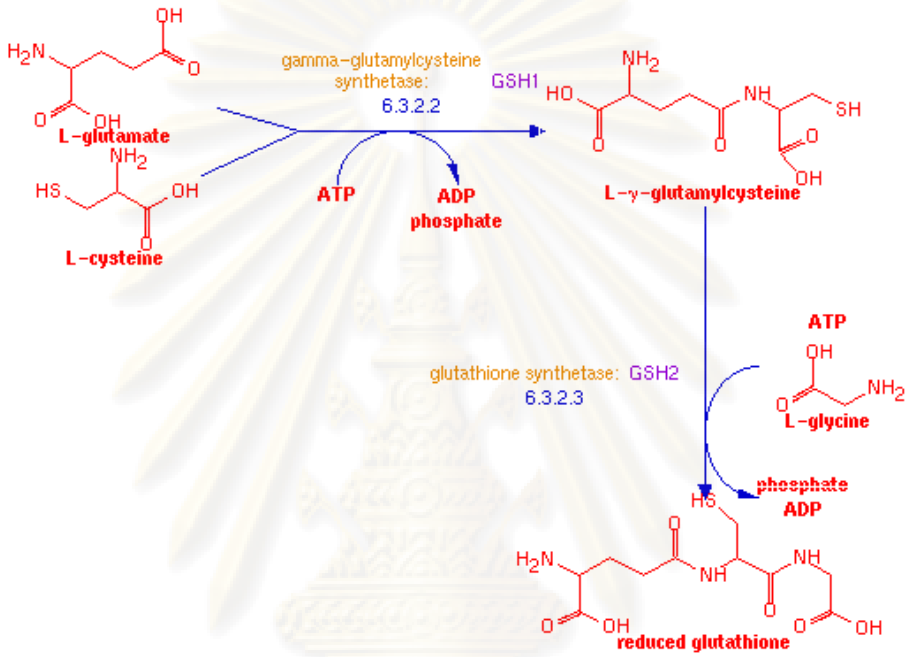


Figure 6: The biosynthesis of GSH.

GSH is known as substrate in both conjugation reactions and reduction reactions, catalyzed by glutathione *S*-transferase enzymes in cytosol, microsomes and mitochondria. In healthy tissue, more than 90% of total glutathione pool is in the reduced form and less than 10% exists in the disulfide form (GSSG). An increased GSSG/GSH ratio is considered indication of oxidative stress. So, the ratio of reduced to oxidized glutathione within cells is often used scientifically as a measure of cellular toxicity.

Glutathione *S*-transferase

Glutathione *S*-transferase (GST) constitutes a large family of multifunctional enzymes involved in GSH conjugation to xenobiotics and aldehydes from lipid peroxidation. Conjugate forms in hepatic cells are often excreted into bile using ATP-dependent pumps.

These enzymes can constitute up to 10% of cytosolic protein in some mammalian organs. GST catalyses the conjugation of reduced glutathione via the sulfhydryl group, to electrophilic centers on a wide variety of substrates. This activity is important for the detoxification of endogenous compounds as well as the metabolism of xenobiotics. GST may also bind toxins and function as transport proteins.

The mammalian GST super-family comprises of cytosolic dimeric isoenzymes (40 to 55 kDa sizes) which have been assigned to at least four generic classes: Alpha, Mu, Pi and Theta. These classes are based upon N-terminal amino acid sequences, substrate specificities, sensitivity to inhibitors, isoelectric point and immunological analysis. Most mammalian GST isoenzymes can catalyse 1-chloro-2, 4-dinitrobenzene (CDNB) so the spectrophotometric assays utilizing this substrate are commonly used to report total GST activity. Mammalian GST isoforms also have cell specific distribution e.g. alpha GST in hepatocytes and pi GST in the biliary tract of the human liver.

Biochemical adaptation

Biochemical adaptation which leads to change into structure, function, regulation and integration of biological molecules and metabolic processes is necessary for two main reasons:

1. All biological molecules and all biochemical reactions are directly susceptible to perturbation by multiple environmental parameters such as temperature, pressure, pH, ionic strength, solute concentrations, water availability, radiation and attack by free radicals.
2. All cells and all organisms, in order to remain viable must maintain an adequate level of energy turnover through an adequate supply of the energy currencies of the cells, primarily adenosine triphosphate (ATP) which is used to drive

thermodynamically unfavorable reactions and reduced nicotinamide adenine dinucleotide phosphate which is used for reductive biosynthetic reactions.

Mechanisms of metabolic regulation

Multiple mechanisms of metabolic regulation employed in biochemical adaptation are shown in Table1.

Table1: Some mechanisms of biochemical adaptation (Storey, 2004).

Enzyme/Protein Level
Changes in protein or enzyme amount
Changes in isoform/isozyme type and properties
Changes in the concentrations of substrates and effectors of enzymes
Changes in the kinetic and regulatory properties of enzymes
Covalent modification of enzymes and proteins
Protein-protein binding and changes in subcellular location
Influence of low-molecular-weight stabilizers
Transcription and translational Levels
Evolution of novel protein or enzyme types
Changes in response elements and transcription factors
Control over protein translation

Enzyme adaptation

Central to biochemical adaptation is the control of enzyme function, and multiple mechanisms have been designed to tailor enzymes for optimal function in the cells/tissues/organisms in which they reside and to provide an appropriate range of responses to deal with metabolic demands and environmental stresses.

Changes in enzyme or protein amount

The amount of each enzyme or protein in a cell is a primary determinant of the capabilities of different cells, tissues and species, and changes in enzyme/protein amount provide the coarse control of metabolism that is a key part of cellular response to many external signals (hormones, environmental stresses, etc.).

Stress-induced changes in enzyme amount

Change in the amount of cellular enzyme redefines the maximum capacity of its catalyzed reaction. Numerous signals or stresses can stimulate changes in the amount of an enzyme or protein in a cell. This coarse control typically occurs over a relatively long time frame. It involves adjustments to protein synthesis (transcription and/or translation) or protein degradation and differs from short-term responses to the same stress that can be achieved via fine control on the activity of individual enzyme molecule.

Changes in enzyme and protein properties

Alterations of enzymes make change in substrate/ligand affinity. Evolutionary time has also produced isoforms of proteins (encoded on different genes yet catalyzing the same reaction) and alloforms (protein variants of a single gene that can have slightly different properties). Isoforms may differ in their tissue distribution, subcellular location, expression patterns at different developmental stages, functional roles and regulatory mechanisms and can play critical roles in defining the metabolic capabilities of different tissues.

Adaptive control of transcription

Change in the amounts of selected proteins in cells is a major mechanism of biochemical adaptation. Regulation of gene transcription and protein translation are integral to biochemical adaptation. Transcriptional control, which can be utilized to achieve biochemical adaptation, including

- Evolution of new genes encoding new protein types that address key adaptive needs.

- Duplication of genes to insert multiple copies in the genome to greatly increase the number of mRNA transcripts that are produced in response to a signal.
- Modification of existing or elaboration of new response elements in the 5' untranslated region of a gene so that transcription of an individual gene can respond to a new transcription factor or to new combination of factors.
- Elaboration of new transcription factors or modification of the stimuli that activate transcription factor synthesis to provide a new or modified transcription factor response to a signal. Modification of transcription factor response (in either type or amount) would typically alter the responses of all target genes under its control to provide a modified yet coordinated response by multiple genes to a signal.
- Modification of one or more elements of the signal transduction pathway leading from cell surface to gene expression that may include: the cell surface receptor, a second messenger metabolite, and one or more protein kinases often linked in a cascade that ultimately regulate transcription factor synthesis. Multiple components in the pathway allow for multiple regulatory inputs that could permit:
    - One signal to be spread out to initiate multiple effects on different aspects of cell metabolism such as up-regulation of selected genes, stimulation of fuel metabolism, and modulation of membrane ion conductance.
    - Diverse signals to activate the same coordinated set of responses or subsets of the responses.
    - Signal response to be modulated by inputs from other extracellular or intracellular sources.

Transcription factor Nrf2

Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) is a basic leucine zipper transcription factor that binds and activates the antioxidant response element (ARE) in the promoters of many antioxidant and detoxification genes.

Under unstressed conditions, the transcription factor Nrf2 interacts with the actin-anchored protein Keap1 (the Kelch-like-ECH-associated protein 1), largely localized in the cytoplasm. It is thought to be continually degraded by the proteasome. This quenching interaction maintains low basal expression of Nrf2-regulated genes. However, upon recognition of chemical signals imparted by oxidative and electrophilic molecules, Nrf2 is released from Keap1, escapes proteasomal degradation and translocates to the nucleus. Numerous signaling events including oxidation of thiols on Keap1, phosphorylation of Nrf2 and nuclear localization/import signals on Keap1 and Nrf2 likely participate in Nrf2 nuclear translocation.

Nrf2 heterodimerizes with small Maf or Jun proteins, binds antioxidant response elements (AREs) in the upstream region of target genes and activates gene transcription, maintaining a counterbalance to Nrf2 and balancing the oxidation level of the intracellular environment. The Nrf2-mediated antioxidant defense is composed of numerous genes involved in cell stress response, drug metabolism, detoxification, and transport (Lee and Surh, 2005; Aleksunes and Manautou, 2007; Kensler, Wakabayashi, and Biswal, 2007) (Figure 7).

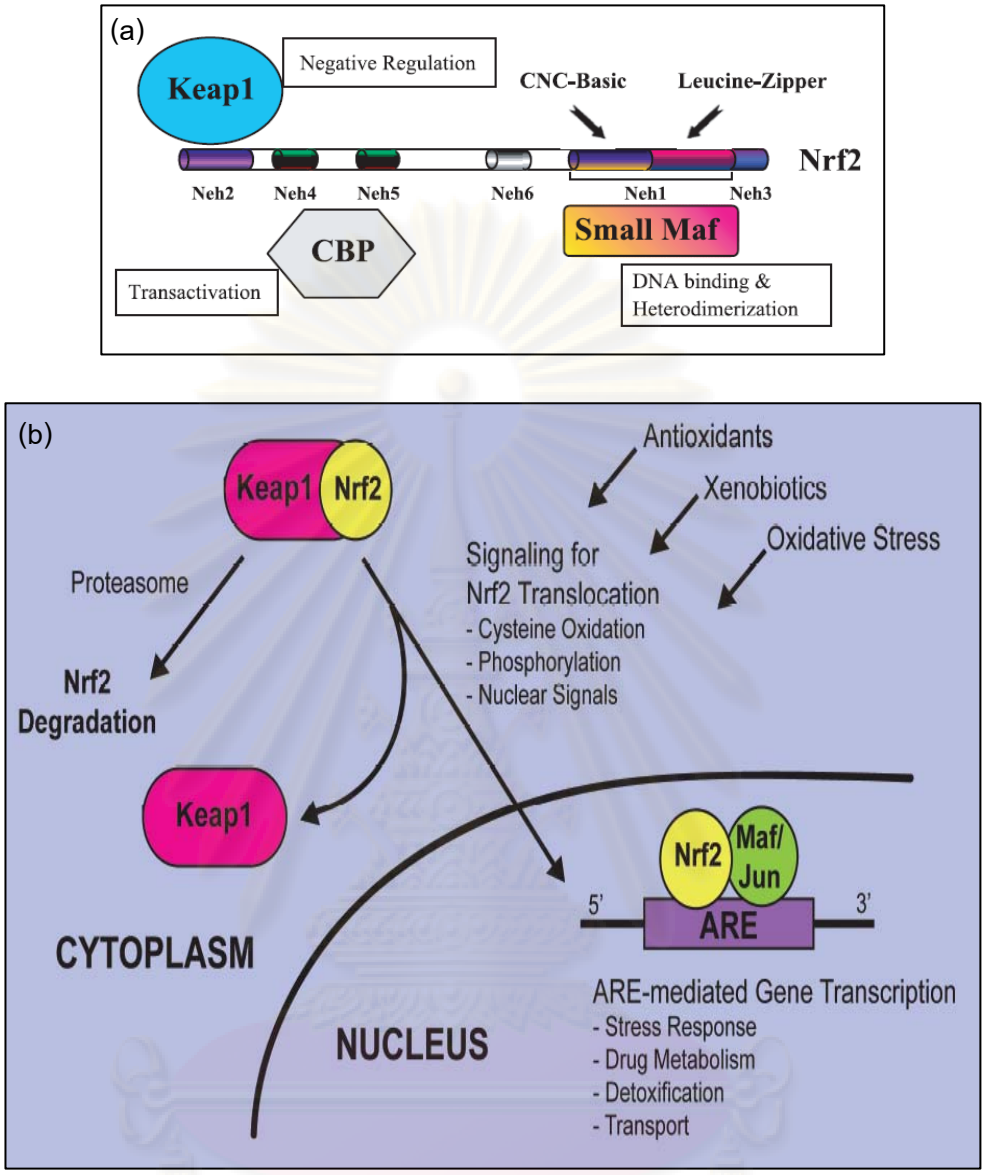
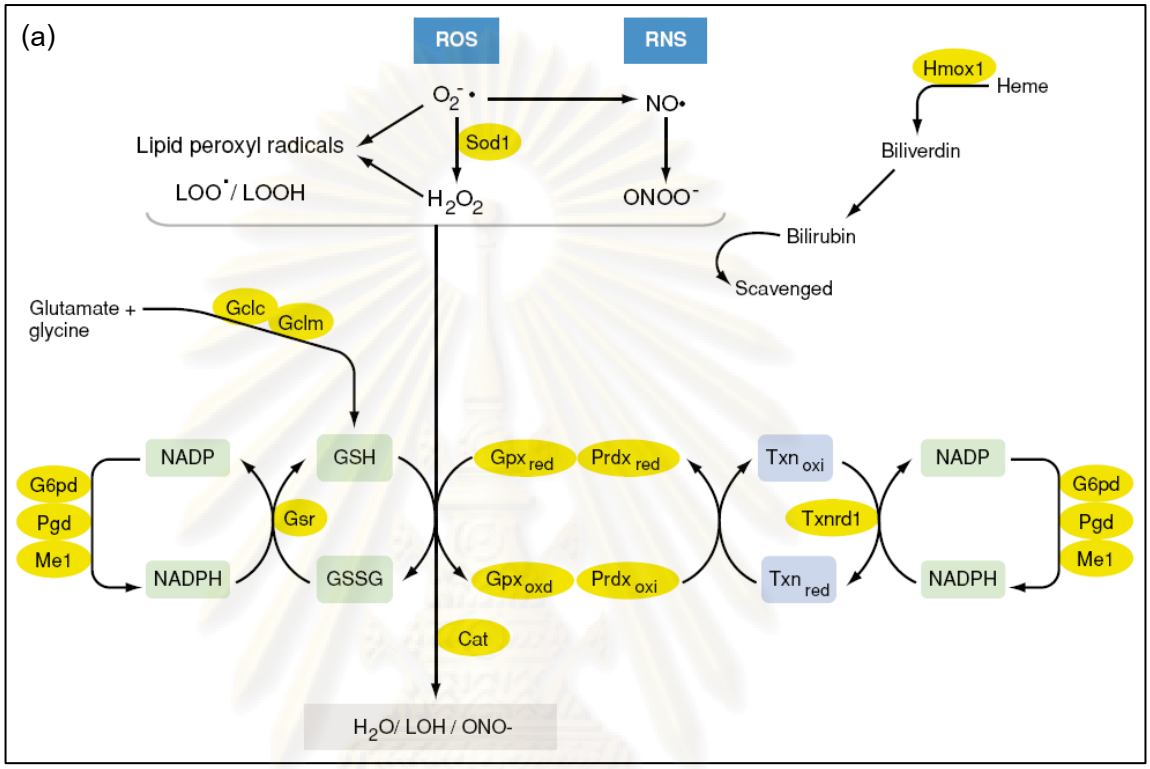


Figure 7: Nrf2 regulatory network; *(a)* Nrf2 has six highly conserved protein regions called Neh1 to Neh6 (Nrf2-ECH homology). At its C terminus, Nrf2 protein has a basic leucine zipper structure for dimerization with small Maf proteins and for binding to the ARE. At its N terminus, Nrf2 has a Neh2 domain, which is most highly conserved among species, and two activation domains, Neh4 and Neh5. Keap1 binds Neh2, whereas Neh4 and Neh5 cooperatively bind CBP (cAMP-responsive element binding protein) to activate transcription, *(b)* Regulation of Nrf2-mediated gene transcription (Itoh, Tong, and Yamamoto, 2004; Aleksunes and Manautou, 2007).

Nrf2 is the primary transcription factor protecting cells from oxidative stress by regulating cytoprotective genes, including the antioxidant glutathione (GSH) pathway, antioxidant proteins and phase II detoxifying enzymes (Figure 8).



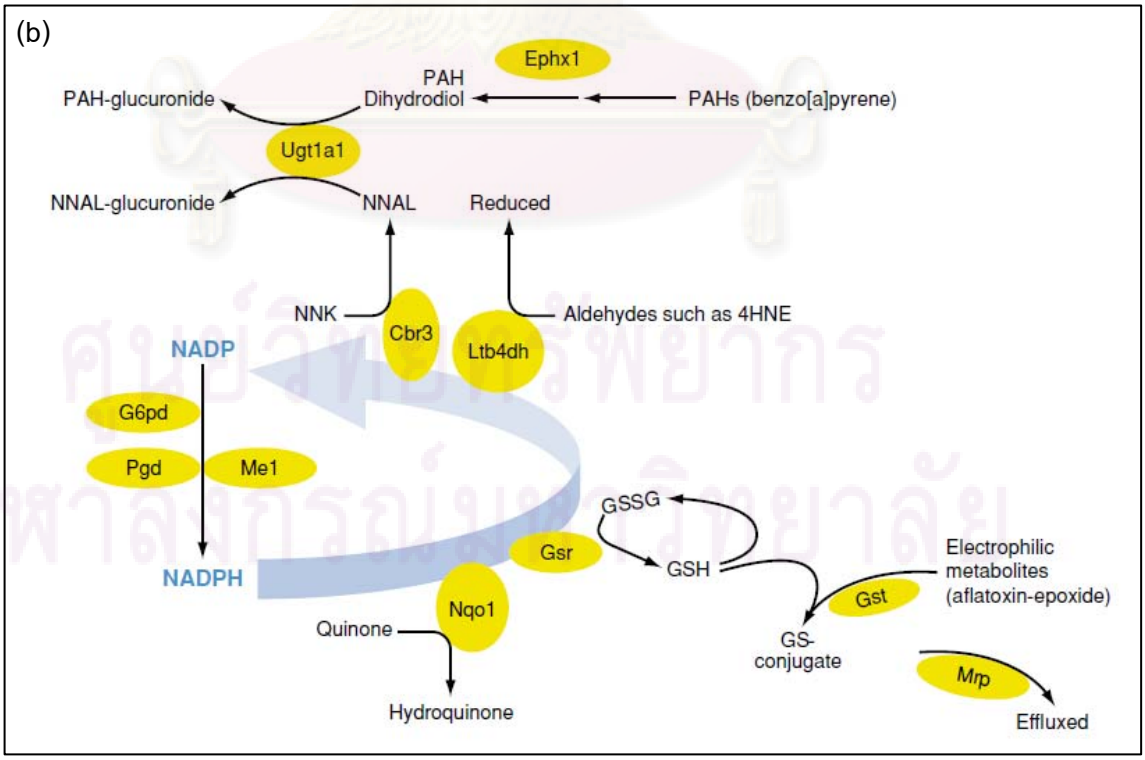


Figure 8: Schematic diagram representing Nrf2-regulated antioxidants and xenobiotic detoxification enzymes. In response to oxidative or electrophilic stress, Nrf2 has been demonstrated to coordinately upregulate expression of *(a)* antioxidants: SOD1, heme oxygenase; and genes associated with glutathione pathway: glutathione peroxidase, glutathione reductase, glutamate-cysteine ligase catalytic subunit (Gclc), and glutamate-cysteine ligase modifier subunit (Gclm); thioredoxin pathway: thioredoxin reductase, peroxiredoxin; as well as NADPH-regenerating enzymes: glucose 6-phosphate dehydrogenase, phosphogluconate dehydrogenase, and maleic enzyme 1; and (*b*) xenobiotic detoxification enzymes: glutathione *S*-transferase, UGT1A1, epoxide hydrolase, carbonyl reductase, LTB4dh, NQO1, and MRP. These antioxidants and xenobiotic detoxification enzymes act in a concerted fashion to attenuate pathological damage caused by ROS, RNS and electrophiles (Kensler, Wakabayashi, and Biswal, 2007).

Upstream signaling pathways regulating Nrf2

Nrf2 activation causes by direct oxidation or covalent modification of thiol groups of Keap1, a cytoplasmic repressor protein. Moreover, the Nrf2-Keap1-ARE signaling can be modulated by post-transcriptional modification of Nrf2 (Figure 9).

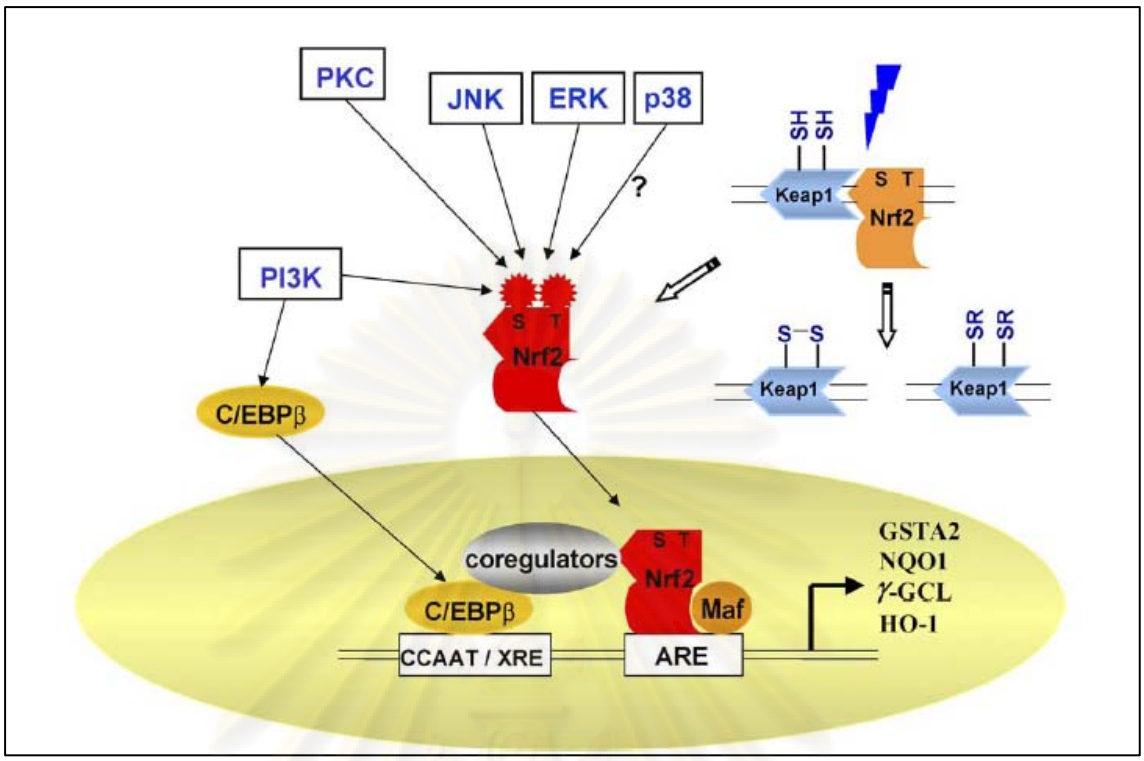


Figure 9: Upstream signaling pathways regulating Nrf2 activation; the Nrf2-Keap1-ARE signaling pathway can be modulated by several upstream kinases including phosphatidylinositol 3-kinase, protein kinase C, and mitogen-activated protein kinases (Lee and Surh, 2005).

Several signaling pathways have been implicated in the activation of Nrf2. Activated PKC may directly phosphorylate Nrf2. Three MAPKs, including extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, may also stimulate the activation of Nrf2. The p38 MAP kinase can either stimulate or inhibit the Nrf2 nuclear translocation depending on the cell type. Alternatively, PI3K can phosphorylate C/EBPb and also Nrf2, inducing its translocation to the nucleus and subsequent binding to the CCAAT sequence of C/EBPb response element within the XRE, in conjunction with Nrf2 binding to ARE. Active C/EBPb may compete with C/EBPa for the C/EBP binding site (Kang et al., 2003).

Many activators, which selected Nrf2-Keap1-ARE signaling pathway, were reported such as oltipraz, anethole dithiolethione, sulforaphane, 6-methylsulphinylhexyl isothiocyanate, curcumin, caffeic acid phenethyl ester, resveratrol, quercetin, 40-bromoflavone (Lee and Surh, 2005; Yao et al., 2007; Rubiolo, Mithieux, and Vega, 2008; Surh, Kundu, and Na, 2008). Some activators trigger the Nrf2 signal transduction by activating upstream kinases via phosphorylation and/or covalent modification/ oxidation of cysteine thiol groups present in Keap1.

Transcription factor Nrf2 and liver protection

Liver plays a major role in metabolism and detoxification. It is particularly frequent target organ of toxicity that is often injured or selective injured by exogenous substances. Transcription factor Nrf2 is essential for protecting cells against xenobiotics and oxidative stress by control expression of a main cellular antioxidant response. Toxicants, carcinogens or some drugs induce organ or tissue damage by varieties of mechanisms. Increased oxidative stress has been implicated in the pathophysiology of many diseases such as ethanol-induced liver disease.

Recently, the protective role of Nrf2 was studied in many researches using knockout animal models and toxicants, which induced liver toxicity such as arsenic, cadmium, carbon tetrachloride, alcohol, acetaminophen, oxfendazole, ferric nitrilotriacetate (Fe-NTA), pentachlorophenol (PCP) and beta-naphthoflavone etc. (Chan, Han, and Kan, 2001; Enomoto et al., 2001; Li, Stein, and Johnson, 2004; Umemura et al., 2006; He, Chen, and Ma, 2008; Kanki et al., 2008; Lamlé et al., 2008; Xu et al., 2008; Dewa et al., 2008; Dewa et al., 2009; Jiang et al., 2009; Liu, Qu, and Kadiiska, 2009). Nrf2 transcription factor can protect the liver from acute and chronic toxicant-mediated damages. In addition, *Nrf2* knockout mice ($Nrf2^{-/-}$) were more sensitive to toxicant-induced tissue/cell injury and displayed more severe pathological changes in tissue/cell when compared to $Nrf2^{+/+}$ mice.

CHAPTER III

MATERIALS AND METHODS

Materials

Animals

Male Wistar rats weighing 180-200 g were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom. Animals were allowed to acclimatize for 7 days before experimentation in animal care facility at Faculty of Pharmaceutical Sciences, Chulalongkorn University, under environmental control conditions: room temperature 25 ± 2 $^{\circ}$C with 12-h light and 12-h dark cycle, relative humidity of approximately 50 ± 20 %. They were received free standard rat pellets and tap water *ad libitum*.

Chemicals

The following chemicals were purchased from Sigma Chemical Co., St. Louis, U.S.A.: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay kits, antioxidant enzyme assay kits for superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase (Cayman chemical, USA), β-actin antibody (HRP) (ab20272, Abcam, Cambridge), bovine serum albumin (BSA), Bradford reagent, 1-chloro, 2,4-dinitrobenzene (CNDB), butylated hydroxytoluene, n-butanol, collagenase, dexamethasone, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2',7'-Dichlorodihydrofluoroscein diacetate (DCFH-DA), 5-5'-dithio-bis-(2-nitrobenzoic acid), ethanol, ethylenediaminetetraacetic acid-disodium salt dihydrate (EDTA), fetal bovine serum (FBS), glutathione peroxidase1 antibody (ab22604, Abcam, Cambridge), glutathione reductase antibody (ab16801, Abcam, Cambridge), GSH reductase (GR), hydrochloric acid (HCl), insulin, methanol, β-nicotinamide adenine dinucleotide phosphate (NADPH), Nrf2 antibody (ab53019, Abcam, Cambridge), penicillin-G sodium, phosphotungstic acid, potassium chloride (KCl), propionaldehyde, rabbit IgG secondary antibody (ab6721, Abcam, Cambridge), reduced glutathione (GSH), superoxide dismutase1 antibody (ab16831, Abcam,

Cambridge), superoxide dismutase2 antibody (ab13533, Abcam, Cambridge), sodium chloride (NaCl), sodium carbonate, sodium laurylsulfate, streptomycin, sulfosalicylic acid, tetraethoxypropane (TEP), 2-thiobarbituric acid, tris(hydroxymethyl)aminomethane, trypan blue solution.

All other reagents were analytical grade and obtained from commercial sources.

Instruments

- Autopipette 10 µl, 100 µl, 200 µl, 1000 µl, 5 ml and 10 ml (BIO-RAD)
- Centrifuge (Kokusan)
- CO_2 incubator (Model:3110 Series, Series II Water Jacketed, ThermoForma)
- Microtubes (Eppendorf)
- Microplate reader (Anthos Labtec HT2,Version 1.22E)
- Microplate reader (Wallac Victor3)
- Mini Trans-blot Electrophoretic Transfer cell (BIO-RAD)
- Pipette tips 10 µl, 200 µl, 1000 µl, 5 ml and 10 ml
- pH meter (Beckman instruments)
- Refrigerated centrifuge (Beckman coulter model Allegra X-12R)
- Shaker (Rocker, Model NB-104, Korea)
- Spectroflorometer (Jasco Model FP-777)
- Surgical equipments
- TC-plate 24 well, TC-plate 96 well (Greiner bio-one)
- Ultra sonicator
- Ultra-low temperature freezer (Forma Scientific)
- UV Spectrophotometer (Jasco model UVDEC 650)
- Vertical Laminar flow (LF-V90 MSSP, HEPA filter)
- Vortex mixer
- 96 well PS microplate black (Greiner bio-one)

Research methodology

1. Extraction, isolation and identification of phyllanthin

Phyllanthin was isolated from *Phyllanthus amarus* which was collected in and around Faculty of Pharmaceutical Sciences, Chulalongkorn University during May to June 2007. Plant was authenticated by Assoc. Prof. Chaiyo Chaichantipyuth at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

The isolation method was modified from Syamasundar et al. (1985). The total fresh plant was pulverized after shade dry and powdered using a laboratory mill. The powder was Soxhlet-extracted repeatedly with 10 L of fresh hexane for 1 day. The solvent was evaporated under reduced pressure at 40°C dried in vacuum. The combined hexane extract (yield 3.5% of dried whole plant) was then subjected to column chromatography with silica gel (60 ± 80 mesh) as stationary phase and N-hexane with an increasing amount of ethyl acetate as mobile phase (yield 0.06% of dried whole plant or 1.7% of hexane extract) (Figure 10). Online thin-layer chromatography was performed for each fraction.

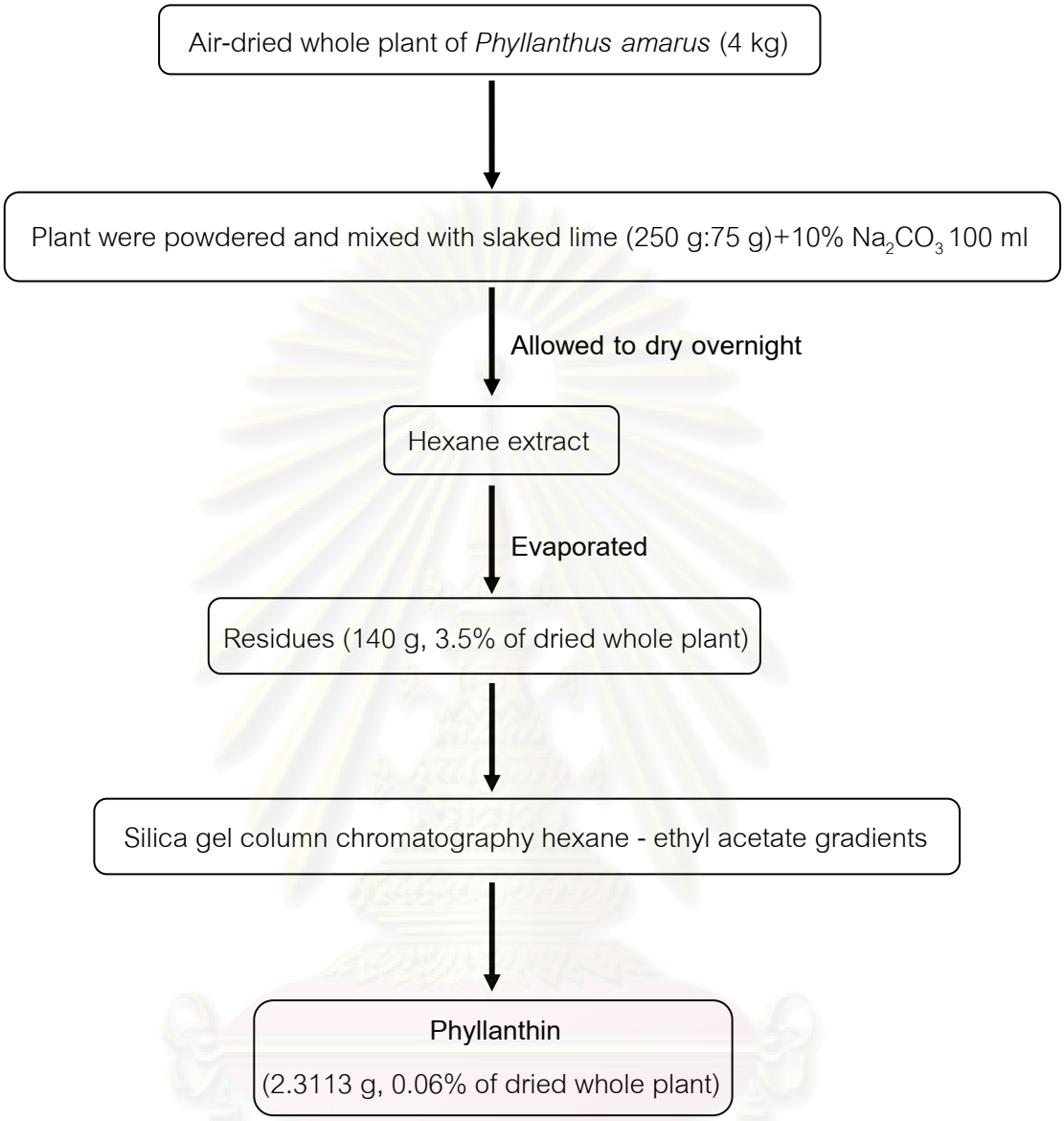


Figure 10: Diagram of the extraction and isolation of phyllanthin.

Analytical identification of phyllanthin

Final purification of phyllanthin was identified by compared with phyllanthin standard reference (Chromadex Inc.) using thin layer chromatography, infrared spectrum and high performance liquid chromatography (HPLC).

Thin layer chromatography (TLC)

TLC was used as primary screening for phyllanthin from each fraction with N-hexane:ethyl acetate (3:2) as mobile phase and anisaldehyde (the sensitive and selective compound) to produce color characterizing the lignans and sterols inherent in hexane extract of *Phyllanthus amarus*.

Chromatographic Conditions

- TLC plates 10 x 10 cm silica gel 60
- Hexane : ethyl acetate (3:2)
- Spray TLC plate with the anisaldehyde.
- Inspect plate in visible light and under UV 366 nm.

Infrared spectrum

Phyllanthin was analyzed using Fourier Transform Infrared Spectrometer (FT-IR) (PerkinElmer (Spectrum One)) at Scientific and Technological Research Equipment Centre, Chulalongkorn University. Infrared light was sent onto or through the sample. Phyllanthin was identified by comparing its IR spectrum with spectra from phyllanthin standard reference (Chromadex Inc.). Fourier Transform IR worked well on small samples and that the results were obtained relatively fast.

High performance liquid chromatography (HPLC)

Phyllanthin was analyzed by HPLC followed the method described by Murugaiyah and Chan (2007).

Chromatographic Conditions

- Column: Mightysil RP-18 GP 250×4.6 (5 µm)
- Column Temperature: 29°C
- Mobile Phase: Acetonitrile:H_2O (55:45 % v/v)
- Flow Rate: 1.4 ml/min
- Detection: 230 nm

2. Primary cultures of rat hepatocyte preparation

Hepatocytes were isolated from male Wistar rat using methods of Berry and Friend (1969) as modified by Stacey and Priestly (1978) and Pramyothin (1986).

- Under ether anesthesia, liver was perfused with Ca^{2+}-free physiological solution via the portal vein.
- When the perfusion of all hepatic lobes was rapidly completed, 40 ml of 0.4% collagenase in the same Ca^{2+}-free physiological solution was added and allowed to digest under the recirculating condition. Flow was maintained at 30-35 ml/min with the pressure head of 20 cm water, and temperature was maintained at 37°C throughout the procedure.
- After perfusion with collagenase (10-15 min), liver was dispersed with blunt spatula in 50 ml of 0.4% fresh collagenase buffer, and incubated at 37°C in a shaker water bath for 10 min. Bovine serum albumin (BSA) was added to give a final concentration of 12 mg/ml and cells were harvested through nylon mesh (150, 250 μm). Hepatocytes were separated from other cells and cellular debris by centrifugation (50 g, 1 min). The cell pellet was washed twice with this fresh physiological medium containing 12 mg/ml BSA and once with the incubation medium.
- Cell viability was estimated by Trypan blue exclusion test using the exclusion index of > 90%.
- Hepatocytes were suspended in Eagle's MEM supplemented with 10% inactivated fetal bovine serum, 10^{-8}M Insulin, 10^{-6}M dexamethasone, penicillin (100 IU/l) and streptomycin (100 μg/ml). Cells were cultured on rat-tail collagen coated 24-well culture plates (Greiner, Germany) at a density of 2 X 10^5 cells/0.8 ml at 37°C in a humidified 5% CO_2-95% air atmosphere. Hepatocytes were used in the experiment after 24 h of culturing (Figure 11).

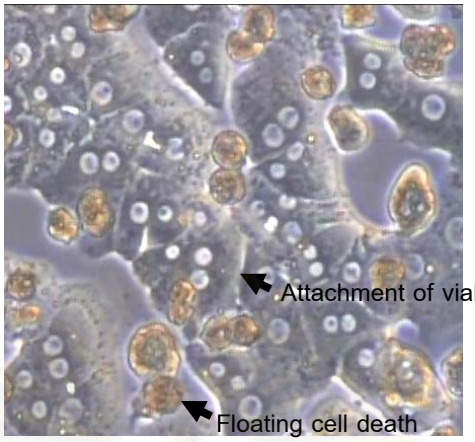


Figure 11: Rat hepatocytes after 24 h culturing.

3. Hepatoprotective study of phyllanthin against ethanol-induced liver cell injury in primary cultures of rat hepatocytes

After 24 h of culturing, hepatocytes were pretreated with phyllanthin for 24 h at concentrations 1, 2, 3 and 4 µg/ml (2.4, 4.8, 7.2 and 9.6 µM) and ethanol 80 µl/ml or DMSO (0.05% v/v, vehicle control) for 2 h (Figure 12).

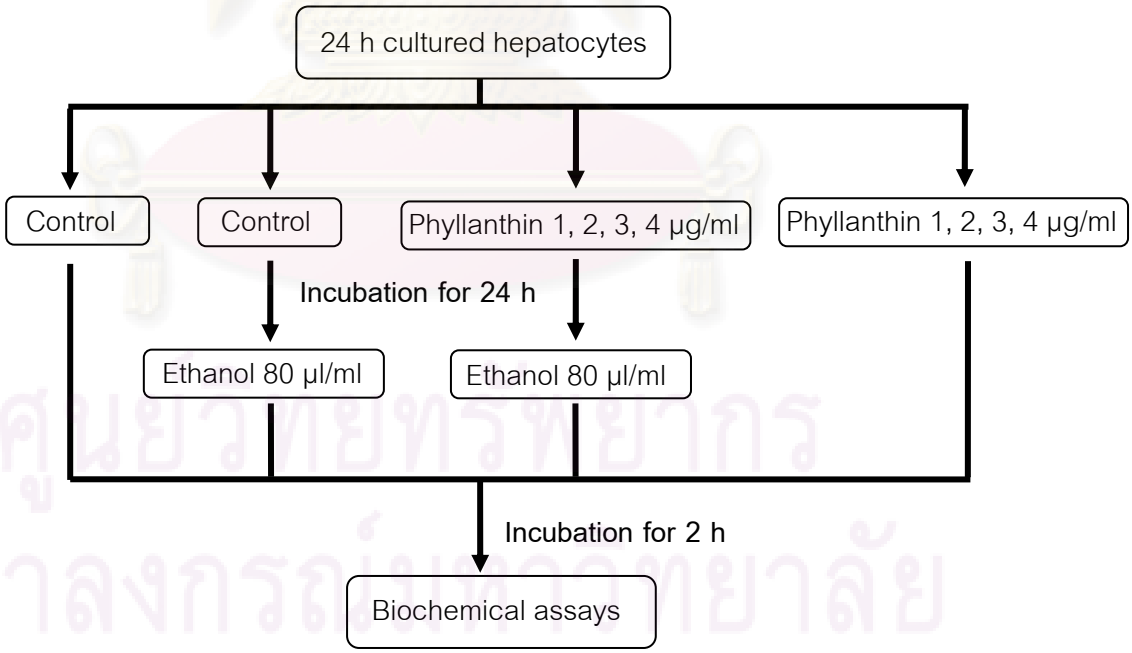


Figure 12: Diagram of hepatoprotective study of phyllanthin in ethanol-treated primary culture of rat hepatocytes.

3.1 Effect of phyllanthin on cytotoxicity in primary culture of rat hepatocytes treated with ethanol

MTT assay

MTT assay was based on the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from a yellow water-soluble dye to a dark blue insoluble formazan product (Mossmann, 1983) (Figure13).

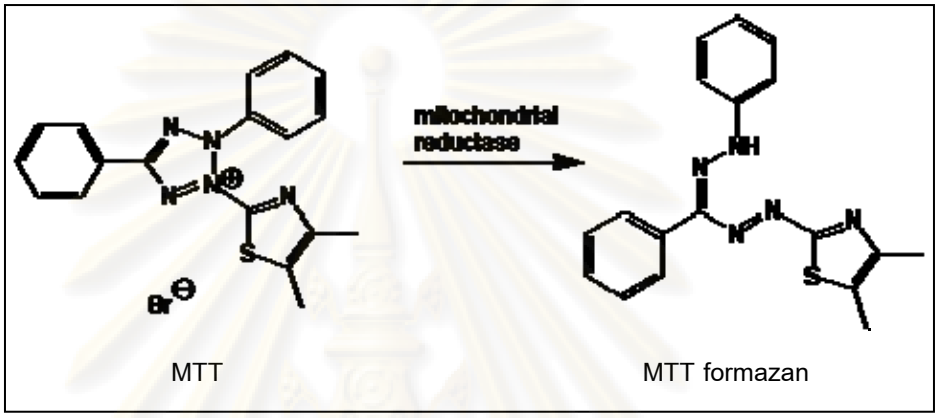


Figure 13: MTT reduction assay.

- 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in phosphate-buffered saline (PBS), pH 7.4 at a concentration of 5 mg/ml and added to the cell culture to the final concentration of 100 µg/ml.
- After 1 h, medium was removed and the remaining MTT crystals were dissolved in 200 µl DMSO.
- Optical density was assessed using a microplate reader (Anthos Labtec HT2, Version 1.21E) at 570/620 nm.

Determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) release

ALT and AST were assayed using kits from CPT diagnostics (IFCC method).

- After treatment, the medium of control or treated-groups was collected.
- Transaminase activity was measured as kinetic reaction using IFCC method.
- The absorbance of reaction was determined at 340 nm by spectrophotometer.

3.2 Effect of phyllanthin on reactive oxygen species (ROS) production in primary culture of rat hepatocytes treated with ethanol

Determination of intracellular reactive oxygen species

2',7'-Dichlorodihydrofluoroscein diacetate (DCFH-DA) was used to assess the generation of intracellular ROS (Wang and Joseph, 1999). The assay is based on the principle that DCFH-DA, a nonpolar and nonfluorescent compound, can diffuse through the cell membrane and deacetylated by cytosolic esterases to yield polar nonfluorescent DCFH (2', 7'-dichlorofluoroscin). DCFH is trapped within the cytoplasm, where it reacts with peroxides to form DCF (2', 7'-dichlorofluorescein), which can be measured by fluorometer at excitation and emission wavelengths of 485 and 530 nm, respectively.

- At the end of treatment, cultured hepatocytes were washed three times with cool PBS, pH 7.4.
- Cells were preincubated with 50 µM DCFH-DA for 45 minutes, 37 °C at dark.
- After treatment, cells were washed three times with cool PBS, pH 7.4.
- Cells were collected using a cell scraper.
- Centrifuged at 5,000 x g at 4°C for 3 minutes. Pellet was resuspened in 200 µl of PBS, pH 7.4.
- The formation of 2',7'-dichlorofluorescein (DCF) was detected under the fluorescence microplate reader (Wallac model 1420) at excitation wavelength of 485 nm and an emission wavelength of 530 nm.

3.3 Effect of phyllanthin on total glutathione levels in primary culture of rat hepatocytes treated with ethanol

Determination of total glutathione levels

Total glutathione levels were measured by Tietze method (1969) using DTNB and glutathione reductase. Glutathione (GSH, reduced form) is generated from glutathione disulfide (GSSG, oxidized form) by glutathione reductase. It reacts with DTNB to produce 2-nitro-5-thiobenzoic acid, a yellow colored product which can be measured by absorbance at 405 nm (Figure 14). This recycling reaction improves the sensitivity of total glutathione detection.

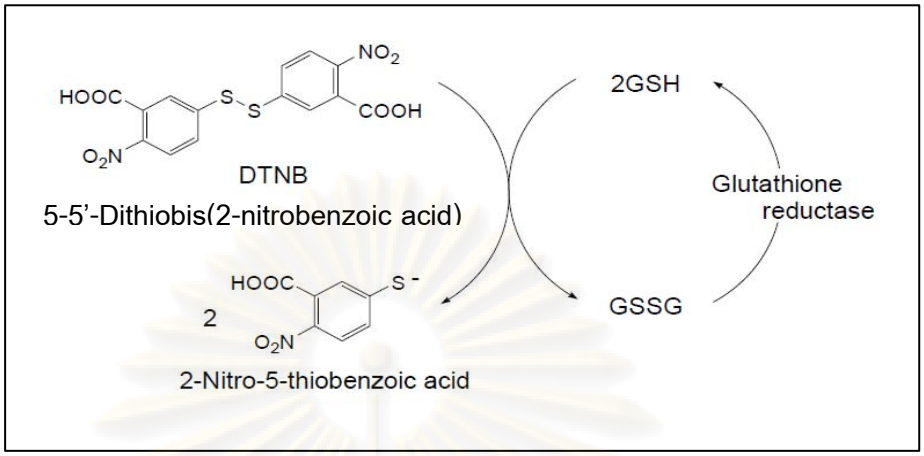


Figure 14: Mechanism of total glutathione quantification.

- After treatment, cultured hepatocytes were washed twice with the cool PBS, pH 7.4.
- The 100 µl of 5% (w/v) sulfosalicylic acid was added and left the plate on ice for 10 minutes.
- Cell suspension was transferred to microtube.
- Centrifuged at 13,000 x g at 4°C for 5 minutes.
- Supernatant was collected for total glutathione determination. By adding 10 µl of supernatant and adjusted with 1 mM EDTA in 0.1 M PBS, pH 7.5 to 100 µl into 96-well plate.
- Next, 100 µl of reaction mixture (0.15 mM 5-5'-dithio-bis-(2-nitrobenzoic acid, 0.2 mM NADPH, 1U GSH reductase) was added.
- Measured the absorbance in the well at 405 nm using the microplate reader (Wallac model 1420) at 30 seconds intervals for 10 minutes
- Total glutathione levels were calculated from standard curve of reduced glutathione (GSH).

3.4 Effect of phyllanthin on lipid peroxidation in primary culture of rat hepatocytes treated with ethanol.

Lipid peroxidation assay for malondialdehyde

Malondialdehyde (MDA) level was measured using a thiobarbituric acid reactive substances (TBARS) assay which modified from Ohkawa et al. (1979).

- At the end of treatment, aspirated the media.
- Added 200 µl of 2% sodium laurylsulfate (for solubilization) and incubated for 30 minutes.
- Cells were scraped and transferred to glass tube.
- Next, 50 µl of 4% butylated hydroxytoluene and 1 ml of 10% phosphotungstic acid were added and mixed.
- Then 1.5 ml of 0.7% thiobarbituric acid were added and heated for 60 minutes at 100°C in a water bath.
- After incubation, the samples were cooled in water.
- Then 4 ml of n-butanol was added.
- The samples were centrifuged at 3,000 x g for 10 minutes.
- The supernatant was measured using a spectrofluorometer (Jusco model FP-777) with excitation at 515 nm and emission at 553 nm.
- MDA level was calculated from standard curve of tetraethoxypropane (TEP)

TEP (Tetraethoxypropane) standard

10 µl of 0.4 M TEP + 990 µl of EtOH

↓

Final concentration of TEP 40 µmol/ml

↓

125 µl of TEP 40 µmol/ml + 50% EtOH/NSS q.s. to 25 ml

↓

TEP 200 µmol/ml

↓

500 µl of TEP 200 µmol/ml + NSS q.s. to 10 ml

↓

*TEP 10 µmol/ml for standard TEP preparation

STD TEP (nmol/ml)	10 nM TEP (µl)	NSS (µl)
1.0	100	900
0.6	60	940
0.4	40	960
0.2	20	980
0.1	10	990

Note: *freshly prepared

1 M TEP = 1 M MDA

3.5 Effect of phyllanthin on the activities of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx)), glutathione *S*-transferase (GST) and ethanol-metabolizing enzymes (alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH)) in primary culture of rat hepatocytes treated with ethanol.

Sample preparation for enzyme activity assays

- After treatment, hepatocytes were washed with PBS, pH 7.4 in order to eliminate dead cells.
- Cells were collected using a cell scraper and centrifuged at 2,000 x g for 10 minutes at 4°C.
- Cell pellets were resuspended in sample buffer and sonicated on ice.
- Centrifuged at 10,000 x g for 15 minutes at 4°C.
- Removed the supernatant for enzyme activity assays and store on ice. If not assaying on the same day, kept the sample at -80°C. They will be stable for at least one month.

Determination of superoxide dismutase activity

Superoxide dismutase (SOD) activity was assayed by kits from Cayman Chemical (USA) utilizing a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and xanthine (Figure 15).

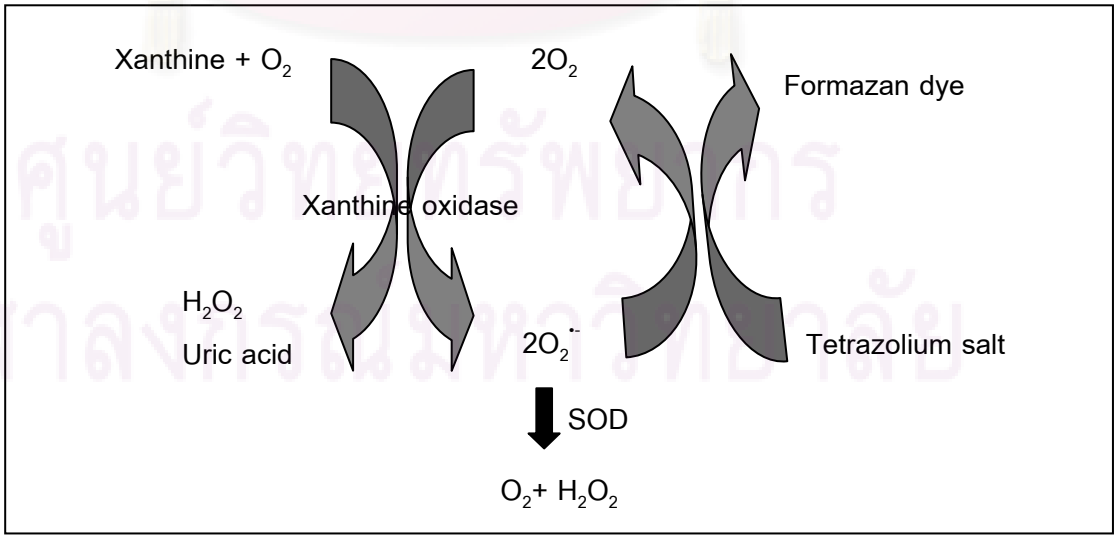


Figure 15: Superoxide dismutase activity.

- To perform the assay

  Blank well: 200 µl of diluted radical detector + 10 µl of sample buffer.

  Positive control well: 200 µl of diluted radical detector + 10 µl of SOD standard.

  Sample well: 200 µl of the diluted radical detector + 10 µl of sample.

- Initiated reactions by adding 20 µl of diluted xanthine oxidase as quickly as possible to all the wells being tested.
- Carefully shook the 96-well plate for a few seconds to mix.
- Incubated the plate on a shaker for 20 minutes at room temperature.
- Read the absorbance at 450 nm using the microplate reader (Anthos Labtec HT2, Version 1.22E).
- The SOD activity was calculated from standard curve. Enzyme activity was expressed as unit per milligram of cellular protein. One unit of SOD activity is defined as the amount of needed to exhibit 50% dismutation of the superoxide radical.

Determination of catalase activity

Catalase (CAT) activity was assayed by kits from Cayman Chemical (USA). CAT is involved in the detoxification of hydrogen peroxide. This enzyme catalyzes the conversion of two molecules of hydrogen peroxide to oxygen and two molecules of water (catalytic activity). CAT also demonstrates peroxidatic activity, in which low molecular weight alcohols can serve as electron donors (Figure 16). While aliphatic alcohols serves as specific substrates for CAT, other enzymes with peroxidatic activity do not utilize these substrates. This method is based on CAT peroxidatic activity using reaction of enzyme with methanol in the presence of an optimal concentration of hydrogen peroxide. The produced formaldehyde is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald, acting as the chromogen) at 540 nm.

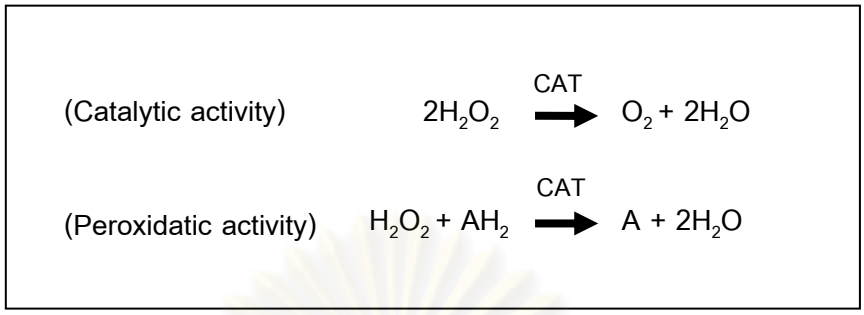


Figure 16: Catalase activity.

- To perform the assay

  Blank well: 100 µl of Assay buffer + 30 µl of methanol + 20 µl of buffer.

  Positive control well: 100 µl of Assay buffer +30 µl of methanol + 20 µl of CAT standard.

  Sample well: 100 µl of Assay buffer + 30 µl of methanol + 20 µl of sample.

- Initiated reactions by adding 20 µl of hydrogen peroxide as quickly as possible to all the wells being tested.
- Incubated the plate on a shaker for 20 minutes at room temperature.
- Added 30 µl of potassium hydroxide to each well to terminate the reaction and then added 30 µl of Purpald (chromagen) to each well.
- Incubated the plate on a shaker for 10 minutes at room temperature.
- Added 10 µl of potassium periodate to each well. Incubated the plate on a shaker for 5 minutes at room temperature.
- Read the absorbance at 540 nm using the microplate reader (Wallac model 1420).
- CAT activity was calculated from standard curve. Enzyme activity was expressed as nmole per minute per milligram of cellular protein. One unit of CAT activity is defined as the amount of enzyme that will generate the formation of 1.0 nmole of formaldehyde per minute at 25°C.

Determination of glutathione reductase activity

Glutathione reductase (GR) activity was assayed by kits from Cayman Chemical (USA). GR catalyzes the NADPH-dependent reduction of glutathione disulfide (GSSG) to GSH (Figure 17). The activity of GR is used as indicator for oxidative stress. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm and is directly proportional to the GR activity.

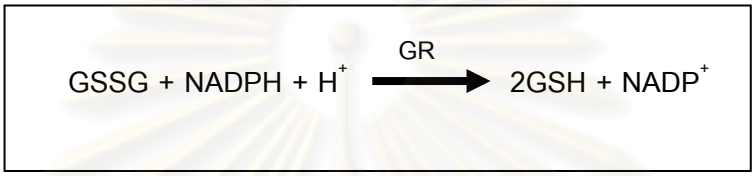


Figure 17: Glutathione reductase activity.

- To perform the assay

  Blank well: 120 µl of Assay buffer + 20 µl of GSSG.

  Positive control well: 100 µl of Assay buffer + 20 µl of GSSG + 20 µl of diluted GR standard.

  Sample well: 100 µl of Assay buffer + 20 µl of GSSG + 20 µl of sample.

- Initiated reactions by adding 50 µl of NADPH as quickly as possible to all the wells being tested.
- Carefully shook the 96-well plate for a few seconds to mix.
- Read the absorbance once every minute at 340 nm using the microplate reader (Wallac model 1420) to obtain at least 5 time points.
- The change in absorbance per minute (ΔA_{340}) was calculated. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$.
- Enzyme activity was expressed as nmole of NADPH consumed per minute per milligram of cellular protein. One unit of GR activity is defined as the amount of enzyme that will generate the oxidation of 1.0 nmole of NADPH to $NADP^+$ per minute at 25°C.

Determination of glutathione peroxidase activity

Cellular glutathione peroxidase (GPx) activity was assayed by kits from Cayman Chemical (USA). GPx catalyzes the reduction of hydrogen peroxide by reduced glutathione. GPx activity is measured indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) produced upon reduction of hydrogen peroxide by GPx, is recycled to its reduced state by GR and NADPH (Figure 18). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm under conditions in which GPx is rate limiting, the rate of decrease in absorbance is directly proportional to the GPx activity.

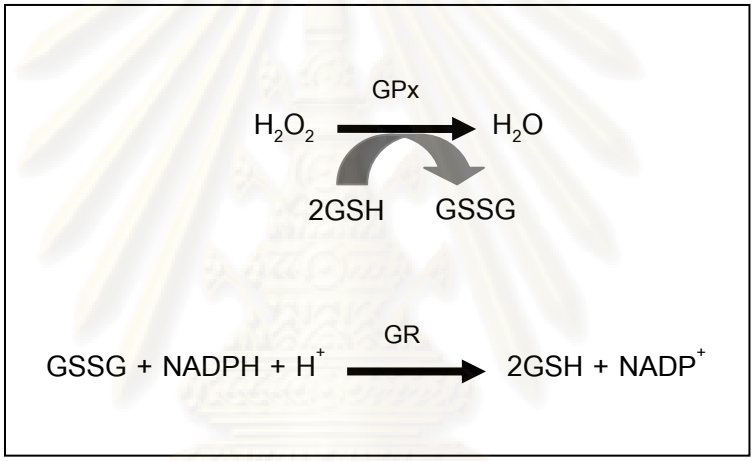


Figure 18: Glutathione peroxidase activity.

- To perform the assay

  Blank well: 120 µl of Assay buffer + 50 µl of co-substrate mixture.

  Positive control well: 100 µl of Assay buffer + 50 µl of co-substrate mixture + 20 µl of diluted GPx standard.

  Sample well: 100 µl of Assay buffer + 50 µl of co-substrate mixture + 20 µl of sample.

- Initiated reactions by adding 20 µl of cumene hydroperoxide as quickly as possible to all the wells being tested.
- Carefully shook the 96-well plate for a few seconds to mix.

- Read the absorbance once every minute at 340 nm using the microplate reader (Wallac model 1420) to obtain at least 5 time points.
- The change in absorbance per minute (ΔA_{340}) was calculated. The reaction rate at 340 nm can be determined using the NADPH extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$.
- Enzyme activity was expressed as nmole of NADPH consumed per minute per milligram of cellular protein. One unit of GPx activity is defined as the amount of enzyme that will generate the oxidation of 1.0 nmole of NADPH to $NADP^{+}$ per minute at 25°C.

Determination of glutathione S-transferase activity

Glutathione *S*-transferase (GST) activity was determined by measuring the conjugation of 1-chloro,2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) (Habig and Jakoby, 1981).

- 20 µl of sample, 20 µl of glutathione and 150 µl of PBS, pH 6.5 were mixed into 96-well plate. Blank was run in absence of sample.
- Initiated reactions by adding 10 µl of 10 mM CDNB as quickly as possible to all the wells.
- Carefully shook the 96-well plate for a few seconds to mix.
- Read the absorbance once every minute at 340 nm using the microplate reader (Wallac model 1420) to obtain at least 5 time points.
- The change in absorbance per minute (ΔA_{340}) was calculated. The reaction rate at 340 nm can be determined using the CDNB extinction coefficient of 0.0096 $\mu M^{-1}cm^{-1}$.
- Enzyme activity was expressed in units of GST activity per mg of protein. One unit of GST activity is defined as the amount of enzyme that synthesizes 1 nmole of product per minute per milligram of cellular protein.

Determination of alcohol dehydrogenase activity

Alcohol dehydrogenase (ADH) activity was performed using the spectrophotometric assay as described by Haseba et al. (2003). ADH is responsible for

catalyzing oxidation of ethanol to acetaldehyde with the reduction of NAD^+ to NADH (Figure 19). Ethanol was used as substrate and measured NADH production by absorbance at 340 nm.

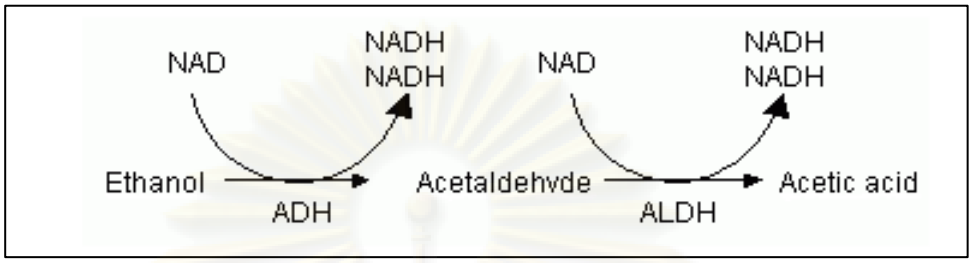


Figure 19: Ethanol metabolism by ADH and ALDH.

- 20 µl of cell extract was added to 180 µl of assay buffer (1.7 mM NAD^+ and 15 mM ethanol (as a substrate) in 0.1 M glycine buffer, pH 10.7).
- The increase in absorbance due to NADH production during oxidation of substrate was measured at 37°C at a wavelength of 340 nm using the microplate reader (Wallac model 1420).
- The assay was blanked by removing the substrate from the reaction medium. The ADH activity was calculated by using the NADH extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$ and was expressed in nmole/mg protein/min.

Determination of aldehyde dehydrogenase activity

Aldehyde dehydrogenase (ALDH) activity was performed using the spectrophotometric assay as described by Boesch et al. (1996), and Moreb et al. (2007). ALDH catalyzes oxidation of acetaldehyde to acetic acid with the reduction of NAD^+ to NADH (Figure 19). Propionaldehyde was used as substrate and NADH production was measured by absorbance at 340 nm.

- 20 µl of cell extract was added to an assay mixture containing 20 mM propionaldehyde and 1 mM NAD^+ in assay buffer (60 mM phosphate, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 8.5).

- The increase in absorbance due to NADH production during oxidation of substrate was calculated by using the NADH extinction coefficient of 0.00622 $\mu M^{-1}cm^{-1}$ at 340 nm. The ALDH activity was expressed in nmole/mg protein/min.

3.6 Effect of phyllanthin on the expression of cytoprotective proteins (superoxide dismutase (Cu-ZnSOD (SOD1), MnSOD (SOD2)), glutathione reductase (GR) and glutathione peroxidase (GPx)) and transcription factor Nrf2 in primary culture of rat hepatocytes treated with ethanol.

Western blot analysis

Whole cell lysates were prepared as described previously by Kim et al. (2003).

- After treatment, cells were washed with 400 µl of 4°C phosphate-buffered saline (PBS; pH 7.4) for two times.
- Added 25 µl of lysis buffer (containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM pefablock and 5 µg/µl aprotinin).
- Cells were scraped into lysis buffer, and the lysates were transferred to Eppendorf tubes. Samples were incubated on ice for 30 minutes.
- The lysates were clarified by centrifugation at 10,000 g for 10 min at 4°C.

Western blot was performed as follows:

- Cellular extracts were separated by 10 or 12% SDS–PAGE and transferred to PVDF membranes (Bio-Rad, München, Germany).
- Blots were blocked in 3% bovine serum albumin in TBST (10 mM Tris, 100 mM NaCl and 0.01% Tween 20) at room temperature for 1 h.
- Membranes were incubated with anti-SOD1 (1:2,000, Abcam), anti-SOD2 (1:5,000, Abcam), anti-GPx (1:4,000, Abcam), anti-GR (1:2,000, Abcam), anti-Nrf2 (1:1,000, Abcam) and β-actin antibodies (HRP, 1:5,000, Abcam) at 4°C overnight.
- After incubation in Rabbit IgG secondary antibody (1:20,000, Abcam), blots were visualized by enhanced chemiluminescence and were exposed to X-ray films (Eastman Kodak). Actin bands were monitored on the same blot to verify

the consistency of protein loading. The staining band was quantified by densitometry using computerized image analysis program (ImageJ, Ver. 1.410).

Protein assay

Protein concentration was determined according to Bradford method (1976) using bovine serum albumin (BSA) as the protein standard. The absorbance at 595 nm was recorded using microplate reader (Anthos Labtec HT2, Version 1.21E).

Statistical analysis

All results were expressed as mean ± S.E. Statistical differences between means were determined using analysis of variance (ANOVA) and Tukey's post hoc test. $P < 0.05$ was considered as statistically significant.

CHAPTER IV

RESULTS

Part A: Effect of 24 h phyllanthin (1, 2, 3, 4 µg/ml) pretreatment on ethanol-treated primary culture of rat hepatocytes

Protective effect of phyllanthin on ethanol-treated primary culture of rat hepatocytes

Protective effect of phyllanthin (1, 2, 3, 4 µg/ml) in acute ethanol toxicity was conducted in primary culture of rat hepatocytes. Biochemical markers for early acute hepatic damage were detected using MTT reduction assay and the release of aminotransferases: alanine aminotransferase (ALT) and aspartate aminotransferase (AST).

After 2 h of ethanol treatment, %MTT was decreased with the increase in cellular leakage of ALT and AST. Pretreatment with phyllanthin (1, 2, 3 µg/ml) 24 h before ethanol, increased % MTT and decreased the release of ALT and AST when compared to effect of ethanol alone (Figures 20-23). Pretreatment with phyllanthin at a dose of 4 µg/ml seemed to have less protective effect compared to the lower concentrations.

Effect of phyllanthin on the generation of ROS, MDA and total glutathione levels in primary culture of rat hepatocytes treated with ethanol

In order to determine the antioxidant potential of phyllanthin on ethanol-induced liver cell injury, the biomarkers of oxidative stress; the generation of intracellular ROS, lipid peroxidation and total glutathione levels were assessed.

Ethanol exposure increased the ROS production and MDA level. The elevations were decreased by phyllanthin pretreatment (1, 2, 3, 4 µg/ml, 24 h before ethanol) (Figures 24-25). The level of total glutathione which reduced in ethanol-treated hepatocytes was brought back to normal by phyllanthin pretreatment (Figure 26).

Effect of phyllanthin on the activities of ethanol-metabolizing enzymes (ADH, ALDH) in primary culture of rat hepatocytes treated with ethanol

Effect of phyllanthin (1, 2, 3, 4 µg/ml) on ethanol metabolism was investigated. Activities of ethanol metabolizing enzymes including cytosolic alcohol dehydrogenase (ADH) and mitochondrial aldehyde dehydrogenase (ALDH) were determined. Pretreatment with phyllanthin showed no effect on ADH activity (Figure 27). And had no effect on the activity of ALDH which was decreased by ethanol treatment (Figure 28).

Effect of phyllanthin on the activities of antioxidant enzymes: *superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx); and glutathione S-transferase (GST) in primary culture of rat hepatocytes treated with ethanol*

Effects of phyllanthin (1, 2, 3, 4 µg/ml) pretreatment against ethanol-induced rat liver cell injury on the activities of major antioxidant enzymes: SOD, CAT, GPx, GR; and GST were investigated.

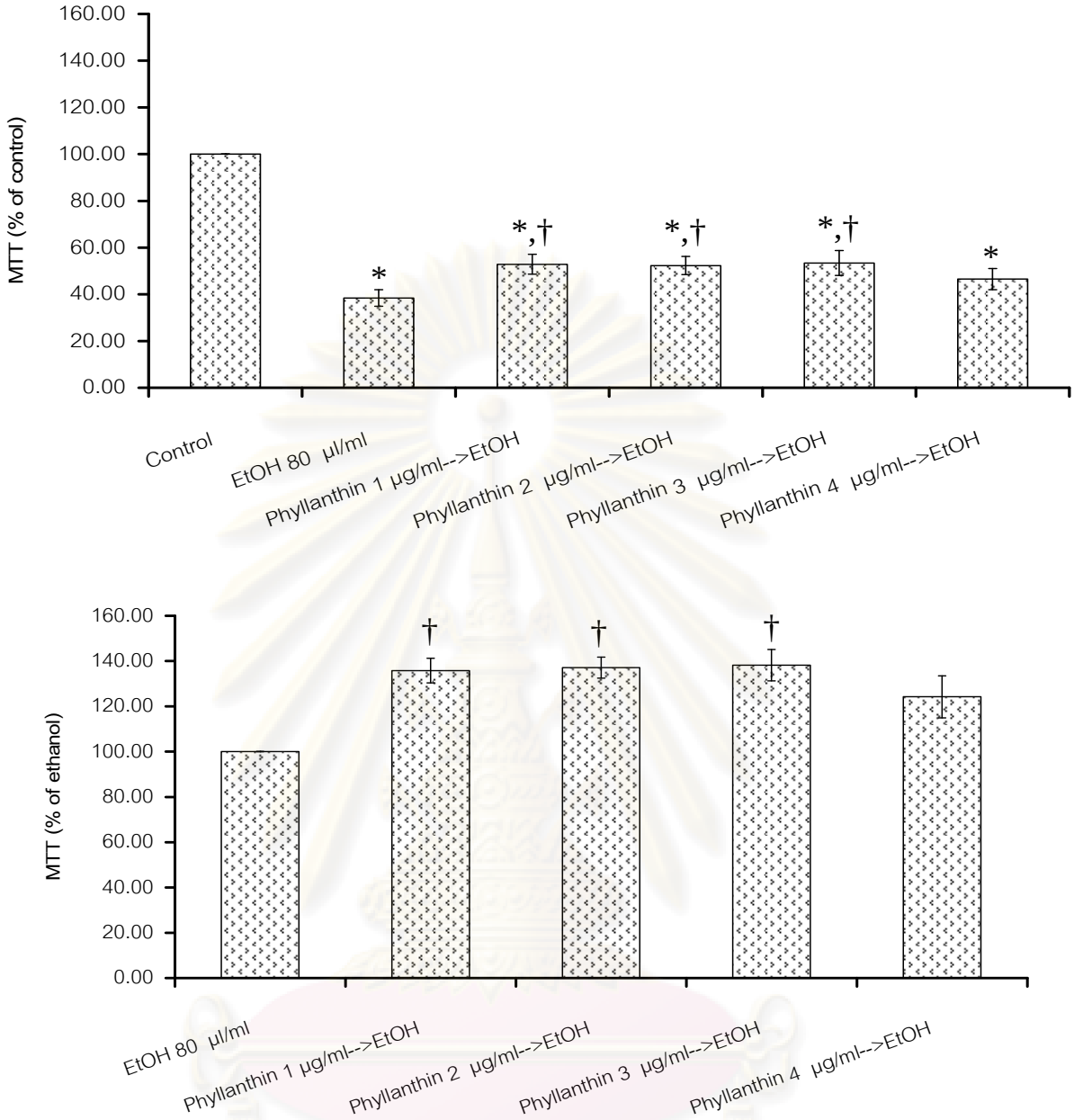
Ethanol decreased the activities of SOD and GR. Pretreatment with phyllanthin brought the activities of SOD and GR back to normal but not the GPx and GST which were increased and decreased respectively by ethanol treatment. There was no change in the activity of CAT (Figures 29-33).

Effect of phyllanthin on the expression of cytoprotective proteins: superoxide dismutases (Cu-ZnSOD (SOD1), MnSOD (SOD2)), glutathione reductase (GR) and glutathione peroxidase (GPx); and transcription factor Nrf2 in primary culture of rat hepatocytes treated with ethanol

The expression of involved cytoprotective proteins and the activation of Nrf2 transcription factor regulated antioxidant and detoxification genes were investigated.

After ethanol treatment there was the reduction of SOD1 (Cu-ZnSOD), GR and GPx proteins with no change in the expression of SOD2 (MnSOD). Pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) 24 h before ethanol increased the expression of SOD1, SOD2, GR and GPx proteins (Figures 34-37).

The reduction of Nrf2 was observed after 2 h of ethanol treatment. Pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) 24 h before ethanol increased the expression of Nrf2 (Figure 38).



Figures 20 and 21: Protective effect of phyllanthin (1, 2, 3, 4 µg/ml) on ethanol (EtOH) treated primary culture of rat hepatocytes, given 24 h before ethanol.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

Note: Using the same data set, % MTT was calculated as 100% for control to see the effect of ethanol in Figure 20 and as 100% for ethanol in Figure 21 to see the improvement after pretreatment with phyllanthin.

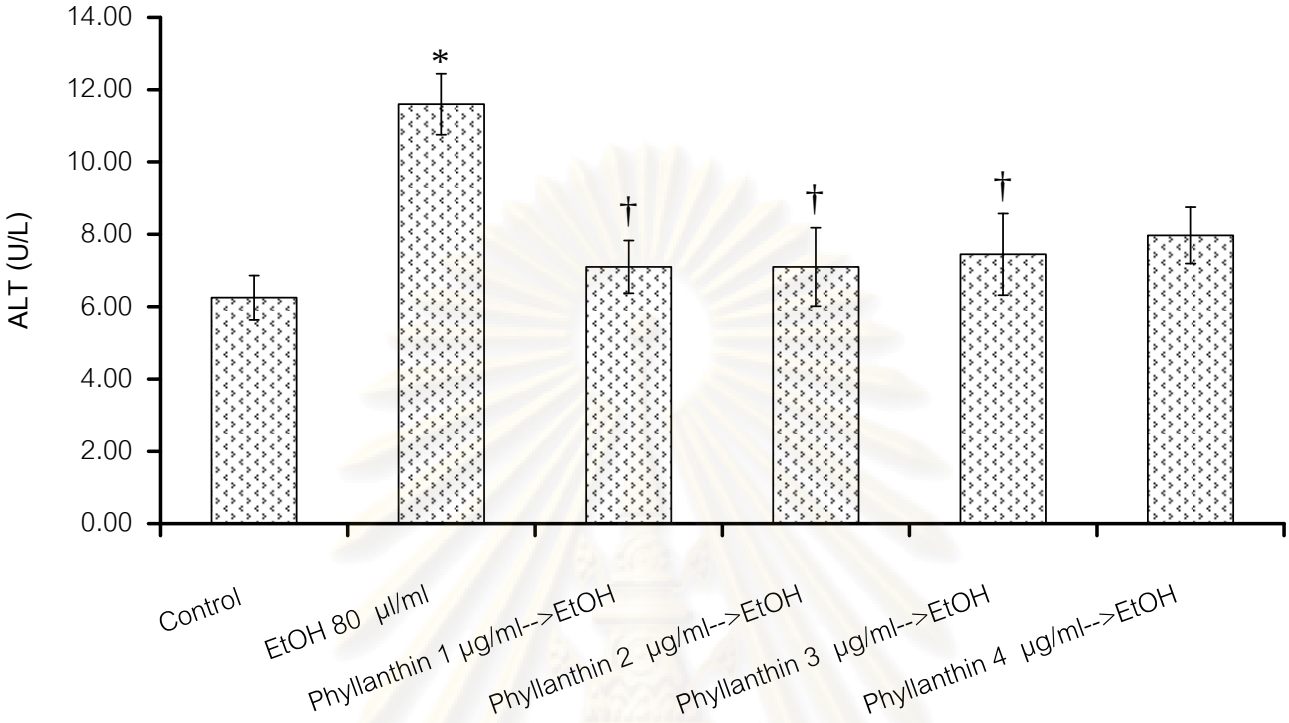


Figure 22: Effect of phyllanthin (1, 2, 3, 4 µg/ml) on the release of alanine aminotransferase (ALT) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

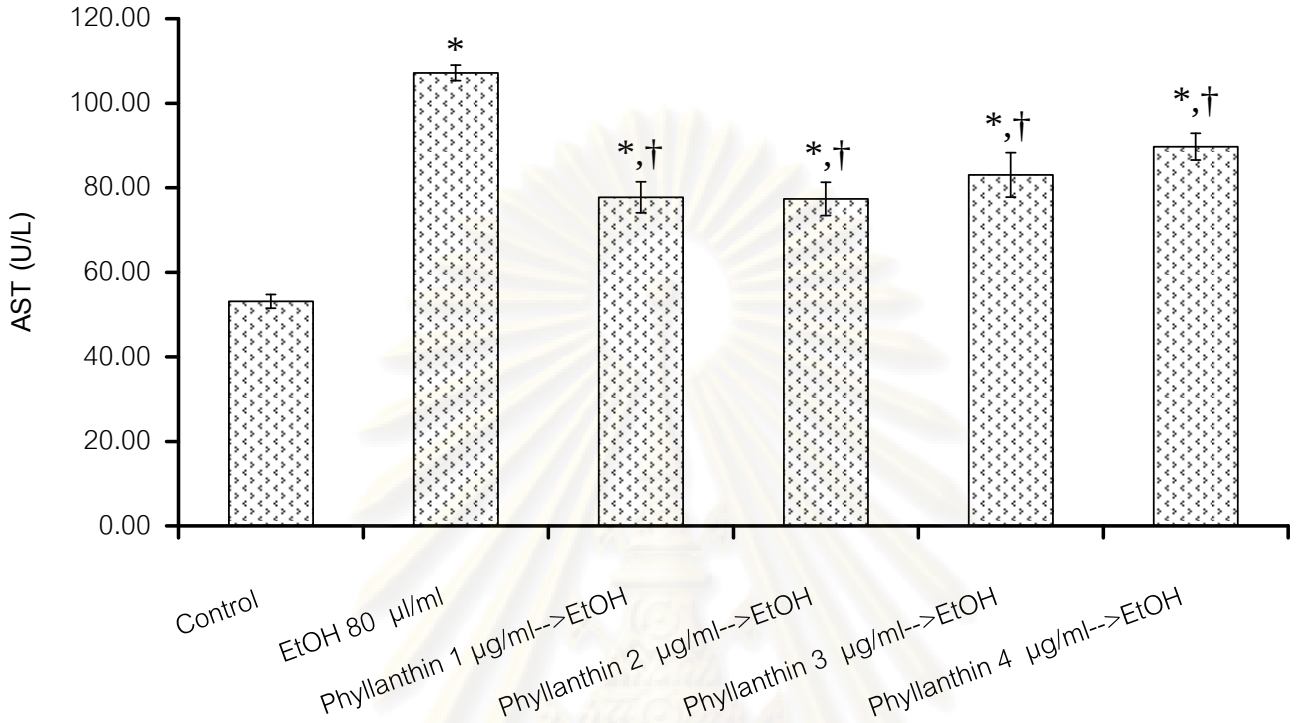


Figure 23: Effect of phyllanthin (1, 2, 3, 4 µg/ml) on the release of aspartate aminotransferase (AST) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

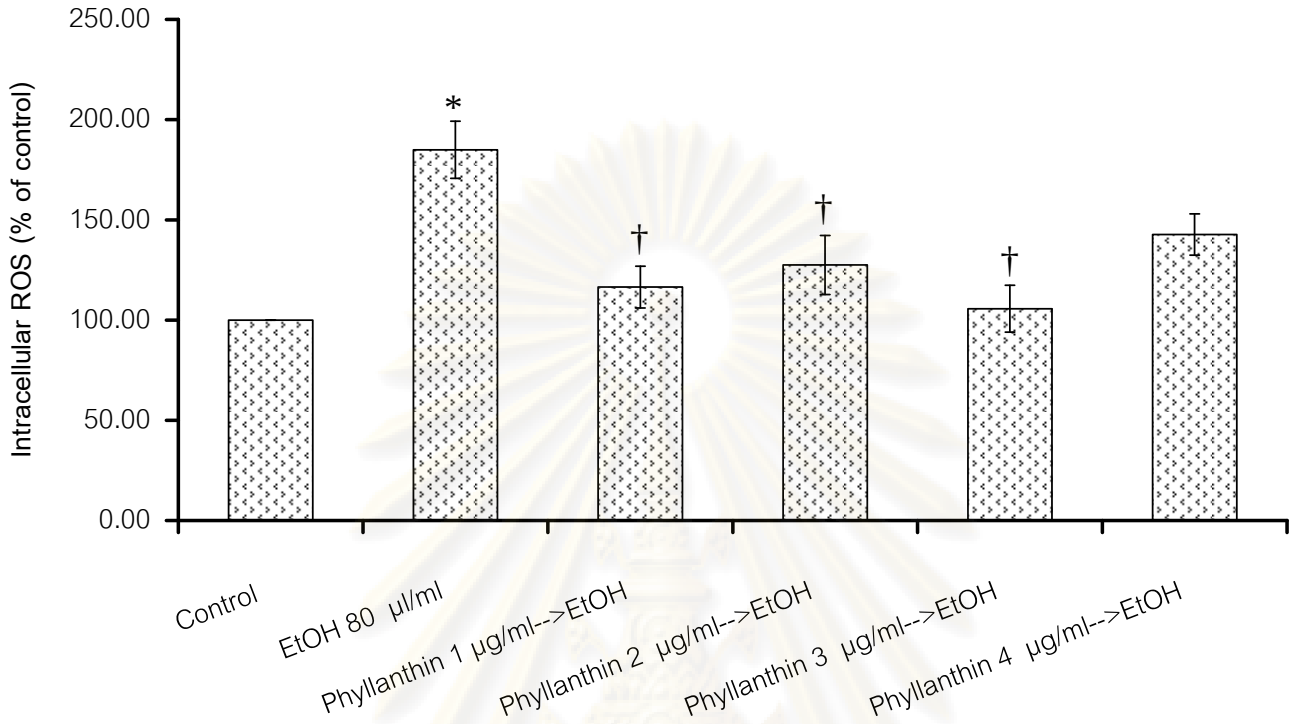


Figure 24: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the generation of intracellular reactive oxygen species (ROS) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

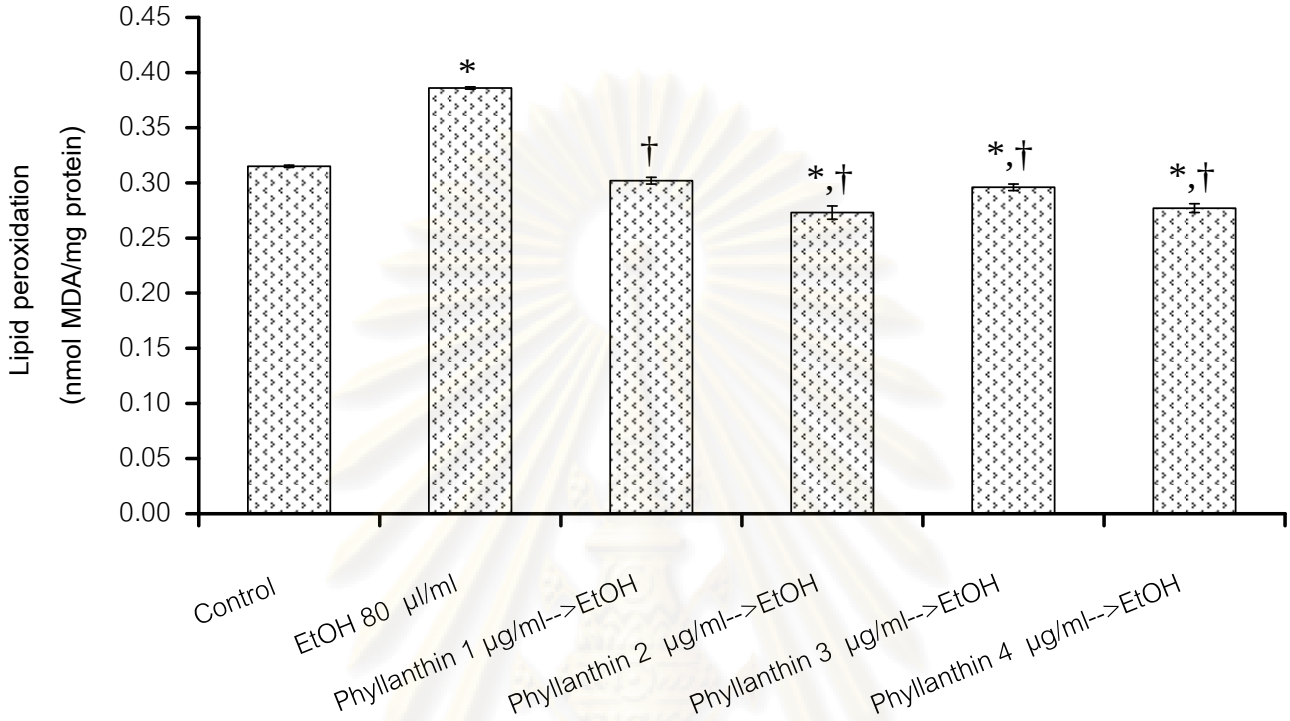


Figure 25: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the production of malondialdehyde (MDA) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

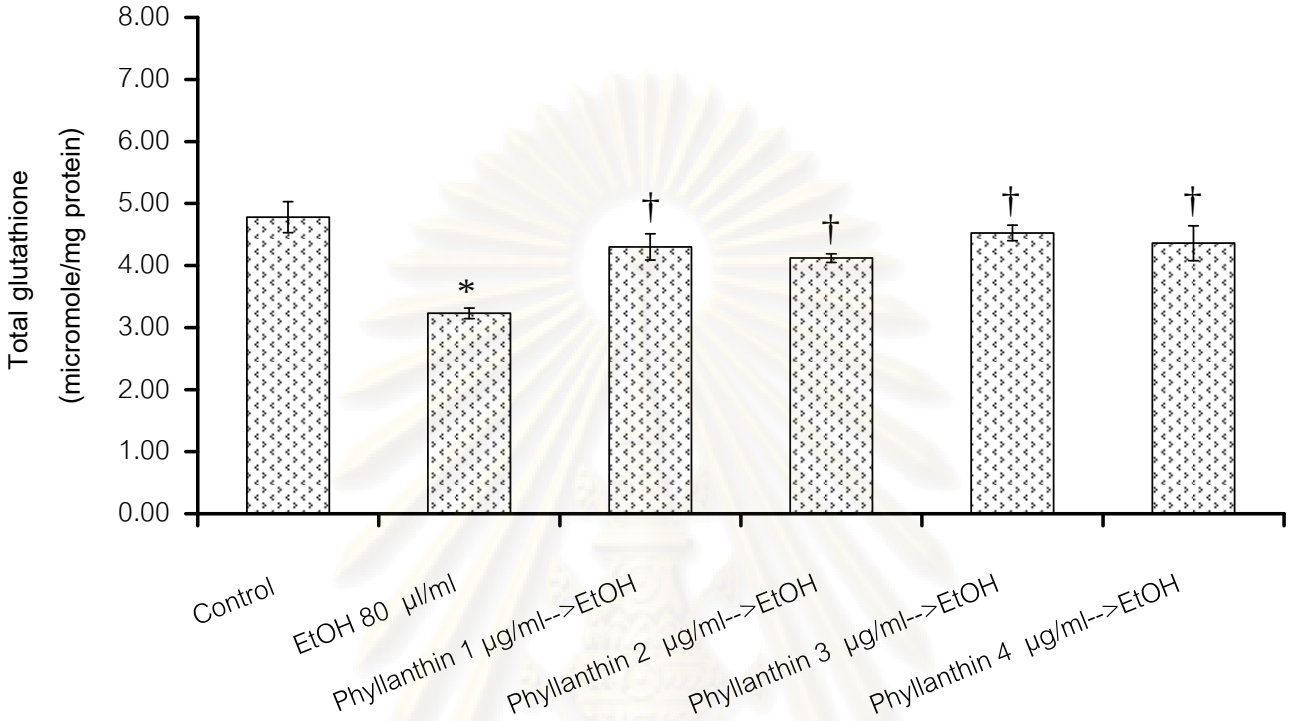


Figure 26: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the level of total glutathione in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

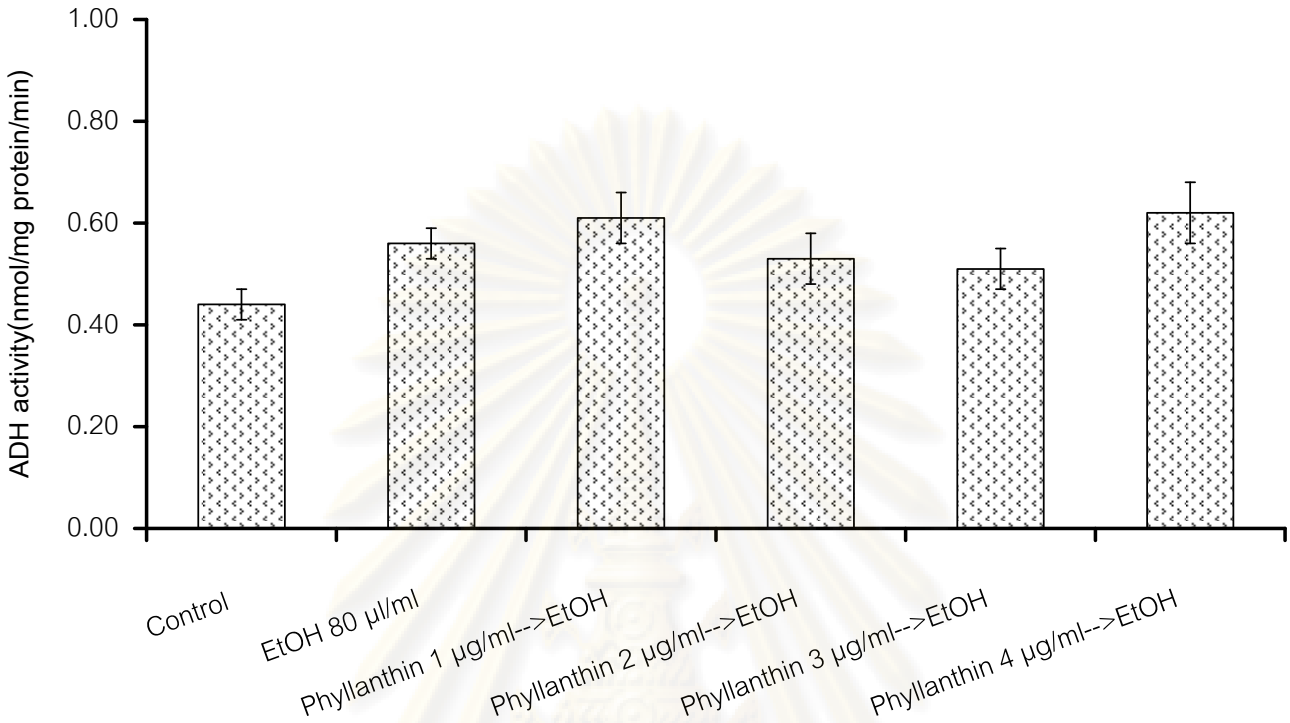


Figure 27: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of alcohol dehydrogenase (ADH) in primary culture of rat hepatocytes treated with ethanol (EtOH).
Values are Mean ± SEM, n=4.

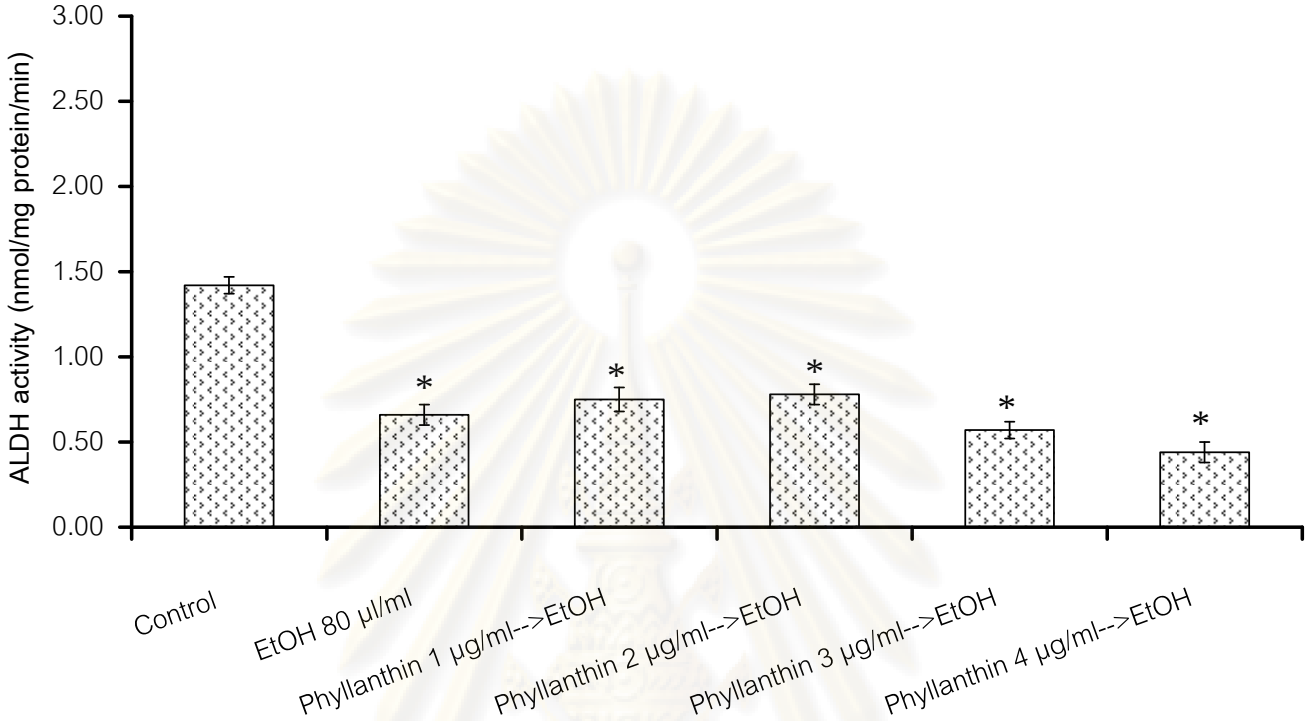


Figure 28: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of aldehyde dehydrogenase (ALDH) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

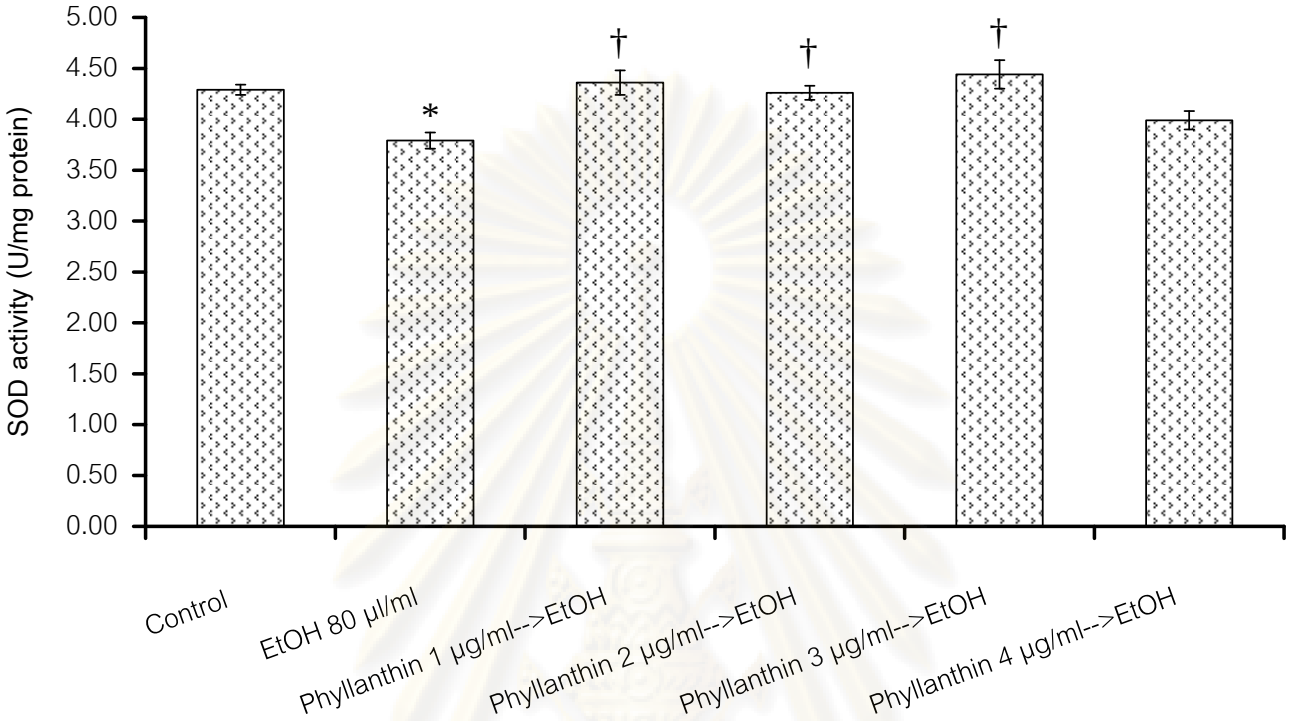


Figure 29: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of superoxide dismutase (SOD) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

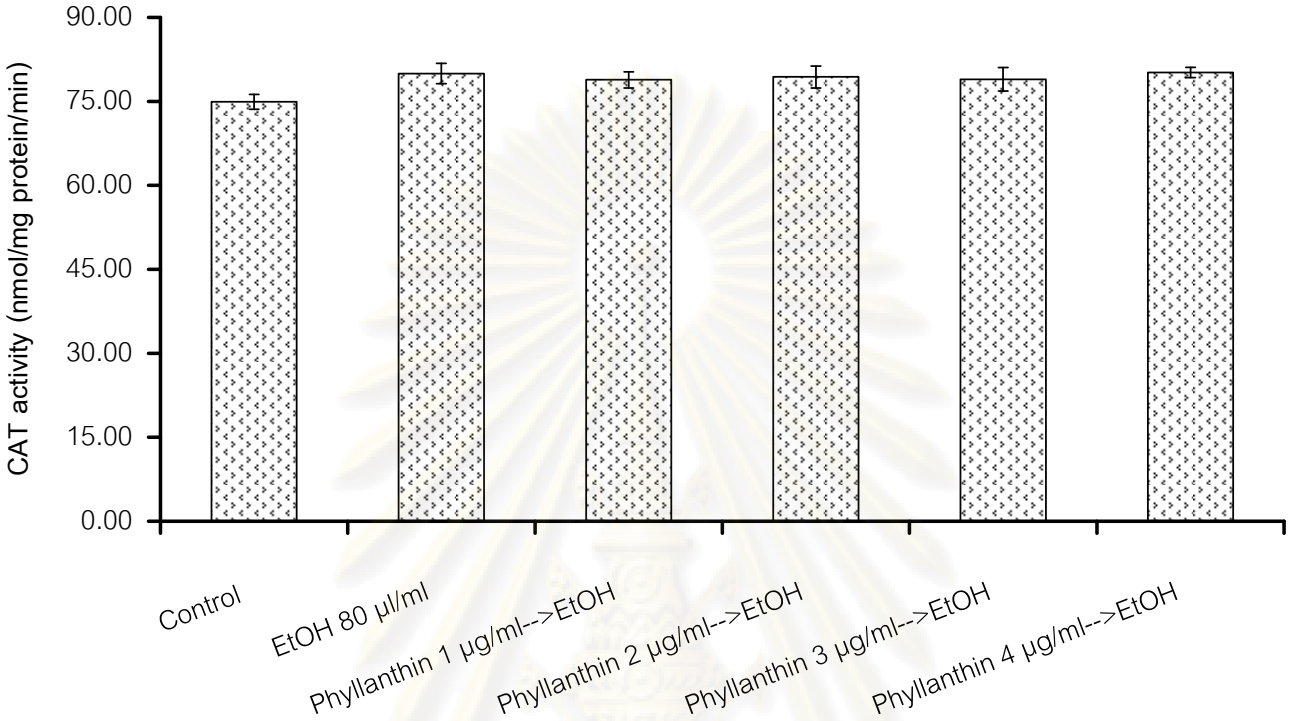


Figure 30: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of catalase (CAT) in primary culture of rat hepatocytes treated with ethanol (EtOH).
Values are Mean ± SEM, n=4.

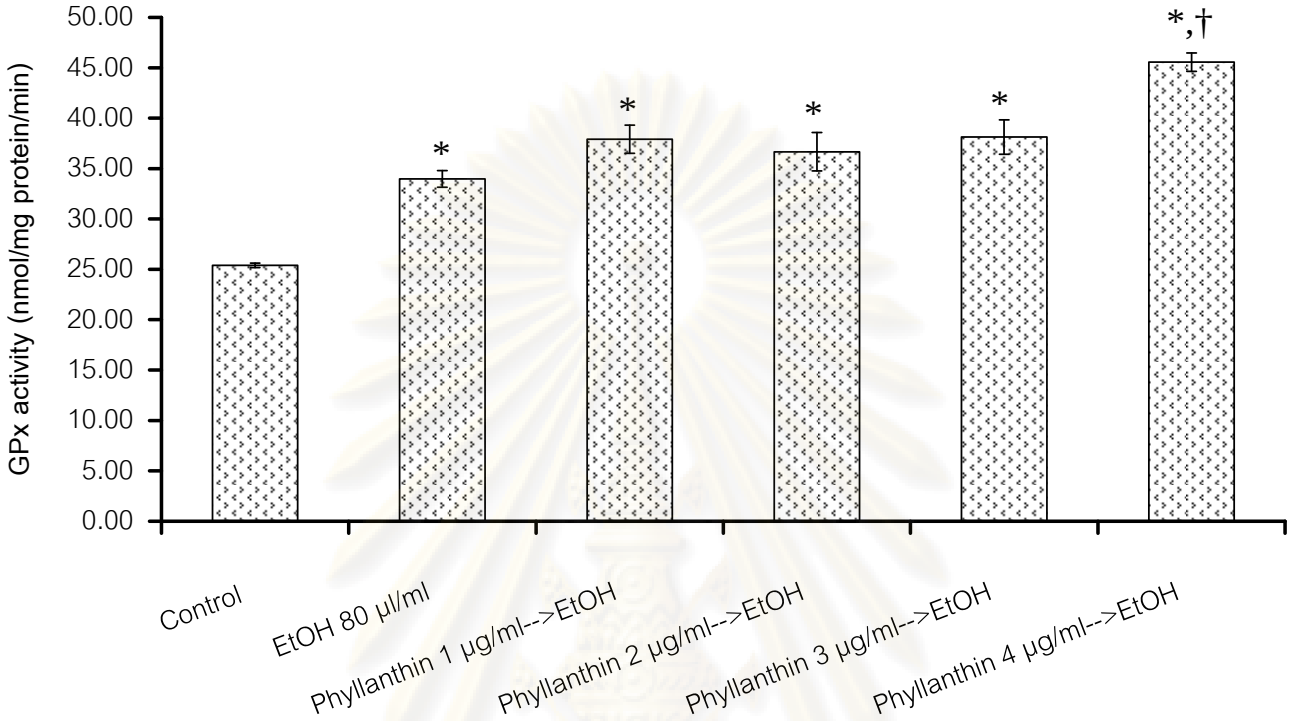


Figure 31: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of glutathione peroxidase (GPx) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

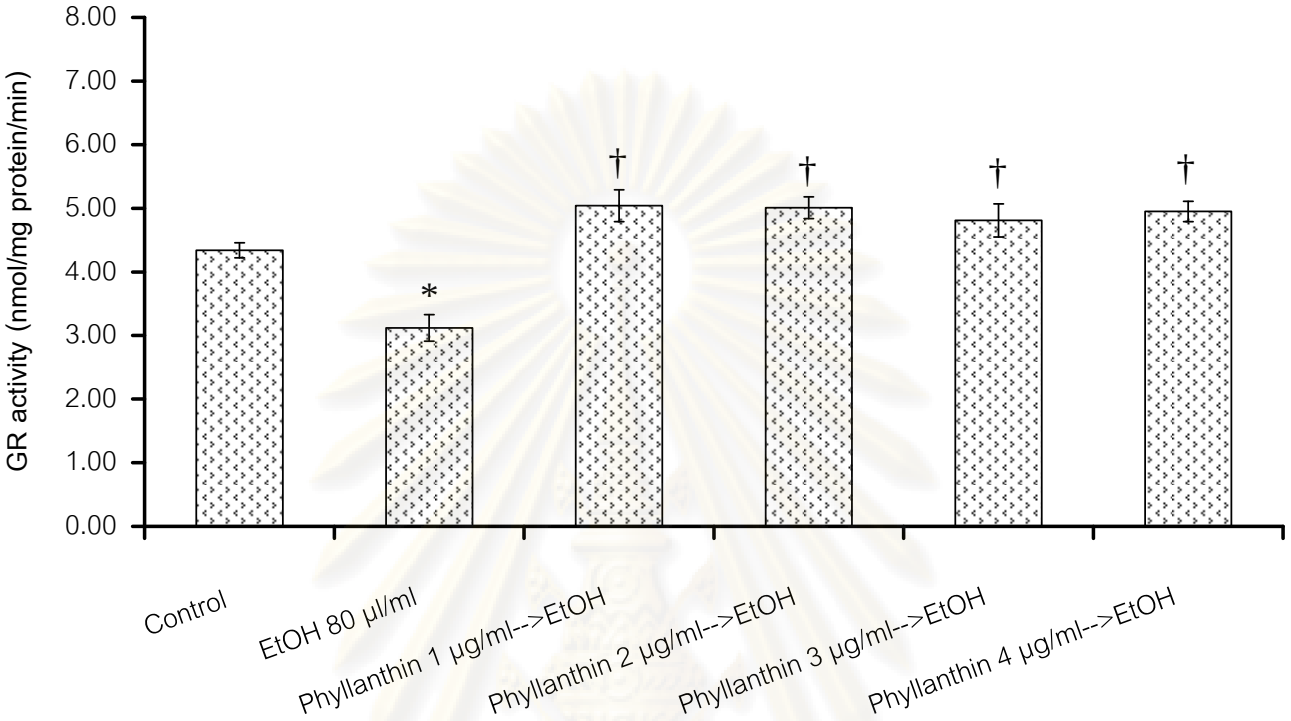


Figure 32: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of glutathione reductase (GR) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

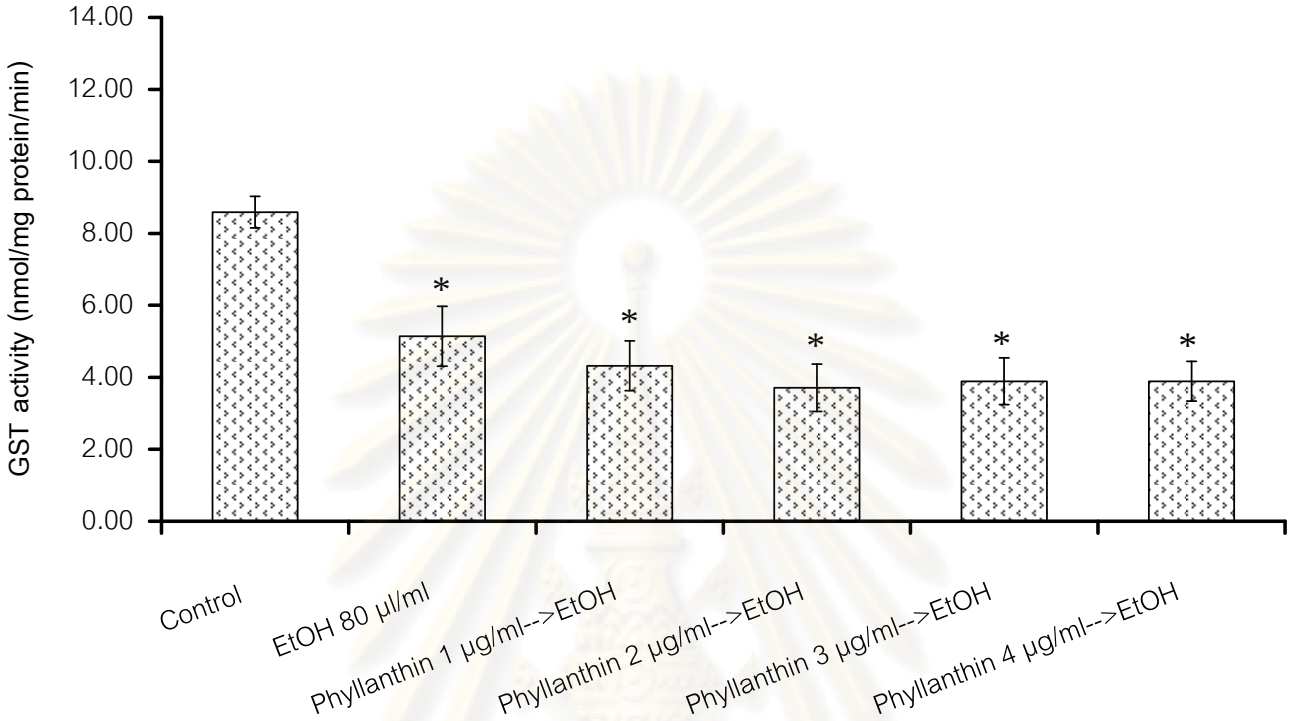


Figure 33: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of glutathione *S*-transferase (GST) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

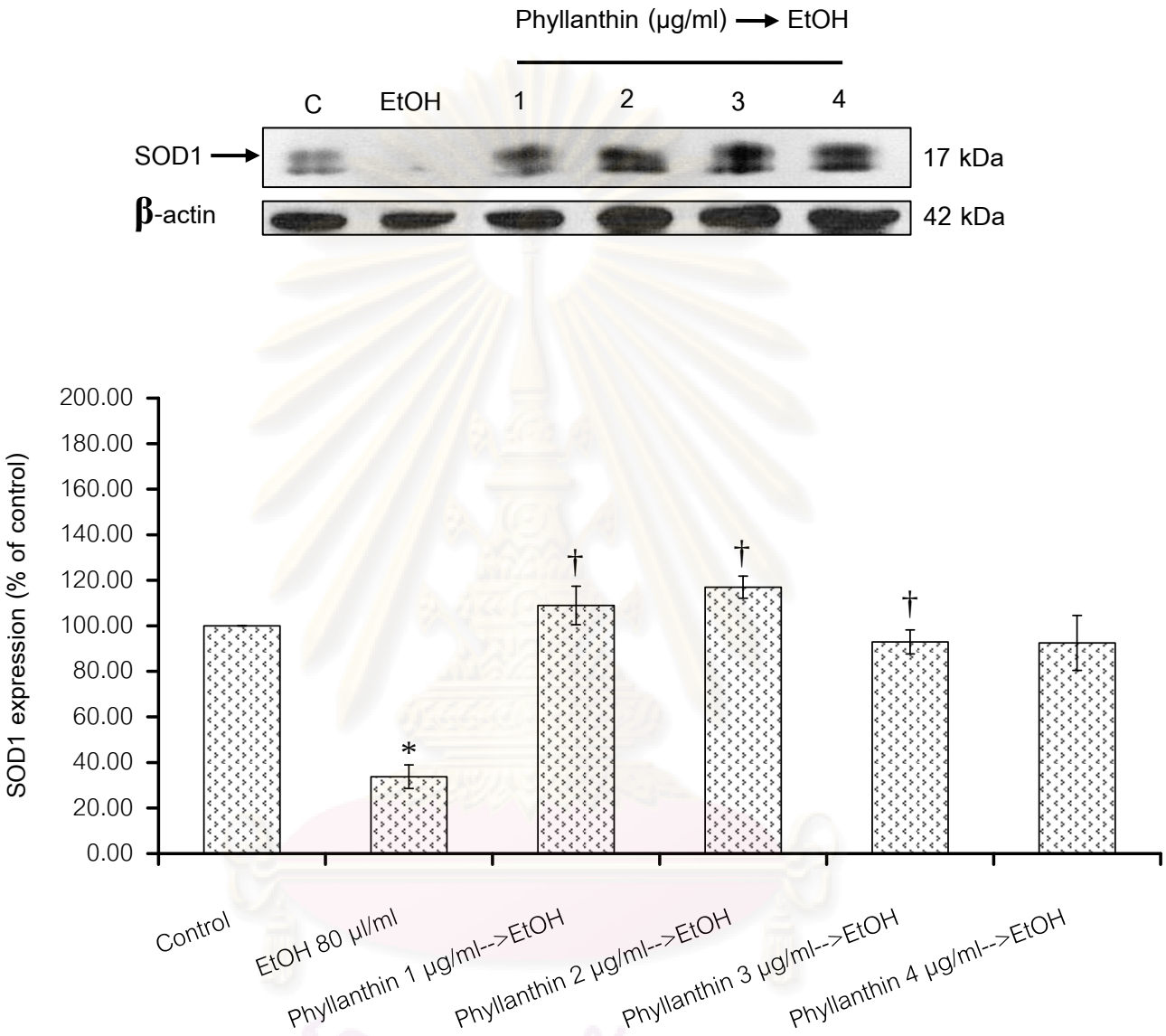


Figure 34: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the protein expression of copper-zinc superoxide dismutase (Cu-ZnSOD, SOD1) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

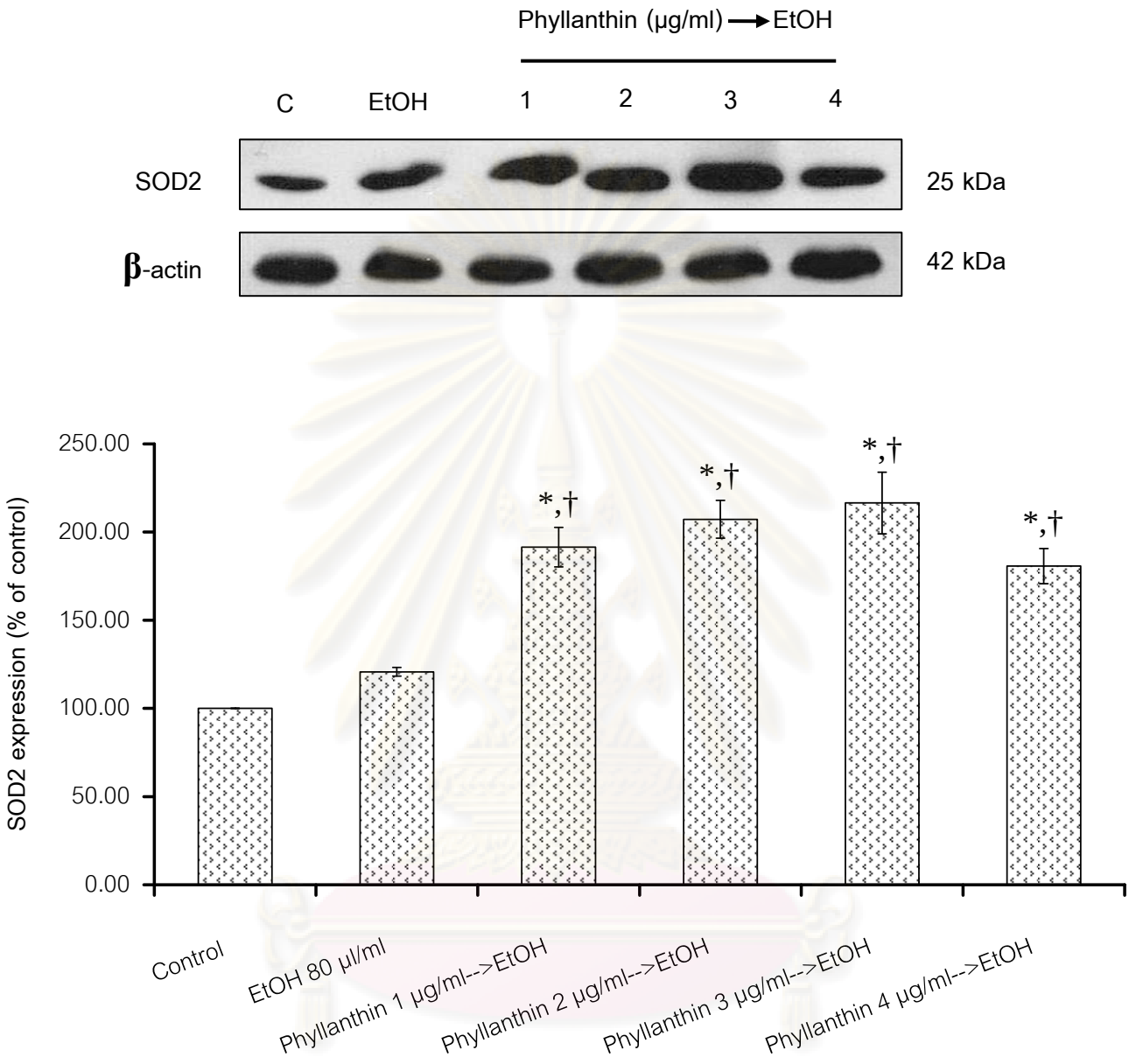


Figure 35: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 μg/ml) on the protein expression of manganese-superoxide dismutase (MnSOD, SOD2) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

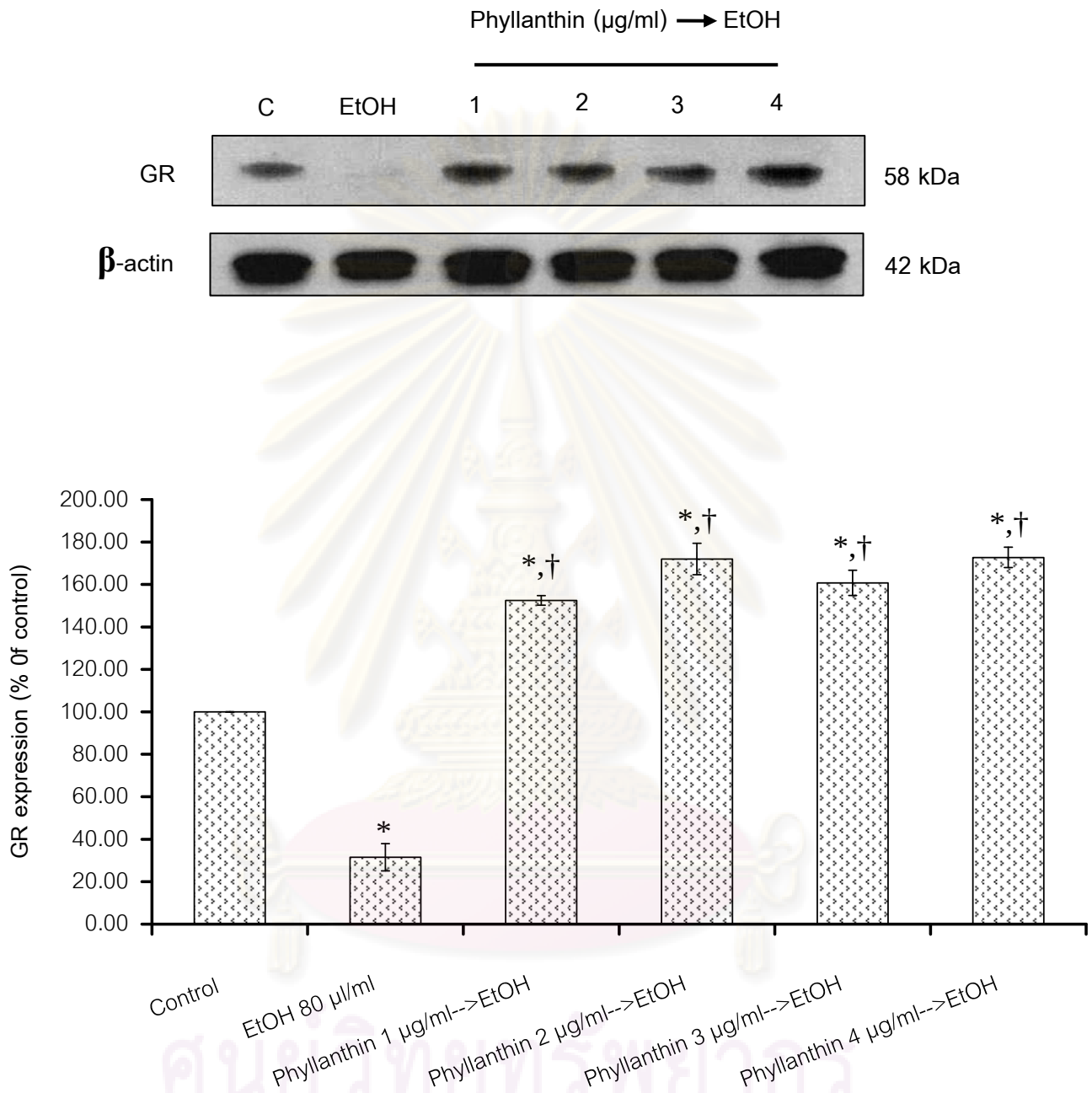


Figure 36: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the protein expression of glutathione reductase (GR) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

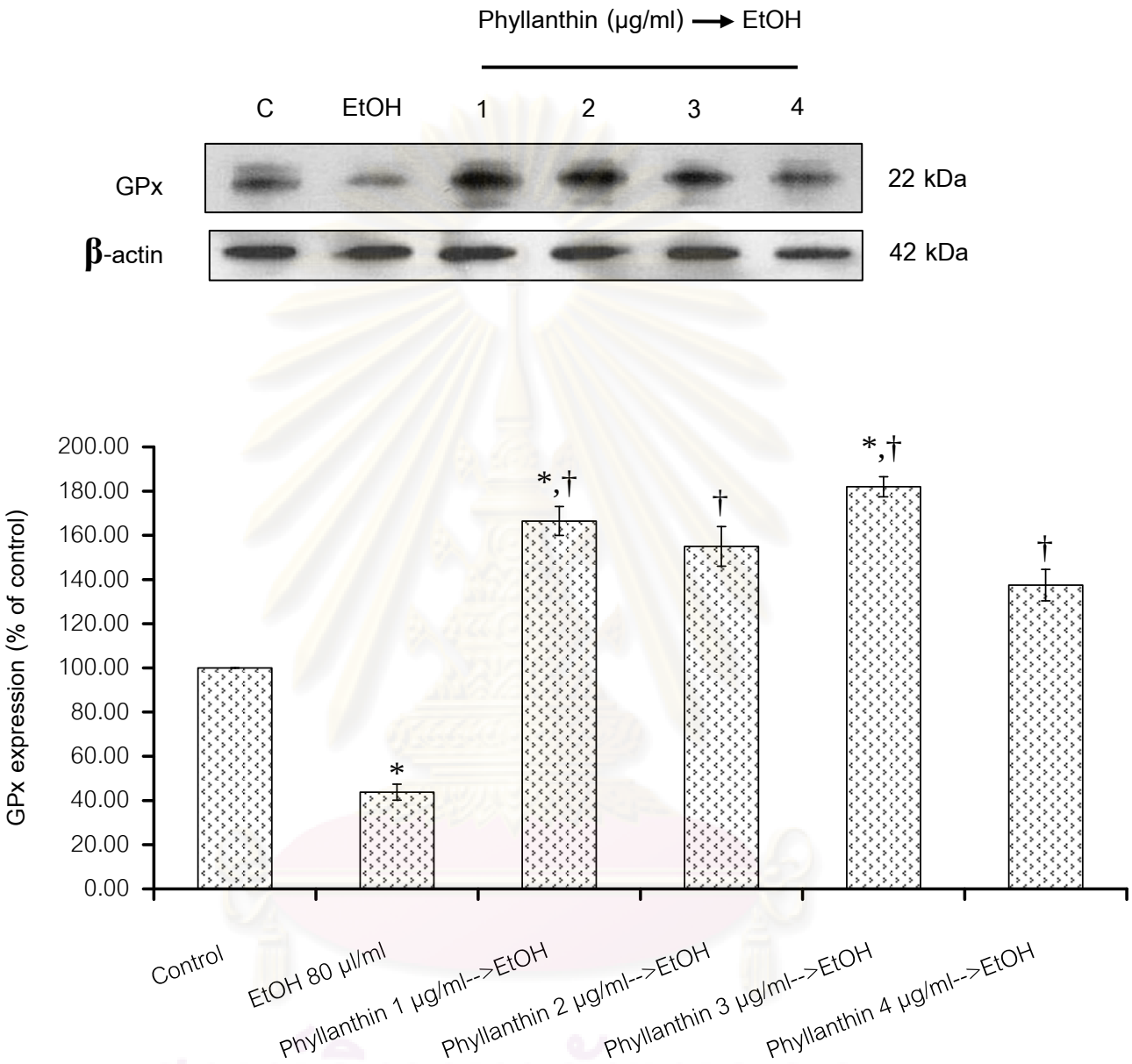


Figure 37: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the protein expression of glutathione peroxidase (GPx) in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

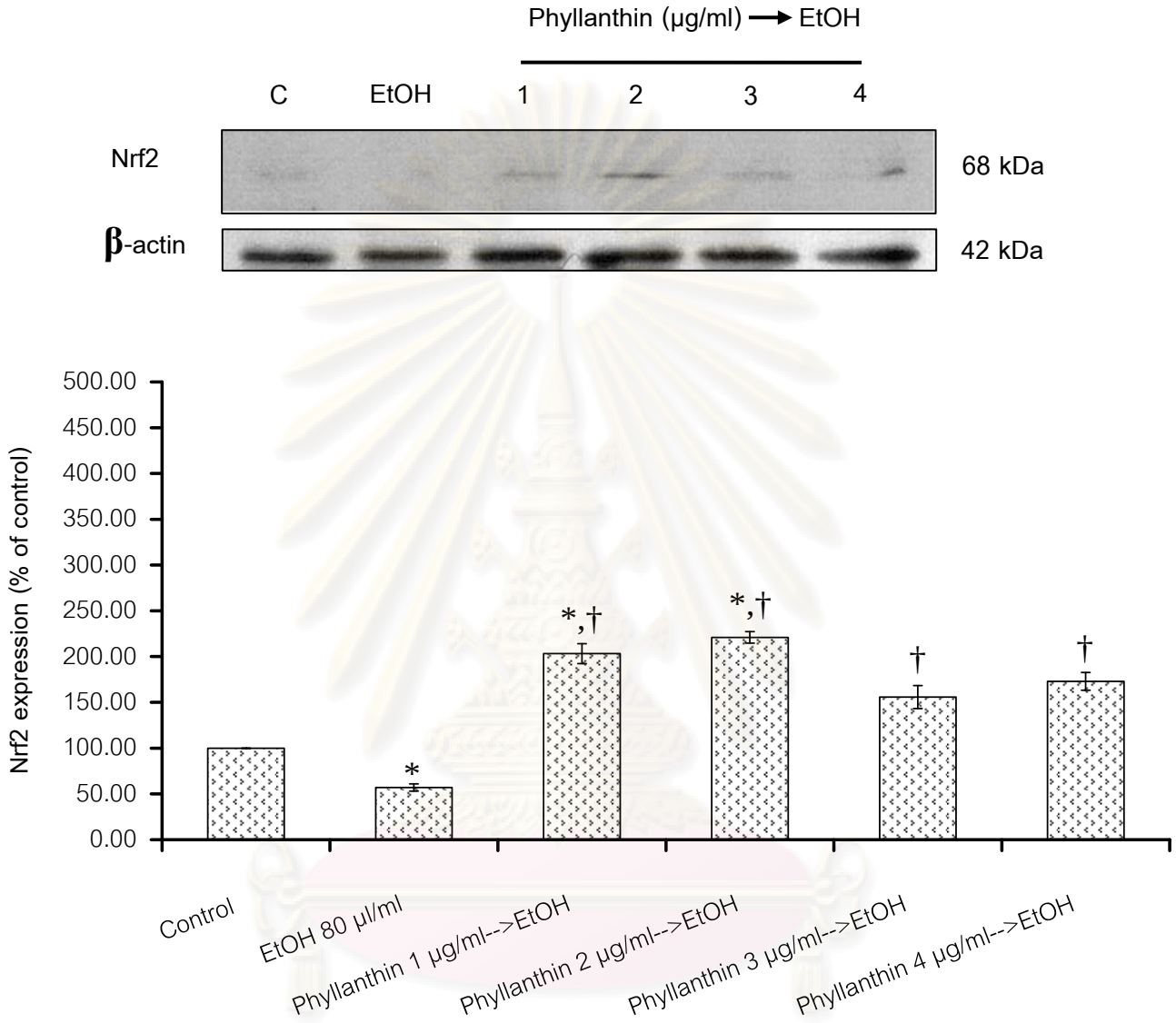


Figure 38: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activation of transcription factor Nrf2 in primary culture of rat hepatocytes treated with ethanol (EtOH).

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

Part B: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) in primary culture of rat hepatocytes

Effect of 24 h pretreatment with phyllanthin in primary culture of rat hepatocytes

Twenty four hours pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) in primary culture of rat hepatocytes showed no cytotoxic effects assessed by MTT reduction assay and the release of ALT and AST (Figures 39-41).

Effect of 24 h pretreatment with phyllanthin on the generation of ROS, MDA and total glutathione level in primary culture of rat hepatocytes

The sign of oxidative damage was not seen with phyllanthin pretreatment.Phyllanthin (1, 2, 3, 4 µg/ml) caused no change in the production of ROS, the levels of malondialdehyde (MDA) and total glutathione (Figures 42-44).

Effect of 24 h pretreatment with phyllanthin on the activities of ethanol-metabolizing enzymes (ADH, ALDH) in primary culture of rat hepatocytes

Phyllanthin (1, 2, 3, 4 µg/ml) had no effect on the activities of ethanol-metabolizing enzymes ADH and ALDH (Figures 45-46).

Effect of 24 h pretreatment with phyllanthin on the activities of antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx); and glutathione S-transferase (GST) in primary culture of rat hepatocytes

The result showed that the activities of SOD, CAT, GPx, GR and GST did not change after 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) (Figures 47-51).

Effect of 24 h pretreatment with phyllanthin on the expression of cytoprotective proteins: superoxide dismutases (Cu-ZnSOD (SOD1), MnSOD (SOD2)), glutathione reductase (GR), glutathione peroxidase (GPx); and transcription factor Nrf2 in primary culture of rat hepatocytes

In primary culture of rat hepatocytes treated with phyllanthin (1, 2, 3, 4 µg/ml) for 24 h, the expression of Nrf2 protein was increased when compared with control (Figure

56). This increase was concomitant with the induction of SOD1 (Cu-ZnSOD), SOD2 (MnSOD), GR and GPx protein expression (Figures 52-55).

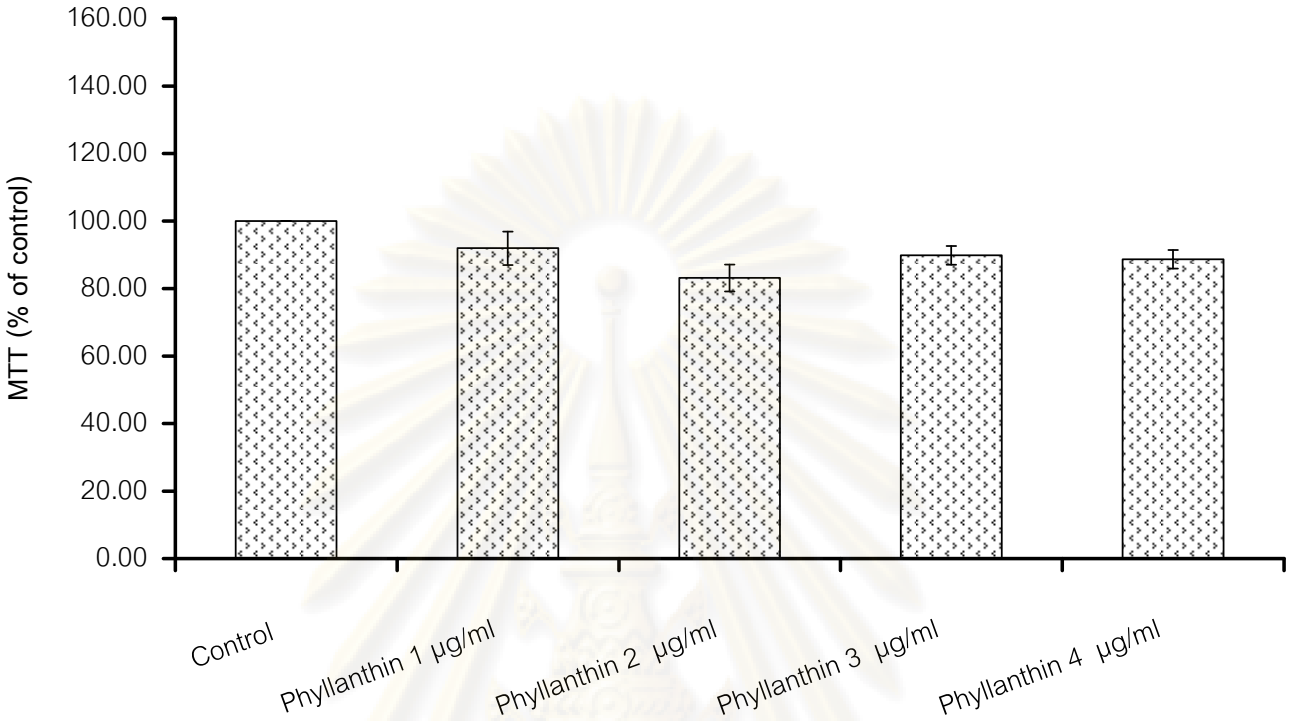


Figure 39: Cytotoxic effect of phyllanthin (1, 2, 3, 4 µg/ml) given 24 h in primary culture of rat hepatocytes.
Values are Mean ± SEM, n=4.

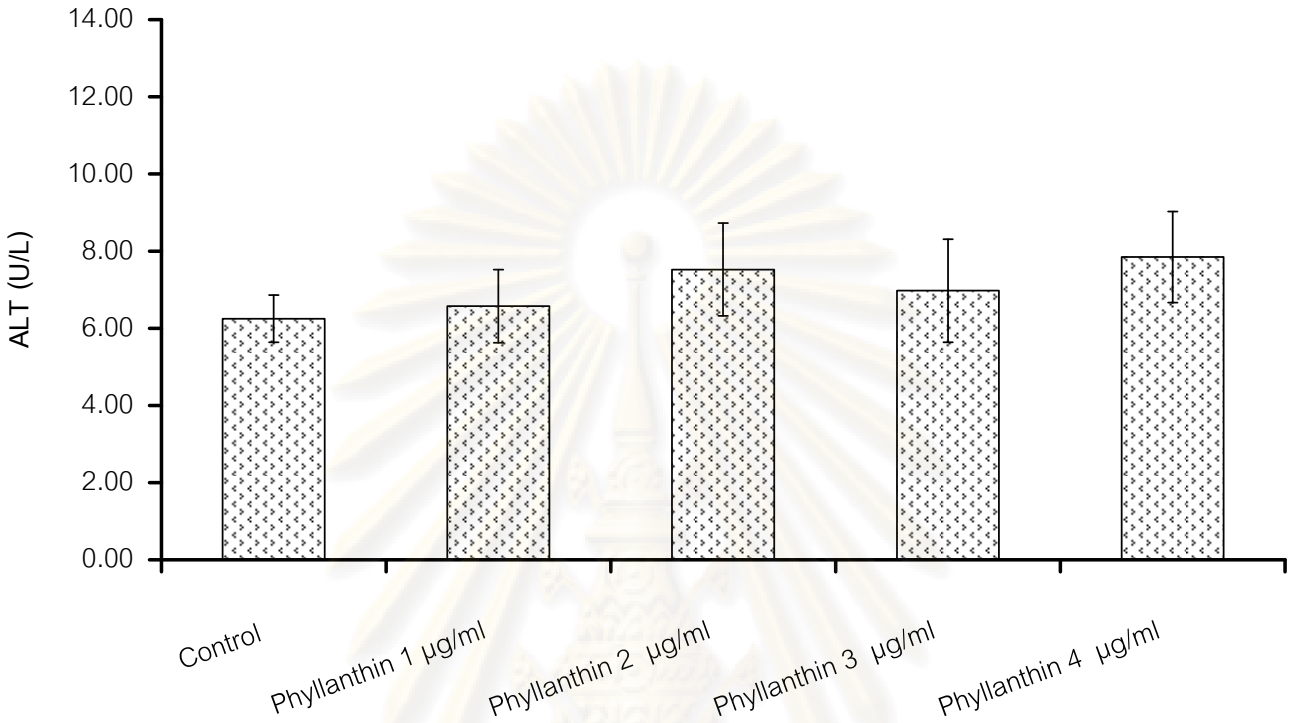


Figure 40: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the release of alanine aminotransferase (ALT) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

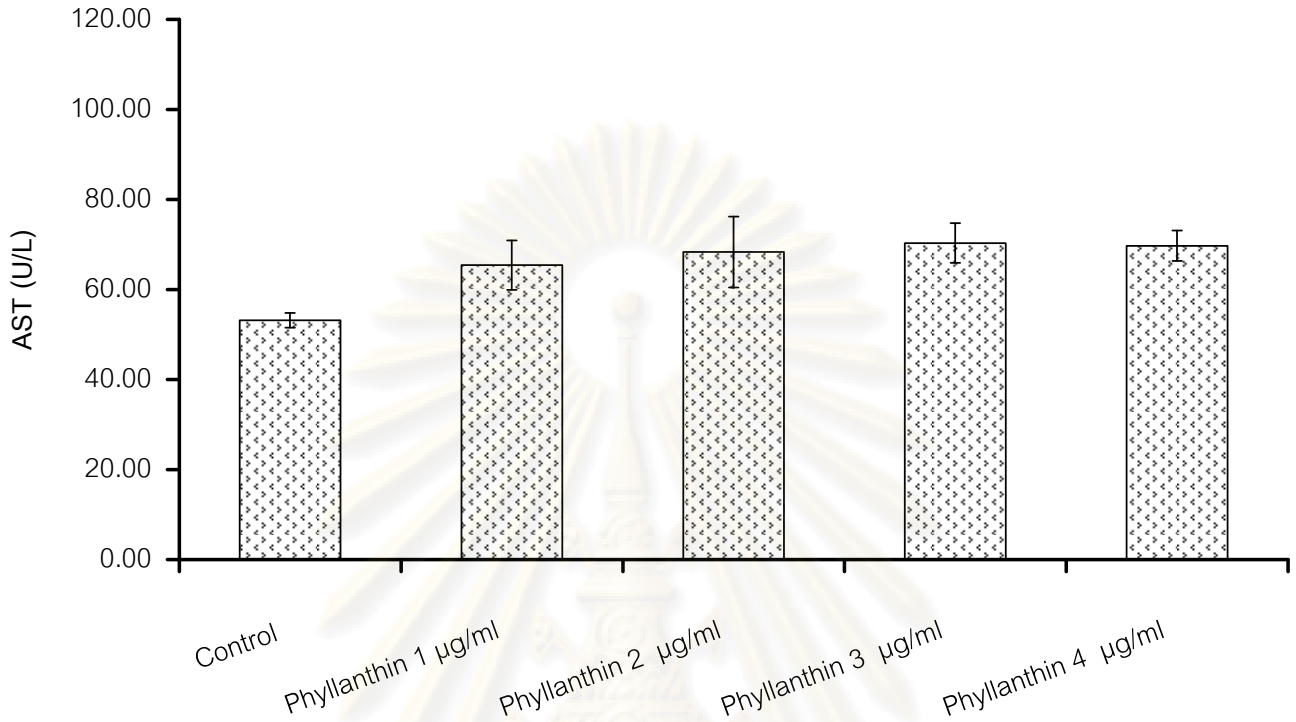


Figure 41: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the release of aspartate aminotransferase (AST) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

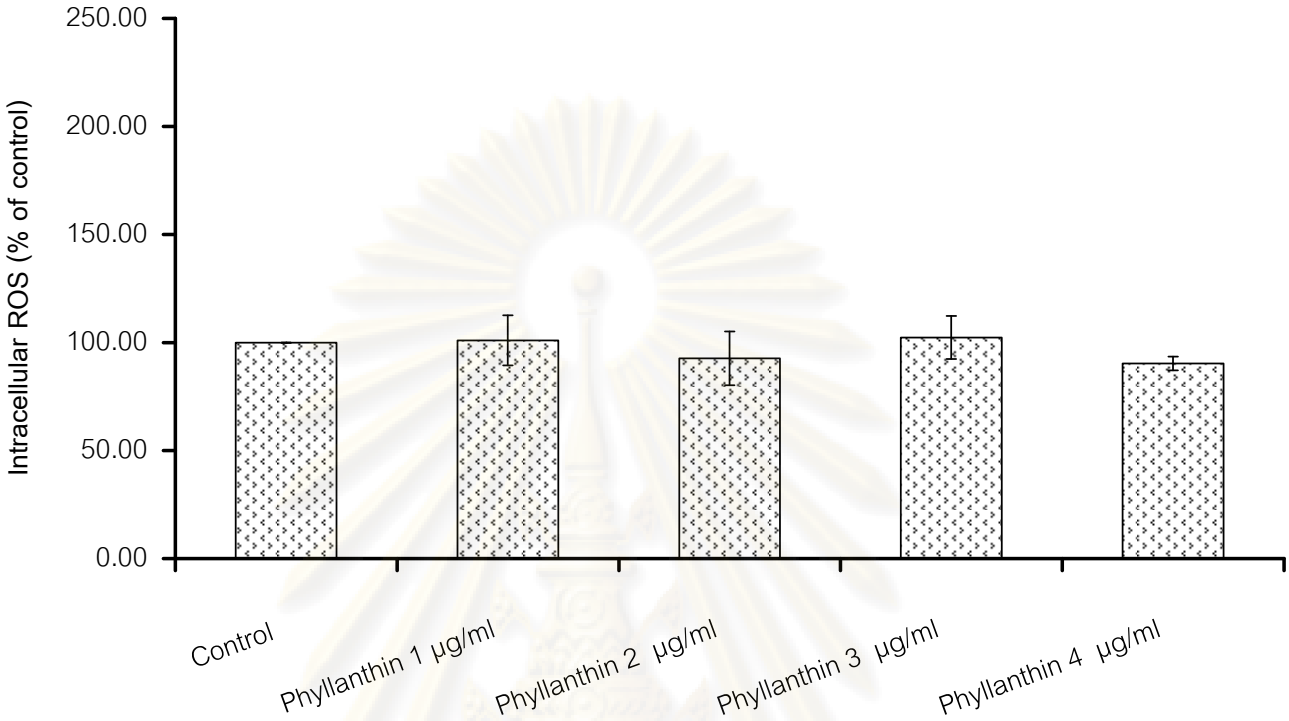


Figure 42: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the generation of intracellular reactive oxygen species (ROS) in primary culture of rat hepatocytes.
Values are Mean ± SEM, n=4.

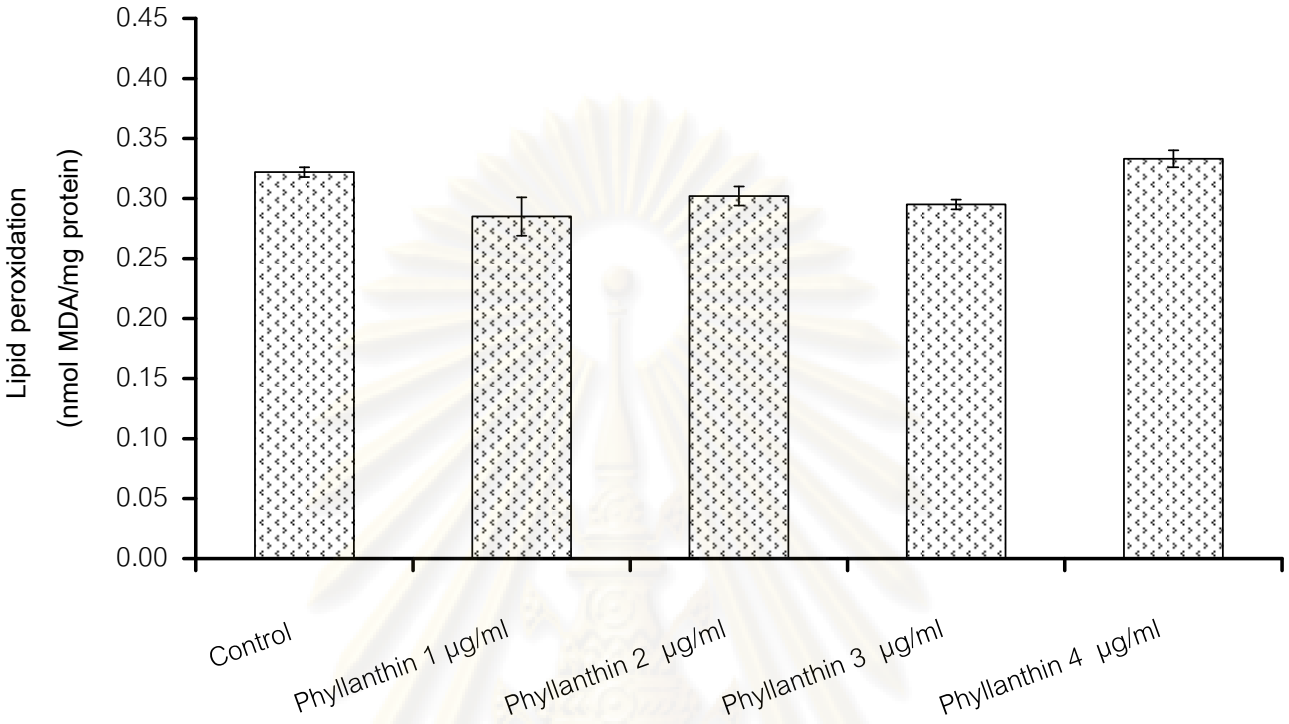


Figure 43: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the production of malondialdehyde (MDA) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

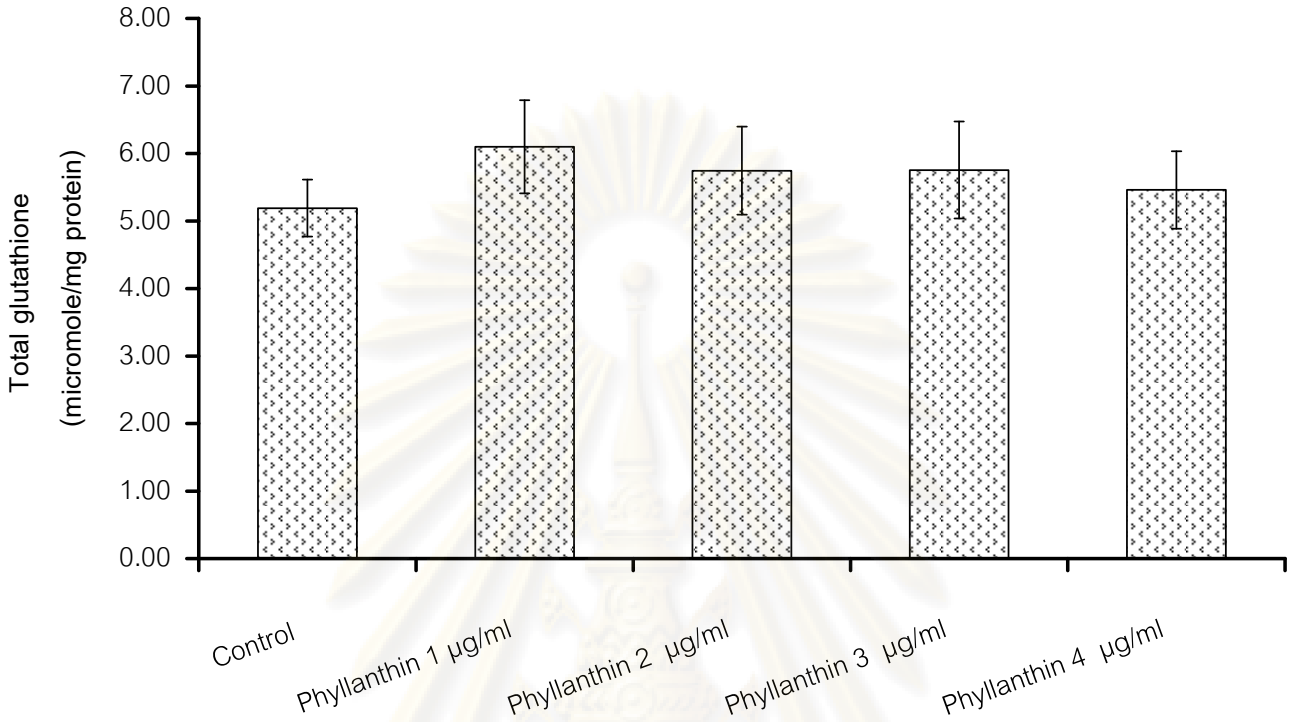


Figure 44: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the level of total glutathione in primary culture of rat hepatocytes. Values are Mean ± SEM, n=4.

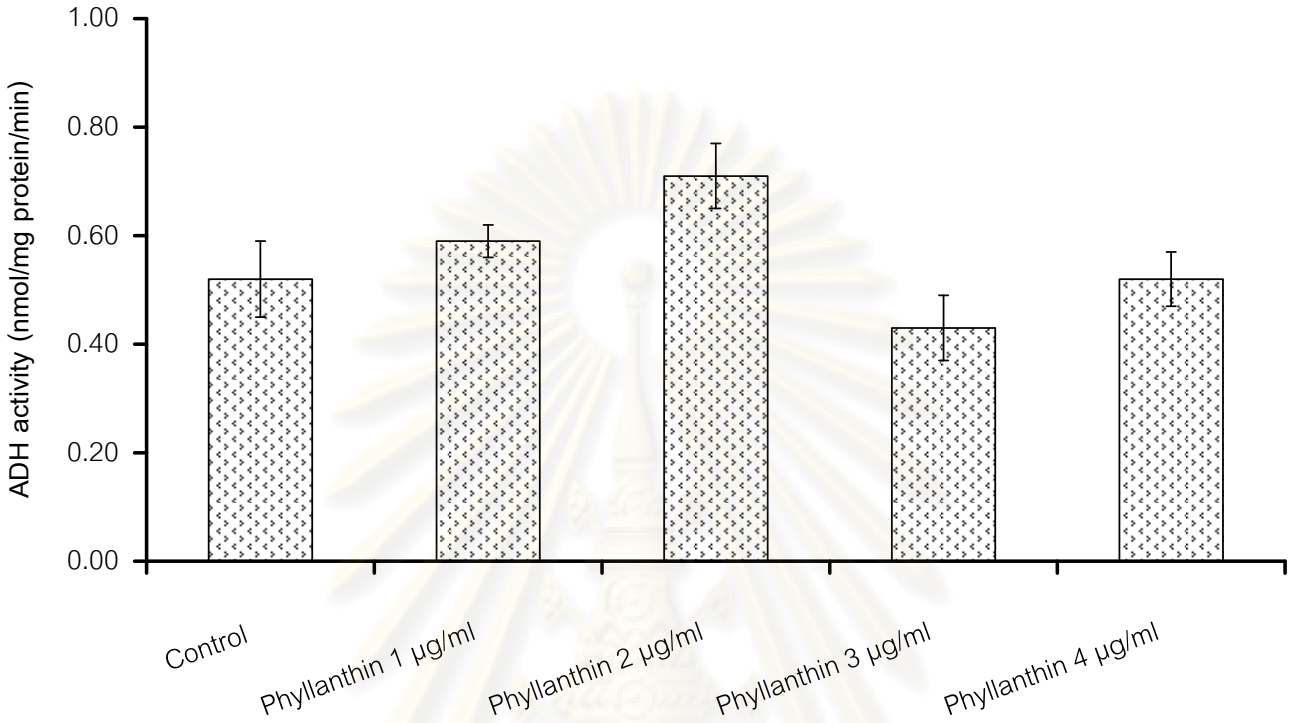


Figure 45: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of alcohol dehydrogenase (ADH) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

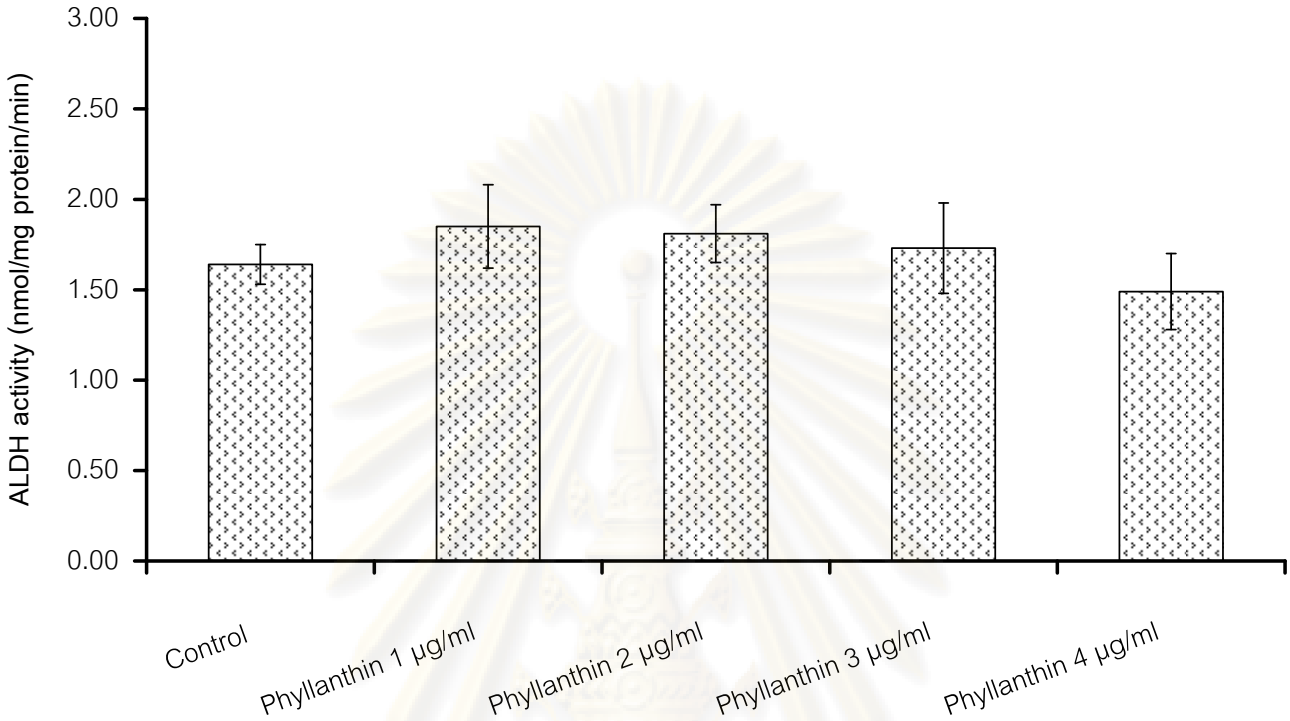


Figure 46: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of aldehyde dehydrogenase (ALDH) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

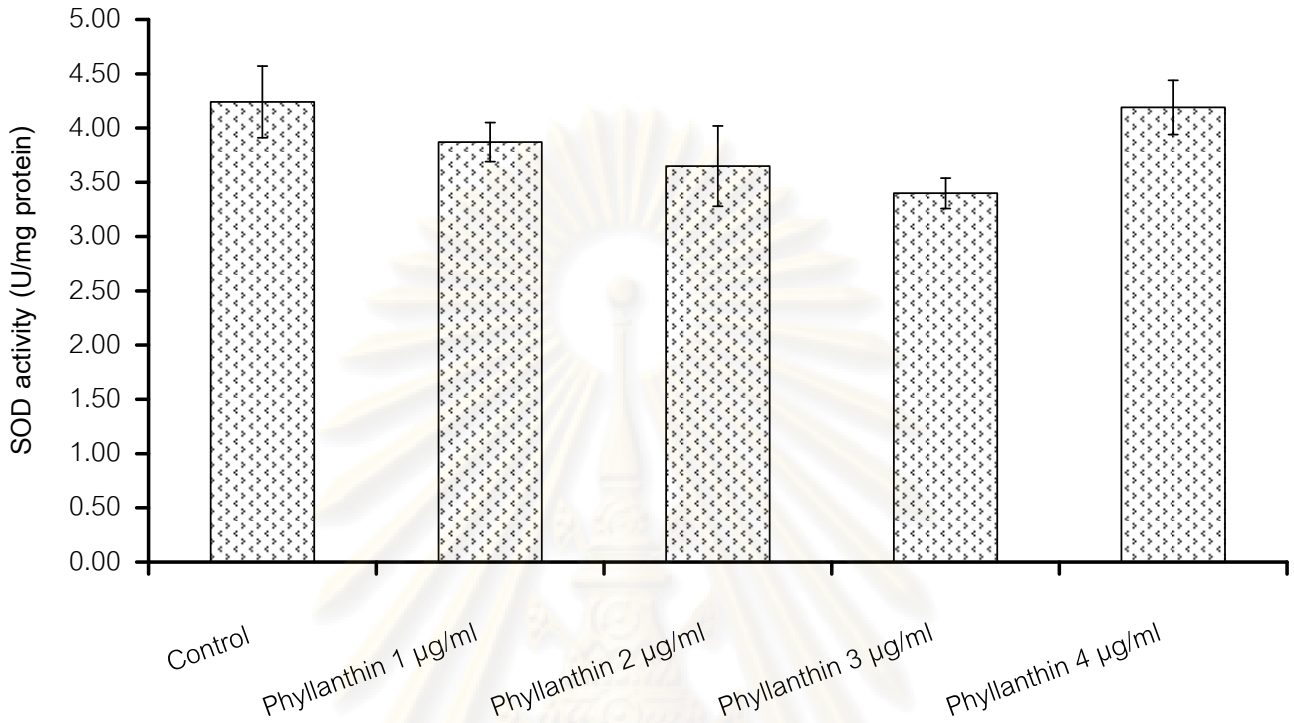


Figure 47: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of superoxide dismutase (SOD) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=3.

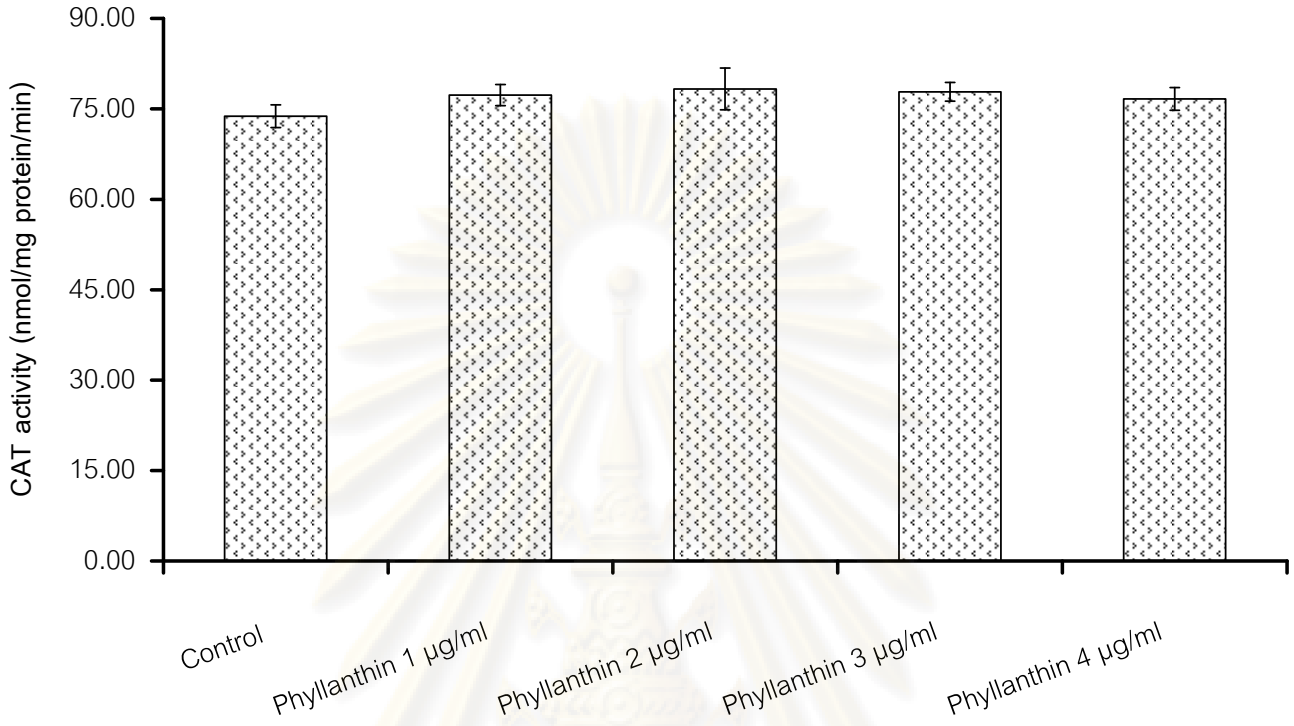


Figure 48: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of catalase (CAT) in primary culture of rat hepatocytes. Values are Mean ± SEM, n=3.

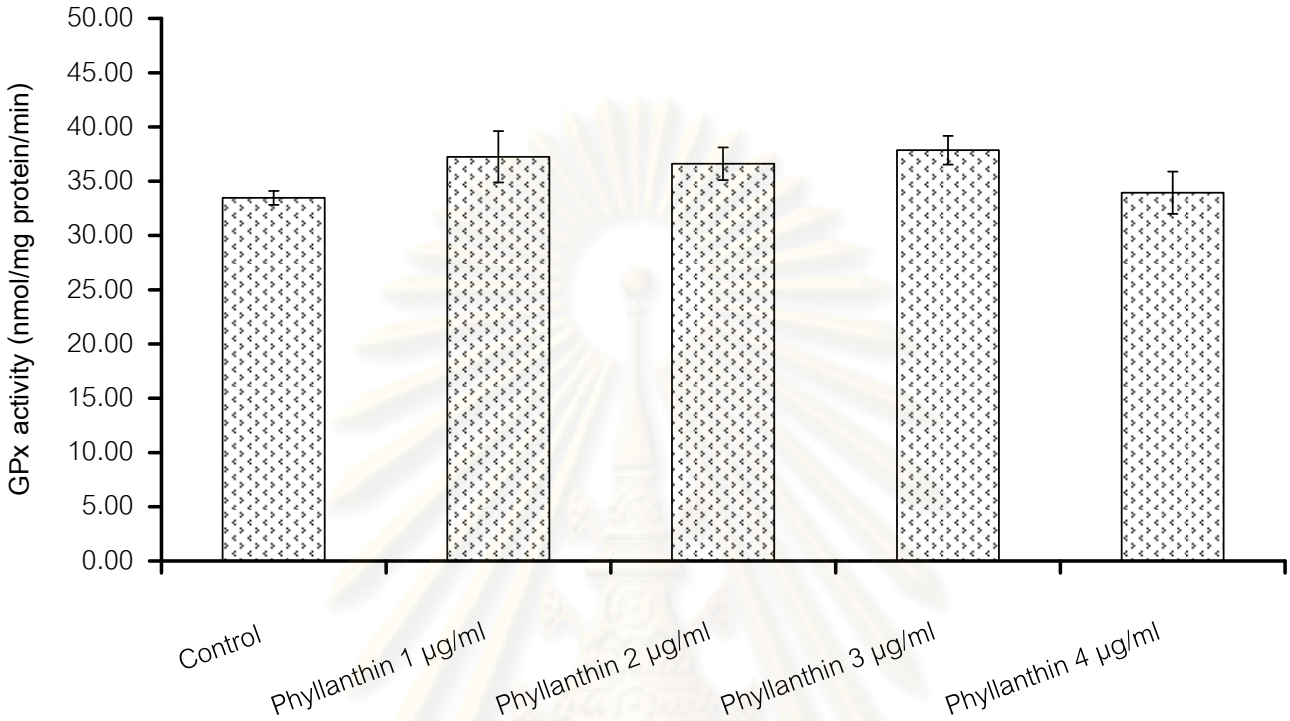


Figure 49: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of glutathione peroxidase (GPx) in primary culture of rat hepatocytes.
Values are Mean ± SEM, n=3.

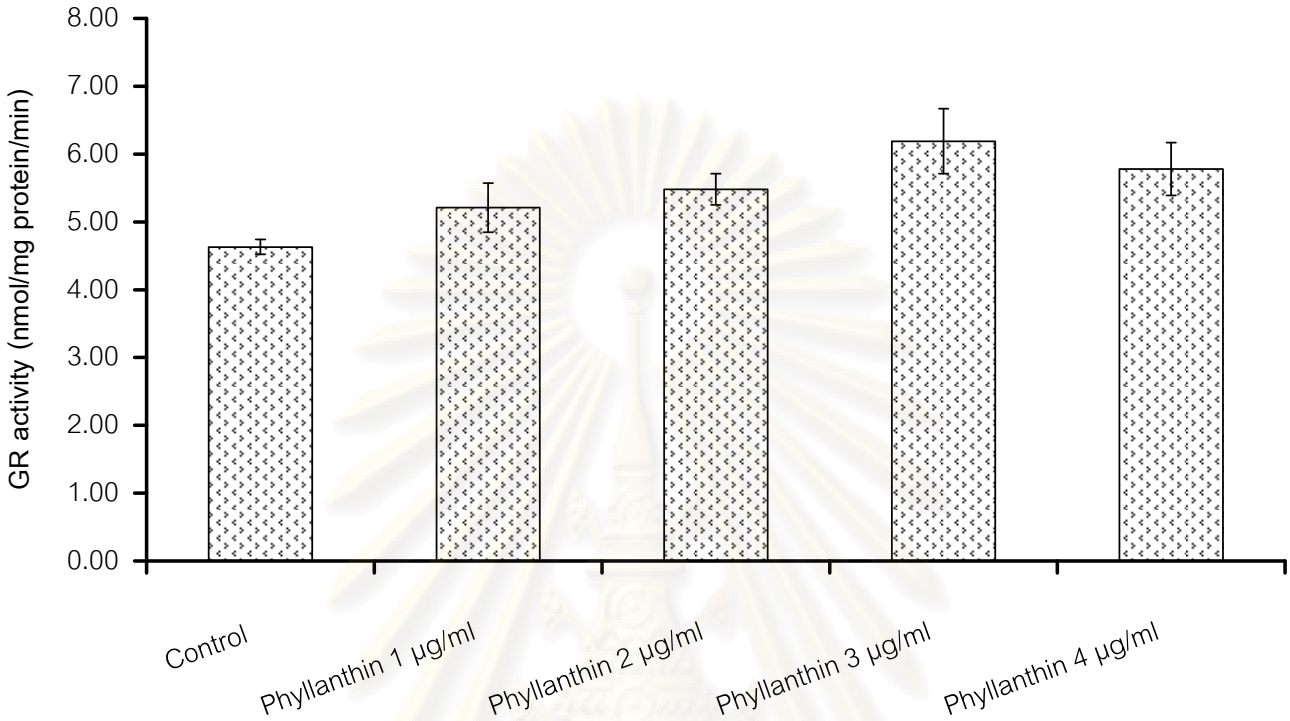


Figure 50: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of glutathione reductase (GR) in primary culture of rat hepatocytes.
Values are Mean ± SEM, n=3.

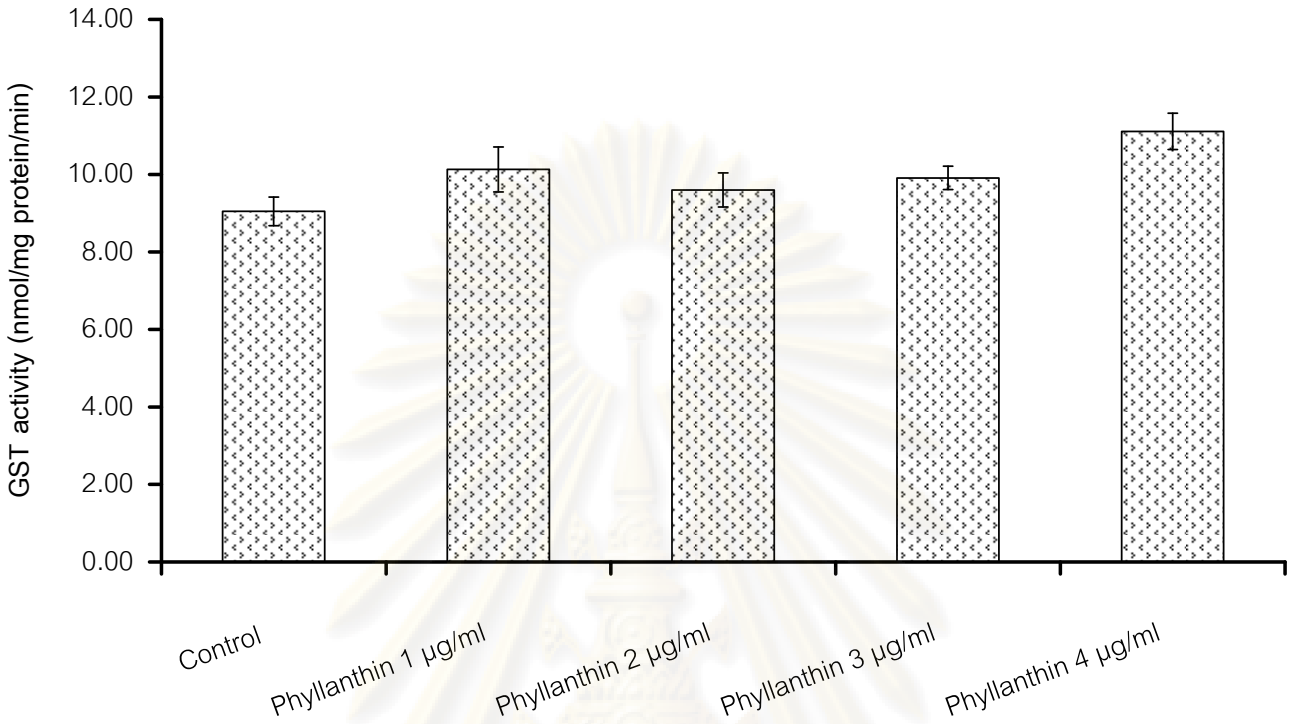


Figure 51: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activity of glutathione *S*- transferase (GST) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=3.

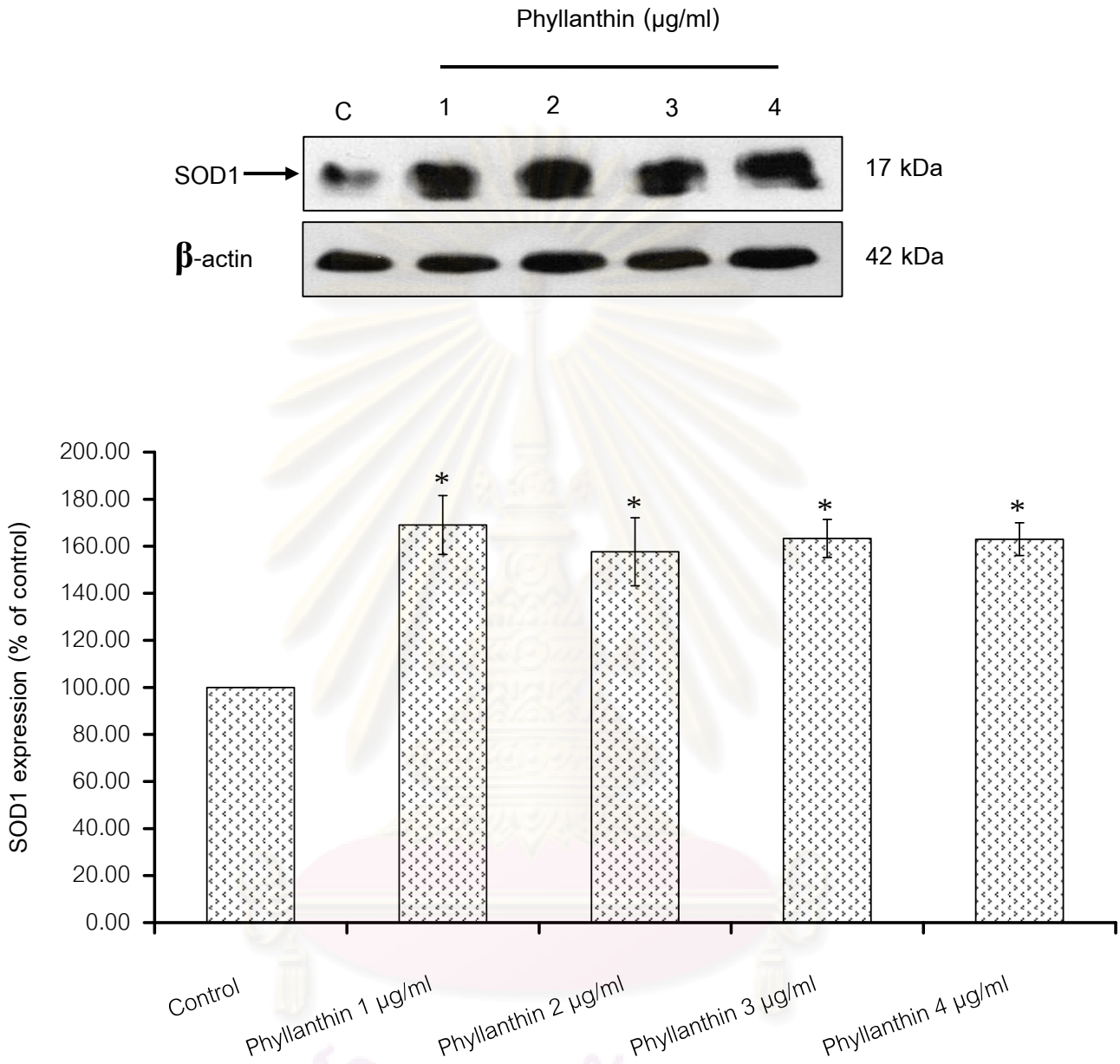


Figure 52: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 μg/ml) on the protein expression of copper-zinc superoxide dismutase (Cu-ZnSOD, SOD1) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

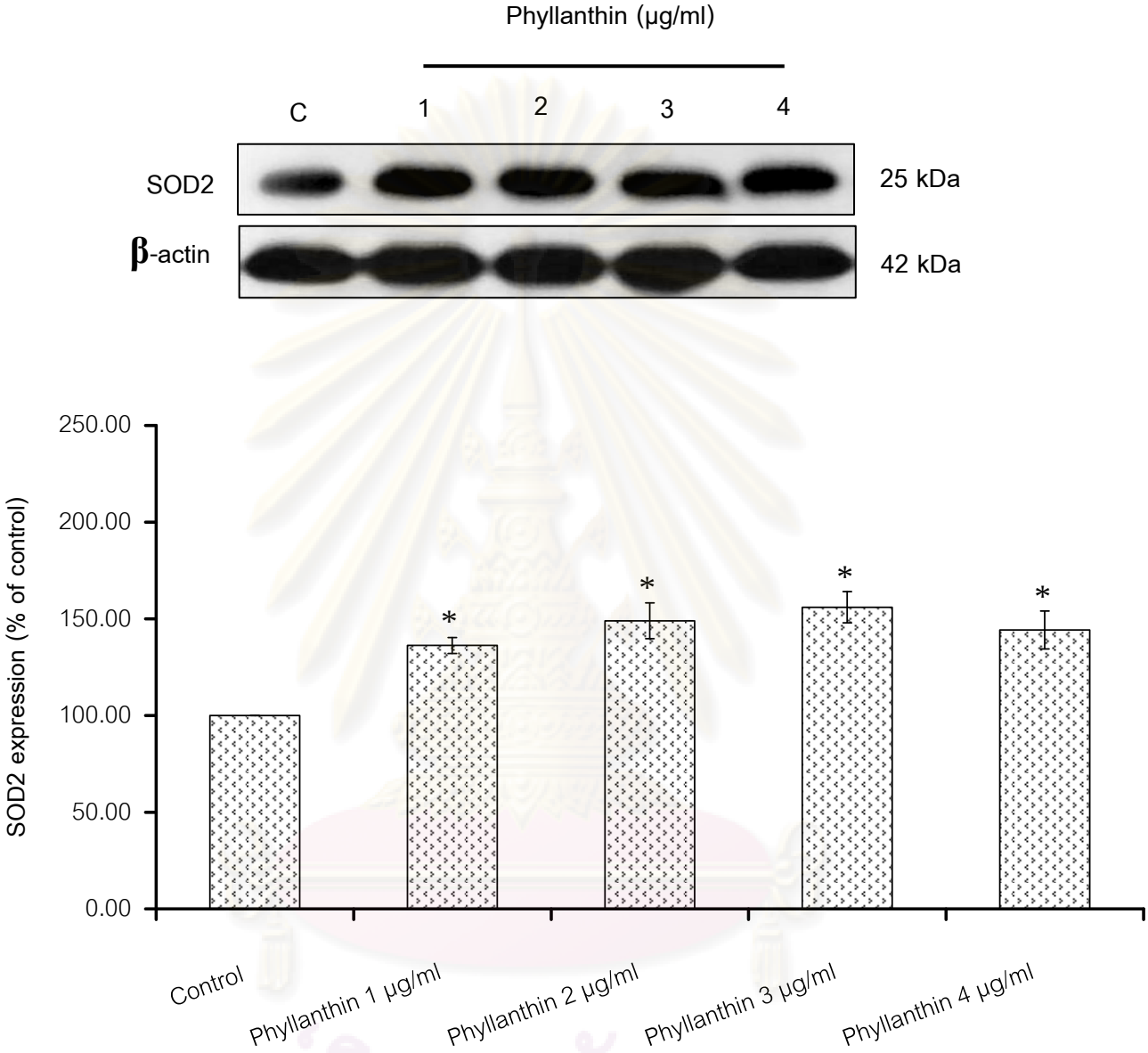


Figure 53: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the protein expression of manganese-superoxide dismutase (MnSOD, SOD2) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

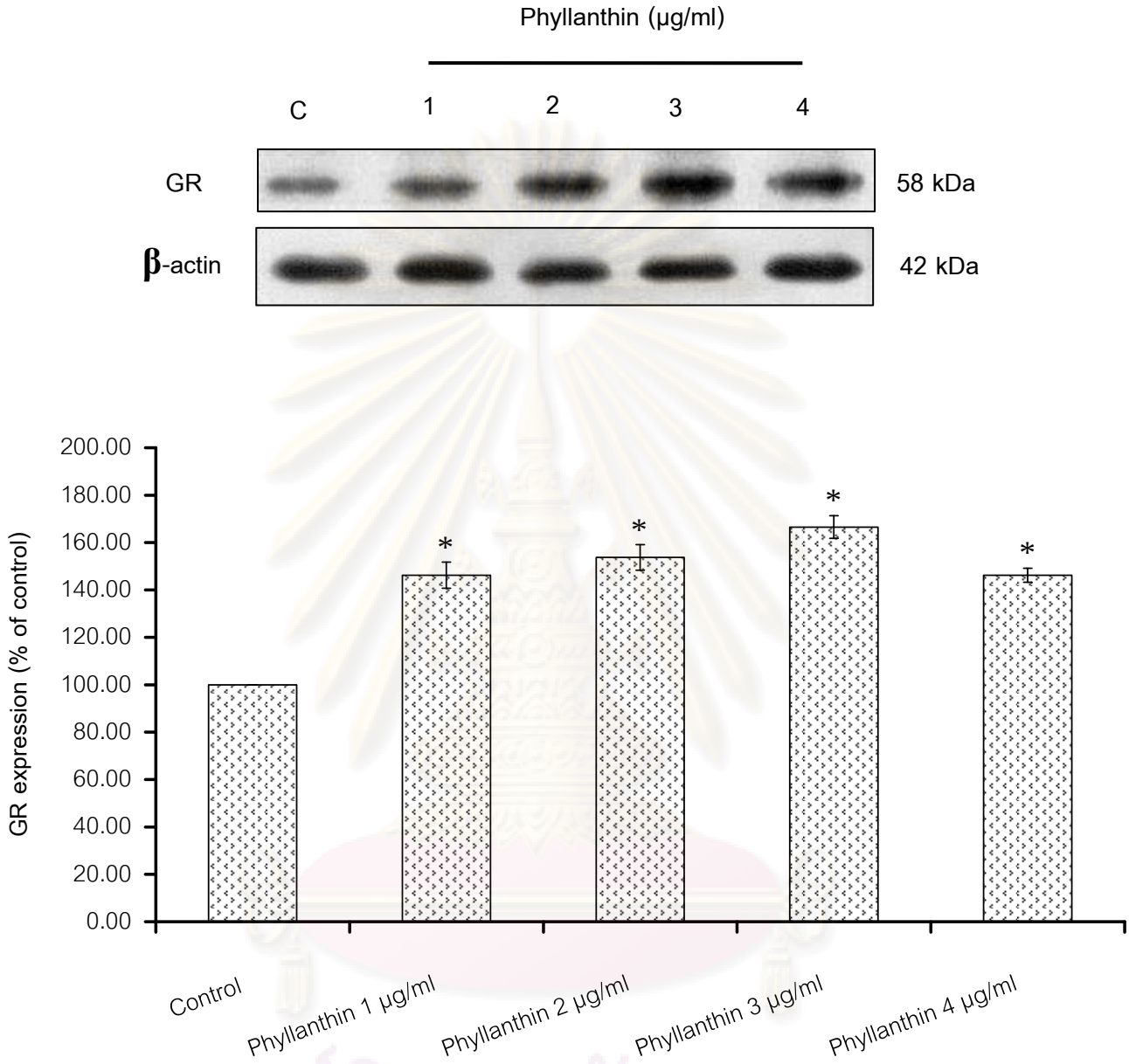


Figure 54: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the protein expression of glutathione reductase (GR) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

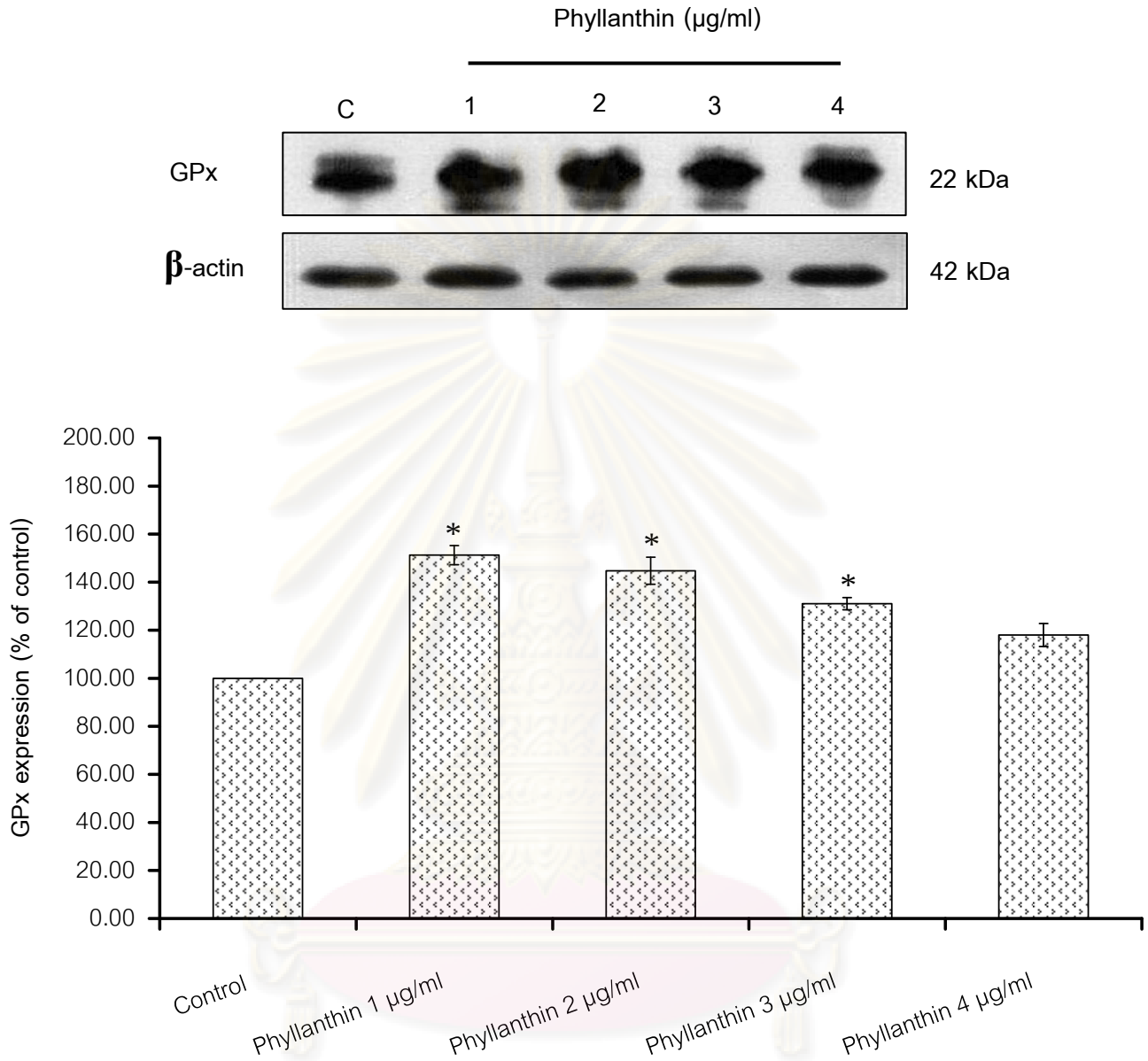


Figure 55: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the protein expression of glutathione peroxidase (GPx) in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

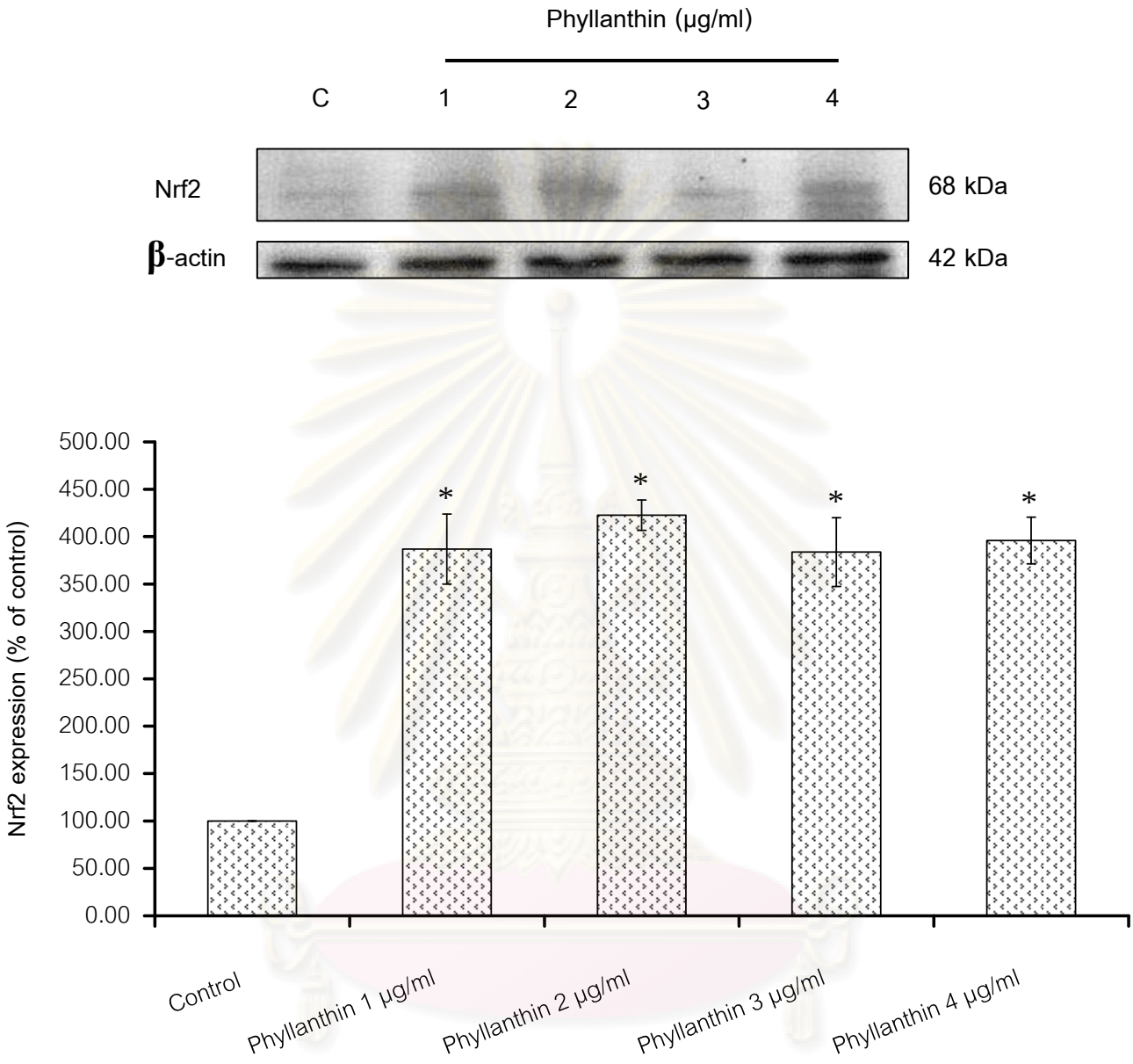


Figure 56: Effect of 24 h pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) on the activation of transcription factor Nrf2 in primary culture of rat hepatocytes.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Primary culture of rat hepatocytes was selected as an *in vitro* model to investigate the direct effect and possible hepatoprotective mechanism of phyllanthin against ethanol induced acute cytotoxicity.

Acetaldehyde and free radical production from ethanol exposure played an initial role prior to the alteration of cellular redox state in ethanol induced cytotoxicity. The excessive production of reactive oxygen species (ROS) from ethanol metabolism and impairment of cellular defense mechanism caused oxidative liver damage (Sergent et al., 2001; Zima et al., 2001; Hoek and Pastorino, 2002; Wu and Cederbaum, 2003). ROS formation exhibited peroxidation of proteins and membrane lipids, depletion of glutathione (GSH) which increased susceptibility to peroxidative damage by ethanol. These causes damaged cell structure and function contributing to acute ethanol-induced necrotic cell death (Dianzini, 1985; Varma et al., 2004).

In the present study, the early acute rat liver cell injury induced by ethanol was detected by using MTT reduction assay which indicates the viability cells and the impairment of membrane integrity as demonstrated by the leakage of certain liver enzymes into the medium. After 2 h exposure to ethanol (80 µl/ml), cell viability was decreased by the decrease in % MTT together with the increase in the leakage of transaminases (ALT & AST) (Figures 20-23). Concurrently, intracellular reactive oxygen species (ROS) was also increased by ethanol exposure (Figure 24) in which ROS triggered oxidative damage as shown by the increase in malondialdehyde (MDA), the product of lipid peroxidation (Figure 25).

Several antioxidant enzymes played major roles on removal of excessive cellular ROS. The most crucial enzymes in the antioxidant system included SOD, CAT, GPx and GR. Impairment of cellular antioxidant activity by ethanol exposure was important and lead to the generation of alcoholic liver disease. Superoxide radicals could inactivate catalase, whereas Cu-ZnSOD was irreversibly inactivated by hydrogen peroxide (H_2O_2),

both enzyme activities were disturbed by overproduction of superoxide anion (Sinet and Garber, 1981; Kono and Fridovich, 1982; Salo et al., 1988).

Acute ethanol-treated rat showed the decrease in activity of copper-zinc superoxide dismutase (Cu-ZnSOD), catalase (CAT) and glutathione *S*-transferase (GST) but not glutathione peroxidase (GPx) (Ribère et al., 1984; Ribère et al., 1985; Kocak-Toker et al., 1985). Long-term exposure to ethanol increased total GST activity as assessed by CDNB conjugation both *in vitro* and *in vivo* studies (Vanhaecke et al., 2000). Recent evidence suggested that ethanol could inhibit glutathione peroxidase and glutathione reductase activities (Luczaj and Skrzydlewska, 2004; Ostrowska et al., 2004; Pushpakiran, Mahalakshmi, and Anuradha, 2004).

In our study in primary culture of rat hepatocytes, ethanol reduced cellular antioxidant capability illustrated by the decrease in level of total glutathione (GSH) (Figure 26) and activities of superoxide dismutase (SOD) (Figure 29), glutathione reductase (GR) (Figure 32) and glutathione *S*-transferase (GST) (Figure 33). The activity of glutathione peroxidase (GPx) was increased by ethanol exposure (Figure 31). GPx may be activated by oxidative stress initiated by ROS resulting in the increased enzyme activity. Catalase (CAT) played minor role for antioxidant activity in rat liver cells (Figure 30). The varieties of the effect of ethanol on antioxidant enzymes activities may depend on the model, diet, amount, and time of ethanol exposure (Bailey et al., 2001; Wu and Cederbaum, 2003).

Phyllanthin was isolated from *Phyllanthus amarus* Schum. et Thonn, one of the best herbs for treating liver diseases. In our previous study, 24 h pretreatment with aqueous extract of *Phyllanthus amarus* showed the hepatoprotective activity against ethanol in rats, based on biochemical and histopathological assessment (Pramyothin et al., 2007). Phyllanthin has been reported to protect liver cells from carbon tetratchloride (CCl_4) and D-galactosamine (Syamasundar et al., 1985). The mechanism of hepatoprotective effect of phyllanthin was through its antioxidant activity (Harish and Shivanandappa, 2006; Fang, Rao, and Tzeng, 2008; Krithika et al., 2009).

In the present study, the exposure time (24 h pretreatment) and concentrations of phyllantin (1, 2, 3, 4 µg/ml) were selected from preliminary study in primary culture of rat hepatocytes as manifested by %MTT and the release of ALT & AST (Figures 39-41).

Precipitation of phyllanthin in DMSO (0.05% v/v) was observed at the concentration more than 100 µg/ml. Phyllanthin at high dose (100 µg/ml) showed no cytotoxicity.

Phyllanthin (1, 2, 3 µg/ml) showed the hepatoprotection by improving cell viability and membrane integrity of rat hepatocytes being treated with ethanol (Figures 20-23). Phyllanthin at dose 4 µg/ml seemed to have lesser effect with slightly toxic on ethanol treated primary culture of rat hepatocytes.

Hepatoprotective effect of *Phyllanthus amarus* and phyllanthin has been reported in CCl_4 induced liver damage. The isolated and purified active principles such as phyllanthin, hypophyllanthin, geraniin and rutin were responsible for the hepatoprotection of *Phyllanthus amarus* by their perfect synergistic combination (Islam et al., 2008). From our previous study, aqueous extract of *Phyllanthus amarus* showed the hepatoprotective activity against ethanol in primary culture of rat hepatocytes and rats. In primary culture of rat hepatocytes, no need for pretreatment of aqueous extract, it was incubated with ethanol for 2 hours, % MTT was improved about 2.5 times of the effect of ethanol alone (Pramyothin et al., 2007). Using the same procedure for phyllanthin in this present study, 24 hours pretreatment was needed for phyllanthin to demonstrate its hepatoprotective activity. This may due to the late effect of phyllanthin. When compared the protective activity of aqueous extract and phyllanthin in improving cell viability, aqueous extract seemed to have more effect suggesting the major involvement of other phenolic compounds inherent in the extract.

The antioxidant effects of phyllanthin on ethanol-induced oxidative damage were shown by the decreased ethanol generating intracellular ROS together with the reduction in lipid peroxidation (Figures 24-25). Twenty four hours pretreatment with these concentrations of phyllanthin (1, 2, 3, 4 µg/ml) produced no oxidative liver cell damage as demonstrated by no change in the production of ROS, lipid peroxidation and total glutathione (Figures 42-44).

Phyllanthin restored the decreased activity by ethanol of major antioxidant enzymes including SOD and GR back to normal (Figures 29, 32). SOD catalyzed dismutation of superoxide anion ($O_2^{\cdot-}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2). The important reactivity of superoxide radical is its capability of initiating free-radical chain reactions. SOD is considered as front line among other antioxidant enzymes in

defense against the potentially free radicals that cause oxidative stress (Mallikarjuna et al., 2008)

Reduced glutathione (GSH) plays crucial roles in physiological functions and protection against oxidative damage by detoxifying electrophilic molecules and scavenging free radicals. Effects of ethanol on total hepatic GSH level were variable. Many studies reported that ethanol depleted GSH levels by generating pro-oxidants as well as inhibiting the mitochondrial glutathione transporter (Vina et al., 1980; Colell et al., 1998; Fernandez-Checa et al., 1993, 1998; Wheeler et al., 2003).

Pretreatment with phyllanthin (1, 2, 3, 4 μg/ml) increased total glutathione levels which was reduced by ethanol back to normal (Figure 26). This may due to the increment of GR activity responsible for the maintenance of cellular GSH homeostasis and regulates GSH/GSSG ratio (Yang, Chan, and Yu, 2006).

Phyllanthin isolated from *Phyllanthus urinaria* showed slightly free radical scavenging activity using DPPH assay when compared to other constituents of this plant (Fang, Rao, and Tzeng, 2008). DPPH radical scavenging activity was influenced by the chemical structure of the radical scavenger (Shizuka and Kawabata, 2005). As previously mentioned, phyllanthin needed time of at least 24 h to demonstrate its hepatoprotection against ethanol in primary culture of rat hepatocytes indicating that hepatoprotective effect of phyllanthin may not involve its direct effect of its own molecule but may involve other regulation of molecular event.

In order to determine the molecular mechanism of 24 h pretreatment with phyllanthin which showed hepatoprotection against ethanol-induced oxidative damage, changes of protein level (antioxidant enzymes) were investigated. The activation of nuclear factor-erythroid-2-related factor 2 (Nrf2), a basic leucine zipper transcription factor which controls the expression of antioxidant and detoxification enzymes, has been focused in this study. In addition the gene expression of antioxidant enzymes affected by ethanol exposure was also determined.

Amount and activity of Nrf2 are regulated at levels of transcription, degradation, translocation and post-translational modifications (Purdom-Dickinson et al., 2007). It is transcriptionally active under both constitutive and stress conditions which induced a subset of cytoprotective proteins during stress adaptations. From our result, an almost

negligible amount of Nrf2 protein was observed in control hepatocytes. After 2 h of ethanol treatment, Nrf2 protein was reduced when compared with control. Twenty four hours pretreatment with phyllanthin (1, 2, 3, 4 μg/ml) before ethanol treatment induced the expression of Nrf2 (Figure 38). This induction of Nrf2 activation was proved to be the effect of phyllanthin pretreatment as shown in Figure 56.

The mechanism of increased Nrf2 activation by phyllanthin is currently not known. Some evidence suggested the multiple mechanisms of signals mediated by one or more of the upstream kinases such as mitogen-activated protein kinases, phosphatidylionositol-3-kinase/Akt, protein kinase C, and casein kinase-2 (Chen et al., 2004; Keum et al., 2006). Recently, several natural products were reported as inducers of Nrf2 activation including curcumin, sulforaphane, garlic organosulfur compounds and resveratrol (Balogun et al., 2003; Chen et al., 2004; Shinkai et al., 2006; Rubiolo, Mithieux, and Vega, 2008). These compounds targeted Keap1 by oxidizing or chemically modifying one or more of its specific cysteine thiols, thereby stabilizing Nrf2. In addition, phosphorylation of specific serine or threonine residues present in Nrf2 by upstream kinases may also facilitate the nuclear localization of Nrf2 (Young-Loon, Kundu, and Hye-Kyung, 2008).

Nrf2 has been shown to have a regulatory role in the inducible expression of a number of enzymes important in protection against reactive oxygen species including NAD(P)H-quinone oxidoreductase-1, heme oxygenase-1, glutamate cysteine ligase, glutathione *S*-transferase, glutathione peroxidase, thioredoxin reductase-1, Cu-Zn superoxide dismutase, catalase etc (Itoh et al., 1997; Chan and Kwong, 2000; Thimmulappa et al., 2002; Kwak et al., 2003; Hu et al., 2006). Nrf2 also plays a critical role in the maintenance of cellular redox homeostasis under both stress and non-stress conditions and is critical for maintaining the GSH redox state via transcriptional regulation of glutathione reductase (Harvey et al., 2009).

In the present study, Western blot analysis showed the reduction of SOD1 (Cu-ZnSOD), GR and GPx protein in hepatocytes after 2 h treatment with ethanol. Ethanol-induced oxidative damage could induce varying degrees of protein cross-linking, protein fragmentation and proteolysis. Ethanol has been reported to interrupt cellular

proteolytic pathways that regulated the quantity and the types of proteins inside cells (Donohue, 2002; Donohue and Osna, 2003).

There was evidence showed that modification of structural protein by free radical especially in the enzyme active site increased proteolytic susceptibility (Salo et al., 1990). Cellular proteins were susceptible to damage by ROS and oxidative modification of proteins may lead to the structural alternation and functional inactivation of many enzymes studied both *in vitro* and *in vivo* (Davies, 1988). These modified proteins were degraded more rapidly than native proteins by proteolytic system (Shoal and Brink, 1992).

The mitochondrial enzyme manganese superoxide dismutase (MnSOD, SOD2) is one of antioxidant defense systems in mammalian cells which plays a pivotal role in safeguarding mitochondria from oxidative stress as well as in regulating intramitochondrial O_2^- concentration. Many studies suggested that mitochondria were primary target of ethanol toxicity. Ethanol-induced oxidative stress was mainly caused by ROS generated by the mitochondrial respiratory chain, which was implicated in the pathogenesis of alcoholic liver disease (Mansouri et al., 2001; Adachi and Ishii, 2002; Albano, 2007). In this present study, change in protein expression of SOD2 (MnSOD) was not observed in ethanol-treated hepatocytes. These occurring may due to cellular adaptive response against ethanol-induced oxidative damage.

However, there was controversy over the cause of the perturbations in hepatic protein metabolism following ethanol exposure. The inconsistent data has been reported on ethanol-induced impairment in liver protein metabolism both protein synthesis and protein degradation (Murty, Verney, and Sidransky, 1980; Morland et al., 1983; Poso, 1987; Donohue, Drey, and Zetterman, 1988).

Pretreatment with phyllanthin (1, 2, 3, 4 µg/ml) 24 h before ethanol exposure markedly increased the expression of SOD1, SOD2, GR and GPx in hepatocytes when compared with ethanol group (Figures 34-37). This induction of antioxidant enzymes (SOD1, SOD2, GR and GPx) was due to the effect of phyllanthin (1, 2, 3, 4 µg/ml) alone as shown in Figures 52-55. However, phyllanthin alone did not change the activities of antioxidant enzymes (SOD, CAT, GR, GPx and GST) when compared with control group (Figures 42-44, 47-51). These results suggested that the antioxidant activity of

phyllanthin against ethanol-induced oxidative damage in rat liver cell may be related to the increase in protein expression of antioxidant enzymes.

Several studies suggested that oxidative stress was an important cause of ethanol-induced cellular damage. Hepatic effect of acute ethanol toxicity also caused oxidative damage via its metabolism (Lieber, 1997). Oxidation of ethanol through the cytosolic alcohol dehydrogenase (ADH) was considered the primary initial step accounting for 90% of ethanol metabolism. This reaction generated acetaldehyde, the potent electrophiles causing cellular damage by reacting with nucleophiles in proteins, phospholipids and nucleic acids to produce adducts. Acetaldehyde was converted to acetate by mitochondrial aldehyde dehydrogenase (ALDH), the NAD^+-dependent enzyme which was the major means of its oxidation in the liver to prevent the formation of adducts from acetaldehyde and protein. The acetaldehyde yield played a role in the production of ethanol-induce hepatic injury. The high levels of acetaldehyde were a consequence of ethanol enhanced alcohol dehydrogenase and/or ethanol/acetaldehyde induced inhibition of aldehyde dehydrogenase (Kamimura, Gaal, and Britton, 1992; Lieber, 1997; Barry, 2006; Fisher, Swaan, and Eddington, 2009).

In the present study, the effect of phyllanthin on the activities of two primary ethanol-metabolizing enzymes ADH and ALDH was also assessed. Ethanol treatment inhibited ALDH activity. Pretreatment with phyllanthin before ethanol did not affect on both enzyme activities (Figures 27-28). Confirmed by pretreatment with phyllanthin alone did not alter in the activities of ADH and ALDH (Figures 45-46). This result indicated that the protective activity of phyllanthin on the ethanol-induced rat liver cell damage did not involve the inhibition of acetaldehyde produced by ethanol oxidation.

In conclusion, phyllanthin exerted the hepatoprotective effect on ethanol-induced oxidative damage in primary culture of rat hepatocytes through its antioxidant activity via the induction of the de novo synthesis of antioxidant enzymes (SOD1, SOD2, GR and GPx) by the activation of transcription factor Nrf2.

This important information may be of therapeutic strategies in attempts to ameliorate or prevent the toxic effect of ethanol. Further study should be performed using *in vivo* model to investigate the protective effect of phyllanthin in ethanol-induced liver toxicity.

REFERENCES

Adachi, M., and Ishii, H. 2002. Role of mitochondria in alcoholic liver injury. Free Radical Biology & Medicine 32(6): 487-491.

Adeneye, AA., Amole, OO., and Adeneye, AK. 2006. Hypoglycemic and hypocholesterolemic activities of the aqueous leaf and seed extract of *Phyllanthus amarus* in mice. Fitoterapia 77(7-8): 511-514.

Adeneye, AA., and Benebo, AS. 2008. Protective effect of the aqueous leaf and seed extract of *Phyllanthus amarus* on gentamicin and acetaminophen-induced nephrotoxic rats. Journal of Ethnopharmacology 118(2): 318-323.

Agrawal, A., Srivastava, S., Srivastava, JN., and Srivasava, MM. 2004. Evaluation of inhibitory effect of the plant *Phyllanthus amarus* against dermatophytic fungi *Microsporum gypseum*. Biomedical and Environmental Sciences 17(3): 359-365.

Albano, E. 2007. Oxidative mechanisms in the pathogenesis of alcoholic liver disease. Molecular Aspects of Medicine 29(1-2): 9-16.

Aleksunes, L.M., and Manautou, J.E. 2007. Emerging Role of Nrf2 in Protecting Against Hepatic and Gastrointestinal Disease. Toxicologic Pathology 35: 459-473.

Amaechina, FC., and Omogbai, EK. 2007. Hypotensive effect of aqueous extract of the leaves of *Phyllanthus amarus* Schum and Thonn (Euphorbiaceae). Acta Poloniae Pharmaceutica 64(6): 547-552.

Bailey, S.M., Patel, V.B., Young, T.A., Asayama, K., and Cunningham, C.C. 2001. Chronic ethanol consumption alters the glutathione/glutathione peroxidase-1 system and protein oxidation status in rat liver. Alcoholism, clinical and experimental research 25: 726-733.

Balogun, E., et al. 2003. Curcumin activates the haem oxygenase-1 gene via regulation of Nrf2 and the antioxidant-responsive element. Biochemical Journal 371: 887-895.

Barry, R.E. 2006. Role of Acetaldehyde in the Pathogenesis of Alcoholic Liver Disease. Addiction 83(12): 1381-1386.

Berry, M.N., and Friend, D.S. 1969. High yield preparation of isolated rat liver parenchymal cells; a biochemical and fine structure study. Journal of Cell Biology 43: 506-520.

Boesch, J.S., Lee, C., and Lindahl, R.G. 1996. Constitutive Expression of Class 3 Aldehyde Dehydrogenase in Cultured Rat Corneal Epithelium. The Journal of Biological Chemistry 271(9): 5150-5157.

Bradford, MM. 1976. Rapid and sensitive method for the quantification of microgram amounts of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248-254.

Cederbaum, AI. 2006. Cytochrome P450 2E1-dependent oxidant stress and upregulation of anti-oxidant defense in liver cells. Journal of Gastroenterology and Hepatology 21(3): S22-S25.

Cederbaum, AI. 2009. Nrf2 and antioxidant defense against CYP2E1 toxicity. Expert opinion on drug metabolism & toxicology 5(10): 1223-1244.

Chan, K., Han, X.D., and Kan, W. 2001. An important function of Nrf2 in combating oxidative stress: detoxification of acetaminophen. Proceedings of the National Academy of Sciences of the United States of America 98: 4611-4616.

Chan, J.Y., and Kwong, M. 2000. Impaired expression of glutathione synthetic enzyme genes in mice with targeted deletion of the Nrf2 basic-leucine zipper protein. Biochemica et Biophysica Acta 1517: 19-26.

Chattopadhyay, P., Agrawal, S.S., and Garg, A. 2006. Liver regenerative effect of *Phyllanthus amarus* Linn. against alcohol induced liver cell injury in partially hepatectomised albino rats. International Journal of Pharmacology 2(4): 426-430.

Chen, C., et al. 2004. Induction of detoxifying enzymes by garlic organosulfur compounds through transcription factor Nrf2: effect of chemical structure and stress signals. Free Radical Biology & Medicine 37(10): 1578-1590.

CIRAD. *Phyllanthus amarus* Schumach. & Thonn. [Online]. (n.d.). Available from: http://plantes-rizieres guyane.cirad.fr/dicotyledones/euphorbiaceae/phyllanthus_amarus [2009, June 20]

Colell, A., et al. 1998. Selective glutathione depletion of mitochondria by ethanol sensitizes hepatocytes to tumor necrosis factor. Gastroenterology 115(6): 1541-1551.

Cunningham, C.C., and Bailey, S.M. 2001. Ethanol consumption and liver mitochondria function. Biological Signals and Receptors 10(3-4): 271-282.

Das, S.K., and Vasudevan, D.M. 2007. Alcohol-induced oxidative stress. Life Sciences 81: 177-187.

Davies, K.J.A. 1988. Proteolytic systems as secondary antioxidant defenses. In: C.K. Chow Editor, Cellular antioxidant defense mechanisms, p. 25. vol. 2 CRC Press, Boca Raton, FL.

Day, C.P. 2006. Alcohol and the liver. Alcohol and drugs 35(1): 22-25.

Dewa, Y., et al. 2008. Beta-Naphthoflavone enhances oxidative stress responses and the induction of preneoplastic lesions in a diethylnitrosamine - initiated hepatocarcinogenesis model in partially hepatectomized rats. Toxicology 244 (2-3): 179-189.

Dewa, Y., et al. 2009. Involvement of oxidative stress in hepatocellular tumor-promoting activity of oxfendazole in rats. Archives of Toxicology 83(5): 503-511.

Dhalwal, K., Biradar, YS., and Rajani, M. 2006. High-performance thin-layer chromatography densitometric method for simultaneous quantitation of phyllanthin, hypophyllanthin, gallic acid, and ellagic acid in *Phyllanthus amarus*. Journal of Association of Official Analytical Chemists International 89(3): 619-623.

Dianzini, MU. 1985. Lipid peroxidation in ethanol poisoning. A critical reconsideration. Alcohol 20: 161-173.

Donohue, T.M., Drey, M.L., and Zetterman, R.K. 1988. Hepatic lysosomal cathepsin activities after acute ethanol administration. Alcohol and Alcoholism 23: 265-270.

Donohue, T.M. 2002. The ubiquitin-proteasome system and its role in ethanol-induced disorders. Addiction Biology 7: 15-28.

Donohue, T.M., and Osna, N.A. 2003. Intracellular Proteolytic Systems in Alcohol-Induced Tissue Injury. Alcohol Research & Health 27(4): 317-324.

Enomoto, A., et al. 2001. High sensitivity of Nrf2 knockout mice to acetaminophen hepatotoxicity associated with decreased expression of ARE-regulated drug metabolizing enzymes and antioxidant genes. Toxicological Science 59: 169-177.

Fang, S.H., Rao, Y.K., and Tzeng, Y.M. 2008. Anti-oxidant and inflammatory mediator's growth inhibitory effects of compounds isolated from *Phyllanthus urinaria*. Journal of Ethnopharmacology 116: 333-340.

Faremi, T.Y., Suru, S.M., Fafunso, M.A., and Obioha, U.E. 2008. Hepatoprotective potentials of *Phyllanthus amarus* against ethanol-induced oxidative stress in rats. Food and Chemical Toxicology 46: 2658-2664.

Fernandez-Checa, J.C., Hirano, T., Tsukamoto, H., and Kaplowitz, N. 1993. Mitochondrial glutathione depletion in alcoholic liver disease. Alcohol 10(6): 469-475.

Fernandez-Checa, J.C., et al. 1998. Oxidative stress: role of mitochondria and protection by glutathione. Biofactors 8(1-2): 7-11.

Fisher, SJ., Swaan, PW., and Eddington, ND. 2009. The Ethanol Metabolite Acetaldehyde Increases Paracellular Drug Permeability In Vitro and Oral Bioavailability In Vivo. The Journal of Pharmacology and Experimental Therapeutics p. 1-31.

Gong, P., and Cederbaum, AI. 2006. Nrf2 is increased by CYP2E1 in rodent liver and HepG2 cells and protects against oxidative stress caused by CYP2E1. Hepatology 43(1): 144-153.

Habig, WH., and Jakoby, WB. 1981. "Assays for differentiation of glutathione *S*-transferases". Methods Enzymology 77: 398-405.

Harikumar, KB., and Kuttan, R. 2006. Inhibition of drug metabolizing enzymes (cytochrome P450) *in vitro* as well as *in vivo* by *Phyllanthus amarus* SCHUM & THONN. Biological & Pharmaceutical Bulletin 29(7): 1310-1313.

Harikumar, KB., and Kuttan, R. 2007. An extract of *Phyllanthus amarus* protects mouse chromosomes and intestine from radiation induced damages. Journal of Radiation Research (Tokyo) 48(6): 469-476.

Harikumar, KB., Kuttan, G., and Kuttan, R. 2009a. Inhibition of viral carcinogenesis by *Phyllanthus amarus*. Integrative Cancer Therapies 8(3): 254-260.

Harikumar, KB., Kuttan, G., and Kuttan, R. 2009b. *Phyllanthus amarus* inhibits cell growth and induces apoptosis in Dalton's lymphoma ascites cells through activation of caspase-3 and downregulation of Bcl-2. Integrative Cancer Therapies 8(2): 190-194.

Harish, R., and Shivanandappa, T. 2006. Antioxidant activity and hepatoprotective potential of *Phyllanthus niruri*. Food Chemistry 95: 180-185.

Harvey, C.J., et al. 2009. Nrf2-regulated glutathione recycling independent of biosynthesis is critical for cell survival during oxidative stress. Free Radical Biology and Medicine 46(4): 443-453.

Haseba, T., Tomita, Y., Kurosu, M., and Ohno, Y. 2003. Dose and time changes in liver alcohol dehydrogenase (ADH) activity during acute alcohol intoxication involve not only class I but also class III ADH and govern elimination rate of blood ethanol. Legal Medicine 5: 202-211.

He, X., Chen, MG., and Ma, Q. 2008. Activation of Nrf2 in defense against cadmium-induced oxidative stress. Chemical Research in Toxicology 21(7): 1375-1383.

Hoek, J.B., and Pastorino, J.G. 2002. Ethanol, oxidative stress, and cytokine-induced liver cell injury. Alcohol 27(1): 63-68.

Hu, R., et al., 2006. Identification of Nrf2-regulated genes induced by chemopreventive isothiocyanate PEITC by oligonucleotide microarray. Life Sciences 79: 1944-1955.

IFCC methods for the measurement of catalytic concentration of enzymes, 1986. Journal of Clinical Chemistry and Clinical Biochemistry 24: 481-495.

Islam, A., Mazumder, U.K., Gupta, M., and Ghosal, S. 2008. Synergistic Effects of Geraniin and Rutin in the Antioxidant Properties of Major Lignans in *Phyllanthus amarus*. Pharmacologyonline 3: 1024-1036.

Itoh, K., et al. 1997. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochemical and Biophysical Research Communications 236: 313-322.

Itoh, K., Tong, K.I., and Yamamoto, M. 2004. Molecular mechanism activating Nrf2-keap1 pathway in regulation of adaptive response to electrophiles. Free Radical Biology and Medicine 36(10): 1208-1213.

Jagetia, C.G. 2007. Radioprotective Potential of Plants and Herbs against the Effects of Ionizing Radiation. Journal of Clinical Biochemistry and Nutrition 40(2): 74-81.

Jiang, T., Huang, Z., Chan, JY., and Zhang, DD. 2009. Nrf2 protects against As (III)-induced damage in mouse liver and bladder. Toxicology and Applied Pharmacology 240(1): 8-14.

Jokelainen, K., Reinke, L., and Nanji, AA. 2001. NF-κB activation is associated with free radical generation and precedes pathological liver injury in experimental alcoholic liver disease. Cytokine 16(1): 36-39.

Joshi, C.S., and Priya, E.S. 2007. β-Glucuronidase Inhibitory Effect of Phenolic Constituents from *Phyllanthus amarus*. Pharmaceutical Biology 45(5): 363-365.

Kamimura, S., Gaal, K., and Britton, RS. 1992. Increased 4-hydroxynonenal levels in experimental alcoholic liver disease: Association of lipid peroxidation with liver fibrogenesis. Hepatology 16(2): 448-453.

Kang, K.W., Cho, I.J., Lee, C.H., and Kim, S.G. 2003. Essential role of phosphatidylinositol 3-kinase-dependent CCAAT/enhancer binding protein beta activation in the induction of glutathione *S*-transferase by oltipraz. Journal of the National Cancer Institute 95: 53-66.

Kanki, K., et al. 2008. A possible role of Nrf2 in prevention of renal oxidative damage by ferric nitrilotriacetate. Toxicologic Pathology 36(2): 353-361.

Kassuya, CA., Leite, DF., de Melo, LV., Rehder, VL., and Calixto, JB. 2005. Anti-inflammatory properties of extracts, fractions and lignans isolated from *Phyllanthus amarus*. Planta Medica 71(8): 721-726.

Kassuya, CA., et al. 2006. Antiinflammatory and antiallodynic actions of the lignan niranthin isolated from *Phyllanthus amarus*. Evidence for interaction with platelet activating factor receptor. The European Journal of Pharmacology 546(1-3): 182-188.

Kensler, TW., Wakabayashi, N., and Biswal, S. 2007. Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway. The Annual Review of Pharmacology and Toxicology 47: 89-116.

Keum, Y-S., et al. 2006. Mechanism of Action of Sulforaphane: Inhibition of p38 Mitogen-Activated Protein Kinase Isoforms Contributing to the Induction of Antioxidant Response Element-Mediated Heme Oxygenase-1 in Human Hepatoma HepG2 Cells. Cancer Research 66: 8804-8813.

Kiemer, AK., Hartung, T., Huber, C., and Vollmar, AM. 2003. *Phyllanthus amarus* has anti-inflammatory potential by inhibition of iNOS, COX-2, and cytokines via the NF-kappaB pathway. Journal of Hepatology 38(3): 289-297.

Kim, SK., Woodcroft, KJ., and Novak, RF. 2003. Insulin and glucagon regulation of glutathione *S*-transferase expression in primary cultured rat hepatocytes. The Journal of Pharmacology and Experimental Therapeutics 305: 353-361.

Kloucek, P., Polesny, Z., Svobodova, B., Vlkova, E., and Kokoska, L. 2005. Antibacterial screening of some Peruvian medicinal plants used in Callería District. Journal of Ethnopharmacology 99(2): 309-312.

Koch, O.R., et al. 2004. Review: Oxidative stress and antioxidant defenses in ethanol-induced cell injury. Molecular Aspects of Medicine 25: 191-198.

Kocak-Toker, N., et al. 1985. Influence of acute ethanol administration on hepatic glutathione peroxidase and glutathione transferase activities in the rat. Pharmacological Research Communications 17: 233-239.

Kono, Y., and Fridovich, I. 1982. Superoxide inhibits catalase. Journal of Biological Chemistry 257: 5751-5754.

Krithika, R., and Verma, RJ. 2009. Mitigation of carbon tetrachloride-induced damage by *Phyllanthus amarus* in liver of mice. Acta Poloniae Pharmaceutica 66(4): 439-444.

Krithika, R., et al. 2009. Isolation, characterization and antioxidative effect of phyllanthin against CCl_4-induced toxicity in HepG2 cell line. Chemico-Biological Interactions 181: 351-358.

Kumar, KB., and Kuttan, R. 2005. Chemoprotective activity of an extract of *Phyllanthus amarus* against cyclophosphamide induced toxicity in mice. Phytomedicine 12(6-7): 494-500.

Kwak, M.K., et al. 2003. Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1-Nrf2 pathway. Identification of novel gene clusters for cell survival. Journal of Biological Chemistry 278: 8135-8145.

Lakari, E. Expression of oxidant and antioxidant enzymes in human lung and interstitial lung diseases. [Online]. (n.d.). Available from: http://herkules.oulu.fi /isbn 951 4266625/html/x494.html [2009, June 5]

Lamlé, J., et al. 2008. Nuclear Factor-Erythroid 2-Related Factor 2 Prevents Alcohol-Induced Fulminant Liver Injury. Gastroenterology 134: 1159-1168.

Lee, J-S., and Surh, Y-J. 2005. Nrf2 as a novel molecular target for chemoprevention. Cancer Letters 224: 171-184.

Leite, DF., et al. 2006. The cytotoxic effect and the multidrug resistance reversing action of lignans from *Phyllanthus amarus*. Planta Medica 72(15): 1353-1358.

Li, J., Stein, T.D., and Johnson, J.A. 2004. Genetic dissection of systemic autoimmune disease in Nrf2-deficient mice. Physiological Genomics 18: 261-272.

Lieber, C.S. 1997. Ethanol metabolism, cirrhosis and alcoholism. Clinica Chimica Acta 257(1): 59-84.

Lieber, C.S., and DeCarli, L.M. 1970. Hepatic microsomal ethanol-oxidizing system. *In vitro* characteristic and adaptive properties *in vivo*. The Journal of Biological Chemistry 245: 2505-2512.

Liu, J., Qu, W., and Kadiiska, MB. 2009. Role of oxidative stress in cadmium toxicity and carcinogenesis. Toxicology and Applied Pharmacology 238(3): 209-214.

Londhe, JS., Devasagayam, TP., Foo, LY., and Ghaskadbi, SS. 2008. Antioxidant activity of some polyphenol constituents of the medicinal plant *Phyllanthus amarus* Linn. Redox Report 13(5): 199-207.

Londhe, JS., Devasagayam, TP., Foo, LY., and Ghaskadbi, SS. 2009. Radioprotective properties of polyphenols from *Phyllanthus amarus* Linn. Journal of Radiation Research (Tokyo) 50(4): 303-309.

Luczaj, W., and Skrzydlewska, E. 2004. Antioxidant properties of black tea in alcohol intoxication. Food and Chemical Toxicology 42: 2045-2051.

Mallikarjuna, K., Chetan, P.S., Reddy, K.S., and Rajendra, W. 2008. Ethanol toxicity: Rehabilitation of hepatic antioxidant defense system with dietary ginger. Fitoterapia 79: 174-178.

Mansouri, A., Demeilliers, C., Amsellem, S., Pessayre D., and Fromenty, B. 2001. Acute Ethanol Administration Oxidatively Damages and Depletes Mitochondrial DNA in Mouse Liver, Brain, Heart, and Skeletal Muscles: Protective Effects of Antioxidants. Journal of pharmacology and experimental therapeutics 298(2): 737-743.

Mazumder, A., Mahato, A., and Mazumder, R. 2006. Antimicrobial potentiality of *Phyllanthus amarus* against drug resistant pathogens. Natural Product Research 20(4): 323-326.

Moreb, J.S., Zucali, J.R., Ostmark, B., and Benson, N.A. 2007. Heterogeneity of aldehyde dehydrogenase expression in lung cancer cell lines is revealed by Aldefluor flow cytometry-based assay. Cytometry B:Clinical Cytometry 72: 281-289.

Morland, J., Bessesen, A., Smith-Kielland, A., and Wallin, B. 1983. Ethanol and protein metabolism in the liver. Pharmacology Biochemistry and Behavior 18(1): 251-256.

Mossmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assays. Journal of Immunologic Method 65: 55-63.

Murty, C.N., Verney, E., and Sidransky, H. 1980. Acute effect of ethanol on membranes of the endoplasmic reticulum and on protein synthesis in rat liver. Alcoholism: Clinical and Experimental Research 4: 93-103.

Murugaiyah, V., and Chan, K-L. 2007. Determination of four lignans in *Phyllanthus niruri* L. by a simple high-performance liquid chromatography method with fluorescence detection. Journal of Chromatography A 1154: 198-204.

Naaz, F., Javed, S., and Abdin, M.Z. 2007. Hepatoprotective effect of ethanolic extract of *Phyllanthus amarus* Schum. et Thonn. on aflatoxin B_1-induced liver damage in mice. Journal of Ethnopharmacology 113: 503-509.

Niemela, O. 1999. Aldehyde-protein adducts in the liver as a result of ethanol induced oxidative stress. Frontiers in Bioscience 4: 506-513.

Notka, F., Meier, GR., and Wagner, R. 2003. Inhibition of wild-type human immunodeficiency virus and reverse transcriptase inhibitor-resistant variants by *Phyllanthus amarus*. Antiviral Research 58(2): 175-186.

Notka, F., Meier, G., and Wagner, R. 2004. Concerted inhibitory activities of *Phyllanthus amarus* on HIV replication *in vitro* and *ex vivo*. Antiviral Research 64(2): 93-102.

Ohkawa, H., Ohishi, N., and Yagi, K. 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95: 351-358.

Ostrowska, J., Luczaj, W., Kasacka, I., Rozanski, A., and Skrzydlewska, E. 2004. Green tea protects against ethanol-induced lipid peroxidation in rat organs. Alcohol 32: 25-32.

Paradis, V., et al. 1996. Cellular and Subcellular Localization of Acetaldehyde-Protein Adducts in Liver Biopsies from Alcoholic Patients. The Journal of Histochemistry and Cytochemistry 44(9): 1051-1057.

Poso, AR. 1987. Ethanol and hepatic protein turnover. Alcohol and Alcoholism 1: 83-90.

Pramyothin, P. 1986. Isolated liver cell preparations. Journal of the National Research Council 18: 1-9.

Pramyothin, P., Ngamtin, C., Poungshompoo, S., and Chaichantipyuth, C. 2007. Hepatoprotective activity of *Phyllanthus amarus* Schum. et. Thonn. extract in ethanol treated rats: *In vitro* and *in vivo* studies. Journal of Ethnopharmacology 114: 169-173.

Purdom-Dickinson, S.E., Sheveleva, E.V., Sun, H., and Chen, Q.M. 2007. Translational Control of Nrf2 Protein in Activation of Antioxidant Response by Oxidants. Molecular Pharmacology 72: 1074-1081.

Pushpakiran, G., Mahalakshmi, K., and Anuradha, C.V. 2004. Protective effects of taurine on glutathione and glutathione-dependent enzymes in ethanol-fed rats. Pharmazie 59: 869-872.

Raphael, KR., and Kuttan, R. 2003. Inhibition of experimental gastric lesion and inflammation by *Phyllanthus amarus* extract. Journal of Ethnopharmacology 87(2-3): 193-197.

Raphael, KR., Ajith, TA., Joseph, S., and Kuttan, R. 2002. Anti-mutagenic activity of *Phyllanthus amarus* Schum & Thonn *in vitro* as well as *in vivo*. Teratogenesis, Carcinogenesis and Mutagenesis 22(4): 285-291.

Raphael, KR., Sabu, MC., and Kuttan, R. 2002. Hypoglycemic effect of methanol extract of *Phyllanthus amarus* Schum & Thonn on alloxan induced diabetes mellitus in rats and its relation with antioxidant potential. Indian Journal of Experimental Biology 40(8): 905-909.

Raphael, KR., Sabu, M., Kumar, KH., and Kuttan, R. 2006. Inhibition of N-Methyl N'-nitro-N-nitrosoguanidine (MNNG) induced gastric carcinogenesis by *Phyllanthus amarus* extract. Asian Pacific Journal of Cancer Prevention 7(2): 299-302.

Rajeshkumar, NV., et al. 2002. Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. Journal of Ethnopharmacology 81(1): 17-22.

Rao, R.K., Seth, A., and Sheth, P. 2004. Recent Advances in Alcoholic Liver Disease I. Role of intestinal permeability and endotoxemia in alcoholic liver disease. American Journal of Physiology-Gastrointestinal and Liver Physiology 286: G881-G884.

Ribère, C., Sinaceur, J., Nordmann, J., and Nordmann R. 1984. Discrepancy between the different subcellular activities of rat liver catalase and superoxide dismutases in response to acute ethanol administration. Alcohol 20: 13-18.

Ribère, C., Sinaceur, J., Sabourault, D., and Nordmann, R. 1985. Hepatic catalase and superoxide dismutases after acute ethanol administration in rats. Alcohol 2: 31-33.

Roma´n, J., et al. 1999. Differential Role of ethanol and acetaldehyde in the induction of oxidative stress in HepG2 cells: effect on transcription factors AP-1 and NF-κB. Hepatology 30: 1473-1480.

Rubiolo, J.A., Mithieux, G., and Vega, F.V. 2008. Resveratrol protects primary rat hepatocytes against oxidative stress damage: Activation of the Nrf2 transcription factor and augmented activities of antioxidant enzymes. European Journal of Pharmacology 591: 66-72.

Sabir, S.M., and Rocha, J.B.T. 2008. Water-extractable phytochemicals from *Phyllanthus niruri* exhibit distinct *in vitro* antioxidant and *in vivo* hepatoprotective activity against paracetamol-induced liver damage in mice. Food Chemistry 111: 845-851.

Salmela, K.S., Kessova, I.G., Tsyrolv, I.B., and Lieber, C.S. 1998. Respective roles of cytochrome P450 2E1, 1A2, and 3A4 in the hepatic microsomal ethanol oxidizing system. Alcoholism: Clinical and Experimental Research 22: 2125-2132.

Salo, DC., Lin, SW., Pacifici, RE., and Davies, KJ. 1988. Superoxide dismutase is preferentially degraded by a proteolytic system from red blood cells following oxidative modification by hydrogen peroxide. Free Radical Biology & Medicine 5(5-6): 335-339.

Salo, DC., Pacifici, RE., Lin, SW., Giulivi, C., and Davies, K.J.A. 1990. Superoxide Dismutase Undergoes Proteolysis and Fragmentation following Oxidative Modification and Inactivation. The Journal of Biological Chemistry 265(20): 11919-11927.

Sarkar, M.K., and Sil, P.C. 2007. Hepatocytes are protected by herb *Phyllanthus niruri* protein isolate against thioacetamide toxicity. Pathophysiology 14: 113-120.

Sergent, O., Griffon, B., Cillard, P., and Cillard, J. 2001. Alcohol and oxidative stress. Pathologie Biologie 49(9): 689-695.

Shinkai, Y., Sumi, D., Fukami, I., Ishii, T., and Kumagai, Y. 2006. Sulforaphane, an activator of Nrf2, suppresses cellular accumulation of arsenic and its cytotoxicity in primary mouse hepatocytes. The Federation of the European Biochemical Societies Letters 580: 1771-1774.

Shizuka, S., and Kawabata, J.J. 2005. Effects of electron-withdrawing substituents on DPPH radical scavenging reactions of protocatechuic acid and its analogues in alcoholic solvents. Tetrahedron 61: 8101-8108.

Shoal, R.S., and Brink, U.T. 1992. Mitochondrial production of pro-oxidants and cellular senescence. Mutation Research 275: 295-304.

Siegmund, S.V., Haas, S., and Singer, M.V. 2005. Animal Models and Their Results in Gastrointestinal Alcohol Research. Digestive Diseases 23: 181-194.

Sinet, PM., and Garber, P. 1981. Inactivation of the human Cu-Zn superoxide dismutase during exposure to $O_2^{-\bullet}$ and H_2O_2. Archives of Biochemistry and Biophysics 212: 411-416.

Sripanidkulchai, B., et al. 2002. Antimutagenic and anticarcinogenic effects of *Phyllanthus amarus*. Phytomedicine 9(1): 26-32.

Stacey, N., and Priestly, B.G. 1978. Dose dependent toxicity of CCl_4 in isolated rat hepatocytes and the effects of hepatoprotective treatments. Toxicology and Applied Pharmacology 45: 29-39.

Storey, K.B. 2004. BIOCHEMICAL ADAPTATION. In B. C. Storey (ed.), FUNCTIONAL METABOLISM: Regulation and Adaptation, pp. 383-413. New Jersey: Wiley-Liss, Inc.

Surh, YJ., Kundu, JK., and Na, HK. 2008. Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals. Planta Medica 74(13): 1526-1539.

Syamasundar, KV., Singh, B., Thakur, RS., Husain, A., Kiso, Y., and Hikino, H. 1985. Antihepatotoxic principles of *Phyllanthus niruri* herbs. Journal of Ethnopharmacology 14(1): 41-44.

Thimmulappa, RK., et al. 2002. Identification of Nrf2-regulated genes induced by the chemopreventive agent sulforaphane by oligonucleotide microarray. Cancer research 62(18): 5196-5203.

Tietze, F. 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Analytical Biochemistry 27: 502-522.

Umemura, T., et al. 2006. A crucial role of Nrf2 in *in vivo* defense against oxidative damage by an environmental pollutant, pentachlorophenol. Toxicological Sciences 90(1): 111-119.

Vanhaecke, T., et al. 2000. Effect of Ethanol on the Expression of Hepatic Glutathione *S*-Transferase: An In Vivo/In Vitro Study. Biochemical Pharmacology 60: 1491-1496.

Varma, P.S., Aruna, K., Rukkumani, R., and Menon, V.P. 2004. Alcohol and thermally oxidized pufa induced oxidative stress: role of *N*-acetyl cysteine. Italian Journal of Biochemistry 53: 10-15.

Vina, J., Estrela, J.M., Guerri, C., and Romero, F.J. 1980. Effect of ethanol on glutathione concentrations in isolated hepatocytes. Biochemical Journal 188: 549-552

Wang, H., and Joseph, J.A. 1999. Quantitating cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radical Biology and Medicine 27: 612-616.

Wheeler, M.D. 2003. Endotoxin and Kupffer Cell Activation in Alcoholic Liver Disease. Alcohol Research & Health 27(4): 300-306.

Wheeler, G.L., Trotter, E.W., Dawes, I.W., and Grant, C.M. 2003. Coupling of the transcriptional regulation of glutathione biosynthesis to the availability of glutathione and methionine via the Met4 and Yap1 transcription factors. The Journal of Biological Chemistry 278: 49920-49928.

Wikipedia. Lipid peroxidation. [Online]. (n.d.). Available from: http://en.wikipedia.org/wiki/Lipid_peroxidation [2009, July 10]

Wu, D., and Cederbaum, AI. 2003. Alcohol, oxidative stress, and free radical damage. Alcohol Research & Health 27(4): 277-284.

Xu, W., et al. 2008. The Nrf2 transcription factor protects from toxin-induced liver injury and fibrosis. Laboratory Investigation 88(10): 1068-1078.

Yao, P., et al. 2007. Quercetin protects human hepatocytes from ethanol-derived oxidative stress by inducing heme oxygenase-1 via the MAPK/Nrf2 pathways. Journal of Hepatology 47(2): 253-261.

Yang, M.S., Chan, H.W., and Yu, L.C. 2006. Glutathione peroxidase and glutathione reductase activities are partially responsible for determining the susceptibility of cells to oxidative stress. Toxicology 226: 126-130.

Young-Loon, S., Kundu, J.K., and Hye-Kyung, N. 2008. Nrf2 as a Master Redox Switch in Turning on the Cellular Signaling Involved in the Induction of Cytoprotective Genes by Some Chemopreventive Phytochemicals. Planta medica 74(13): 1526-1539.

Zima, T., et al. 2001. Oxidative Stress, Metabolism of Ethanol and Alcohol-Related Diseases. Journal of Biomedical Science 8: 59-70.

Zima, T., and Kalousova, M. 2005. Oxidative Stress and Signal Transduction Pathways in Alcoholic Liver Disease. Alcoholism: Clinical and Experimental Research 29(11): 110S-115S.

APPENDIX

Analytical identification of phyllanthin

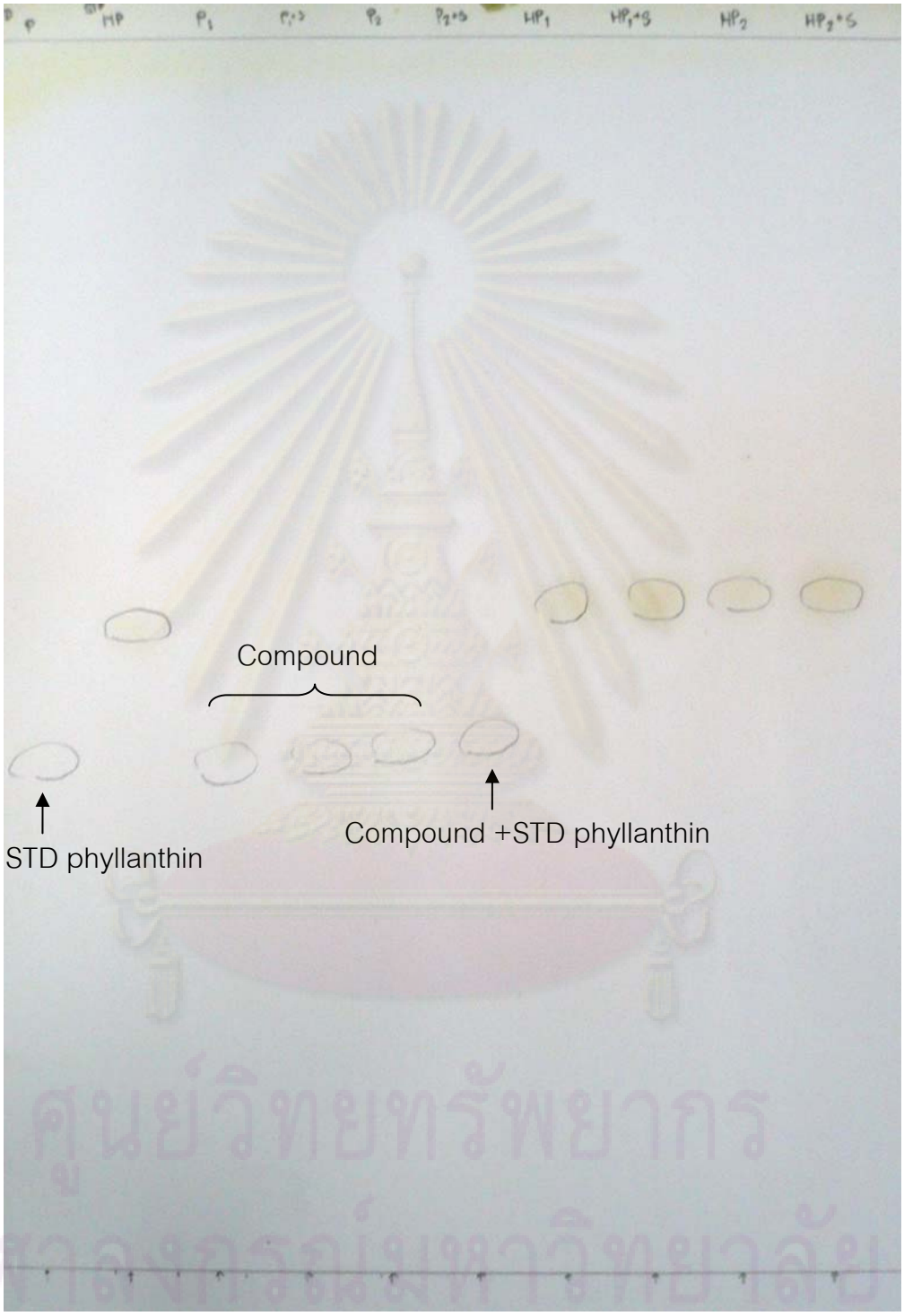


Figure A1: Analytical identification of phyllanthin isolated from *Phyllanthus amarus* by thin layer chromatography (TLC).

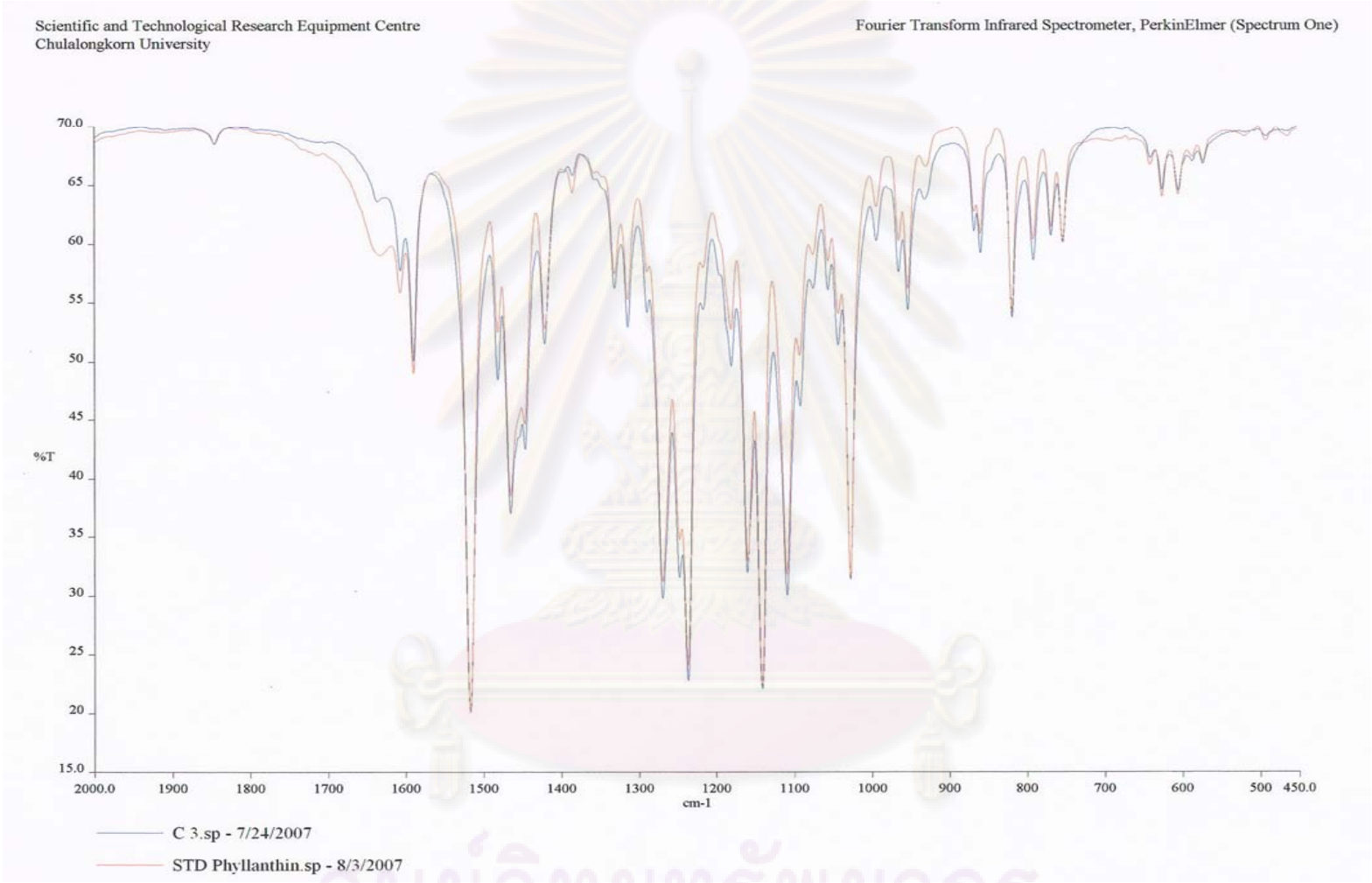


Figure A2: Analytical identification of phyllanthin isolated from *Phyllanthus amarus* by FT-IR (compared with phyllanthin standard in red line).

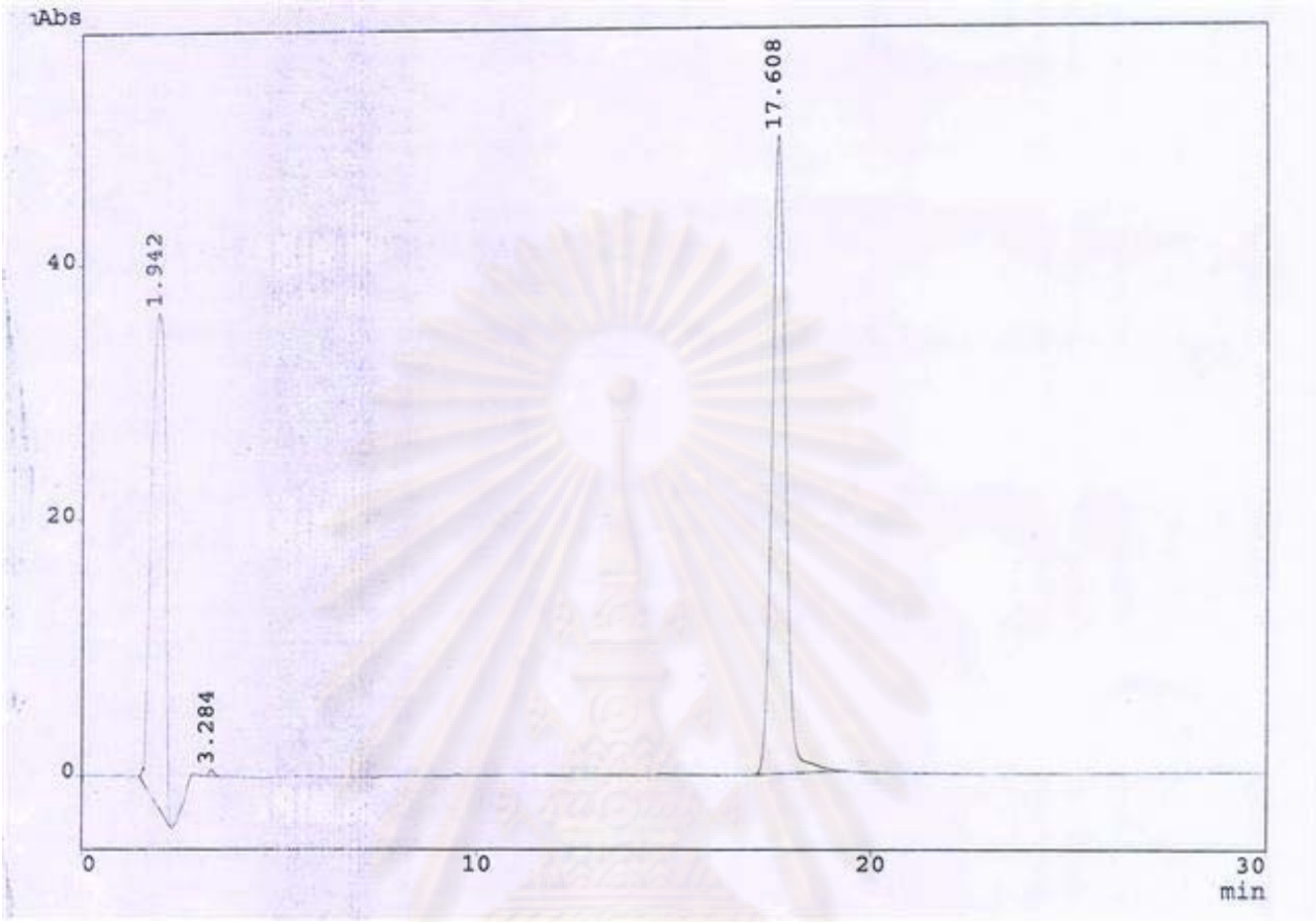


Figure A3: High performance liquid chromatography chromatogram of phyllanthin standard (Chromadex Inc.) + phyllanthin which was isolated from *Phyllanthus amarus*.

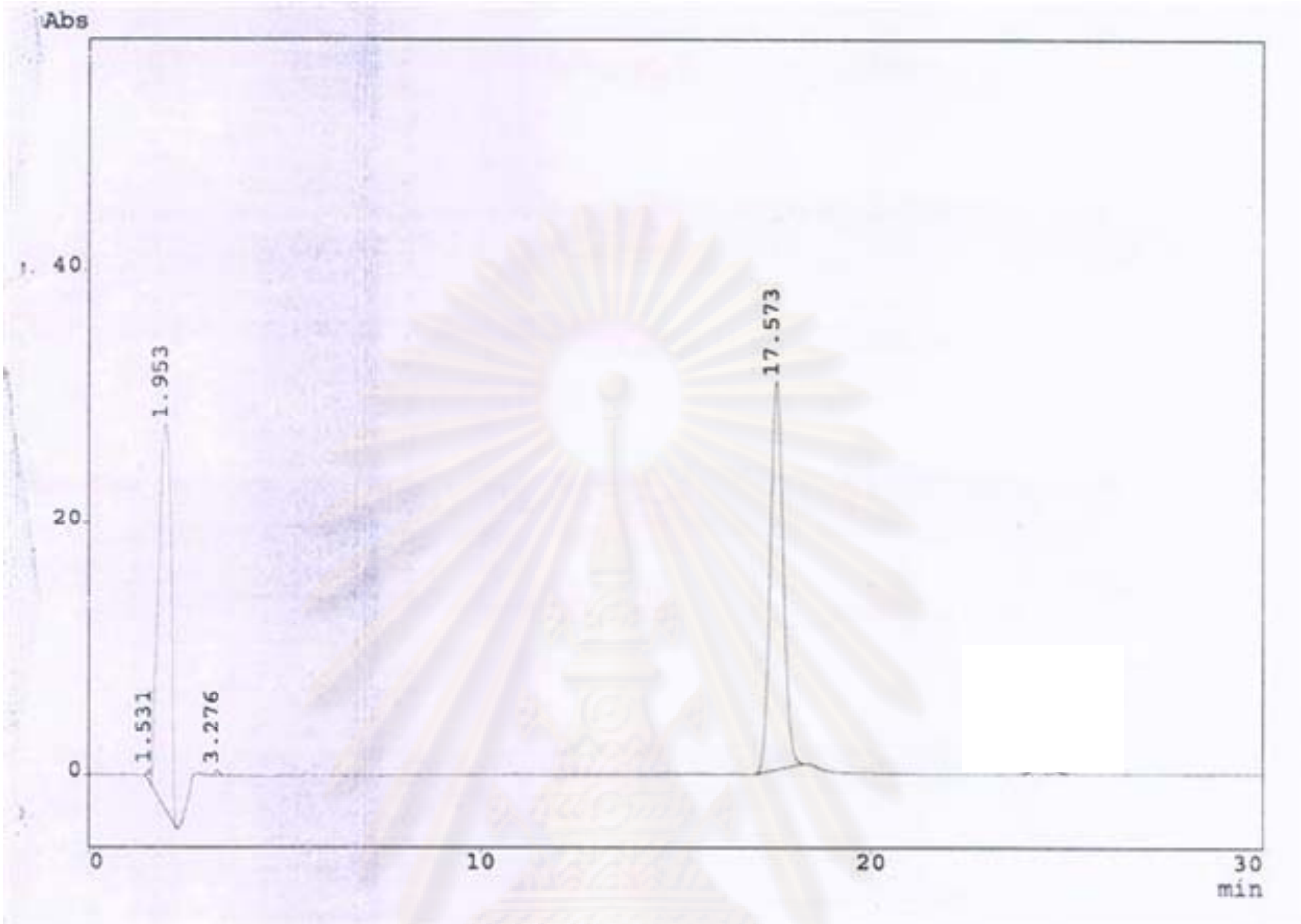


Figure A4: High performance liquid chromatography chromatogram of phyllanthin which was isolated from *Phyllanthus amarus*.

Table A1: Protective effect of phyllanthin on ethanol-treated primary culture of rat hepatocytes.

Group	MTT (% of control)	MTT (% of EtOH)	ALT (U/L)	AST (U/L)
Control	100.00	-	6.25 ± 0.61	53.15 ± 1.63
EtOH 80 µl/ml	38.42 ± 5.53*	100.00	11.60 ± 0.84*	107.18 ± 1.82*
Phyllanthin 1 µg/ml-->EtOH	52.88 ± 9.26*,†	135.78 ± 5.41†	7.10 ± 0.73†	77.75 ± 3.67*,†
Phyllanthin 2 µg/ml-->EtOH	52.30 ± 6.88*,†	137.10 ± 4.66†	7.10 ± 1.09†	77.38 ± 3.94*,†
Phyllanthin 3 µg/ml-->EtOH	53.40 ± 8.30*,†	138.20 ± 6.93†	7.45 ± 1.13†	83.05 ± 5.24*,†
Phyllanthin 4 µg/ml-->EtOH	46.53 ± 4.52*	124.23 ± 9.28	7.98 ± 0.78	89.73 ± 3.16*,†

MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ALT=alanine aminotransferase; AST=aspartate aminotransferase.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

124

Table A2: Effect of phyllanthin on the generation of ROS, MDA and total glutathione levels in primary culture of rat hepatocytes treated with ethanol.

Group	Intracellular ROS (% of control)	MDA (nmol MDA/mg protein)	Total GSH (μmol/mg protein)
Control	100.00	0.315 ± 0.001	4.78 ± 0.25
EtOH 80 μl/ml	185.00 ± 14.28*	0.386 ± 0.001*	3.23 ± 0.09*
Phyllanthin 1 μg/ml-->EtOH	116.50 ± 10.43†	0.302 ± 0.003†	4.30 ± 0.21†
Phyllanthin 2 μg/ml-->EtOH	127.50 ± 14.75†	0.273 ± 0.006*,†	4.12 ± 0.07†
Phyllanthin 3 μg/ml-->EtOH	105.67 ± 11.68†	0.296 ± 0.003*,†	4.53 ± 0.13†
Phyllanthin 4 μg/ml-->EtOH	142.67 ± 10.28	0.277 ± 0.004*,†	4.36 ± 0.28†

ROS=reactive oxygen species; MDA=malondialdehyde; Total GSH=Total glutathione.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

Table A3: Effect of phyllanthin on the activities of ethanol-metabolizing enzymes (ADH, ALDH) in primary culture of rat hepatocytes treated with ethanol.

Group	ADH activity (nmol/mg protein/min)	ALDH activity (nmol/mg protein/min)
Control	0.44 ± 0.03	1.42 ± 0.05
EtOH 80 µl/ml	0.56 ± 0.03	0.66 ± 0.06*
Phyllanthin 1 µg/ml-->EtOH	0.61 ± 0.05	0.75 ± 0.07*
Phyllanthin 2 µg/ml-->EtOH	0.53 ± 0.05	0.78 ± 0.06*
Phyllanthin 3 µg/ml-->EtOH	0.51 ± 0.04	0.57 ± 0.05*
Phyllanthin 4 µg/ml-->EtOH	0.62 ± 0.06	0.44 ± 0.06*

ADH=alcohol dehydrogenase; ALDH=aldehyde dehydrogenase.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

Table A4: Effect of phyllanthin on the activities of antioxidant enzymes: SOD, CAT, GPx, GR; and GST in primary culture of rat hepatocytes treated with ethanol.

Group	SOD activity (U/mg protein)	CAT activity (nmol/mg protein/min)	GPx activity (nmol/mg protein/min)	GR activity (nmol/mg protein/min)	GST activity (nmol/mg protein/min)
Control	4.29 ± 0.05	74.93 ± 1.33	25.39 ± 0.22	4.34 ± 0.12	8.59 ± 0.44
EtOH 80 µl/ml	3.79 ± 0.08*	79.97 ± 1.83	33.97 ± 0.83*	3.12 ± 0.21*	5.14 ± 0.83*
Phyllanthin 1 µg/ml-->EtOH	4.36 ± 0.12†	78.85 ± 1.46	37.91 ± 1.39*	5.04 ± 0.25†	4.32 ± 0.69*
Phyllanthin 2 µg/ml-->EtOH	4.26 ± 0.07†	79.37 ± 1.97	36.67 ± 1.90*	5.01 ± 0.17†	3.71 ± 0.66*
Phyllanthin 3 µg/ml-->EtOH	4.44 ± 0.14†	78.96 ± 2.10	38.13 ± 1.70*	4.81 ± 0.26†	3.89 ± 0.65*
Phyllanthin 4 µg/ml-->EtOH	3.99 ± 0.09	80.19 ± 0.91	45.57 ± 0.91*,†	4.95 ± 0.16†	3.89 ± 0.55*

SOD=superoxide dismutase; CAT=catalase; GPx=glutathione peroxidase; GR=glutathione reductase; GST=glutathione *S*-transferase.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

Table A5: Effect of phyllanthin on the expression of cytoprotective proteins: SOD1 (Cu-ZnSOD), SOD2 (MnSOD), GR, GPx; and transcription factor Nrf2 in primary culture of rat hepatocytes treated with ethanol.

Group	Nrf2 expression (% of control)	SOD1 expression (% of control)	SOD2 expression (% of control)	GR expression (% of control)	GPx expression (% of control)
Control	100.00	100.00	100.00	100.00	100.00
EtOH 80 µl/ml	57.00 ± 3.89*	33.75 ± 5.17*	120.75 ± 2.46	31.50 ± 6.41*	43.75 ± 3.59*
Phyllanthin 1 µg/ml-->EtOH	203.25 ± 10.97*,†	109.00 ± 8.43†	191.50 ± 11.21*,†	152.50 ± 2.25*,†	166.50 ± 6.55*,†
Phyllanthin 2 µg/ml-->EtOH	221.00 ± 6.25*,†	117.00 ± 4.81†	207.25 ± 10.71*,†	172.00 ± 7.36*,†	155.00 ± 8.97†
Phyllanthin 3 µg/ml-->EtOH	155.75 ± 12.57†	93.00 ± 5.28†	216.50 ± 17.45*,†	160.75 ± 5.94*,†	182.00 ± 4.55*,†
Phyllanthin 4 µg/ml-->EtOH	173.00 ± 9.76†	92.50 ± 12.05	180.75 ± 9.93*,†	172.75 ± 4.79*,†	137.50 ± 7.12†

Nrf2=nuclear factor-erythroid 2 p45-related factor 2; SOD1=copper-zinc superoxide dismutase; SOD2=manganese-superoxide dismutase; GR= glutathione reductase; GPx=glutathione peroxidase.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

†Significant difference at $p<0.05$ from ethanol treatment.

128

Table A6: Effect of 24 h pretreatment with phyllanthin in primary culture of rat hepatocytes.

Group	MTT (% of control)	ALT (U/L)	AST (U/L)
Control	100.00	6.25 ± 0.61	53.15 ± 1.63
Phyllanthin 1 µg/ml	91.93 ± 4.95	6.58 ± 0.95	65.40 ± 5.48
Phyllanthin 2 µg/ml	83.13 ± 3.98	7.53 ± 1.20	68.33 ± 7.89
Phyllanthin 3 µg/ml	89.83 ± 2.82	6.98 ± 1.34	70.30 ± 4.41
Phyllanthin 4 µg/ml	88.65 ± 2.76	7.85 ± 1.18	69.73 ± 3.37

MTT=3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ALT=alanine aminotransferase; AST=aspartate aminotransferase.

Values are Mean ± SEM, n=4.

Table A7: Effect of 24 h pretreatment with phyllanthin on the generation of ROS, MDA and total glutathione levels in primary culture of rat hepatocytes.

Group	Intracellular ROS (% of control)	MDA (nmol MDA/mg protein)	Total GSH (μmol/mg protein)
Control	100.00	0.322 ± 0.004	5.19 ± 0.42
Phyllanthin 1 μg/ml	101.00 ± 11.57	0.285 ± 0.016	6.10 ± 0.69
Phyllanthin 2 μg/ml	92.67 ± 12.39	0.302 ± 0.008	5.75 ± 0.65
Phyllanthin 3 μg/ml	102.33 ± 9.94	0.295 ± 0.004	5.76 ± 0.72
Phyllanthin 4 μg/ml	90.33 ± 3.18	0.333 ± 0.007	5.46 ± 0.57

ROS=reactive oxygen species; MDA=malondialdehyde; Total GSH=Total glutathione.

Values are Mean ± SEM, n=4.

Table A8: Effect of 24 h pretreatment with phyllanthin on the activities of ethanol-metabolizing enzymes (ADH, ALDH) in primary culture of rat hepatocytes.

Group	ADH activity (nmol/mg protein/min)	ALDH activity (nmol/mg protein/min)
Control	0.52 ± 0.07	1.64 ± 0.11
Phyllanthin 1 µg/ml	0.59 ± 0.03	1.85 ± 0.23
Phyllanthin 2 µg/ml	0.71 ± 0.06	1.81 ± 0.16
Phyllanthin 3 µg/ml	0.43 ± 0.06	1.73 ± 0.25
Phyllanthin 4 µg/ml	0.52 ± 0.05	1.49 ± 0.21

ADH=alcohol dehydrogenase; ALDH=aldehyde dehydrogenase.

Values are Mean ± SEM, n=4.

Table A9: Effect of 24 h pretreatment with phyllanthin on the activities of antioxidant enzymes: SOD, CAT, GPx, GR; and GST in primary culture of rat hepatocytes.

Group	SOD activity (U/mg protein)	CAT activity (nmol/mg protein/min)	GPx activity (nmol/mg protein/min)	GR activity (nmol/mg protein/min)	GST activity (nmol/mg protein/min)
Control	4.24 ± 0.33	73.78 ± 1.86	33.46 ± 0.65	4.63 ± 0.11	9.05 ± 0.37
Phyllanthin 1 µg/ml	3.87 ± 0.18	77.28 ± 1.75	37.25 ± 2.38	5.21 ± 0.36	10.13 ± 0.58
Phyllanthin 2 µg/ml	3.65 ± 0.37	78.30 ± 3.45	36.61 ± 1.50	5.48 ± 0.23	9.60 ± 0.44
Phyllanthin 3 µg/ml	3.40 ± 0.14	77.83 ± 1.54	37.86 ± 1.32	6.19 ± 0.48	9.91 ± 0.30
Phyllanthin 4 µg/ml	4.19 ± 0.25	76.64 ± 1.87	33.94 ± 1.94	5.78 ± 0.39	11.11 ± 0.47

SOD=superoxide dismutase; CAT=catalase; GPx=glutathione peroxidase; GR=glutathione reductase; GST=glutathione *S*-transferase.

Values are Mean ± SEM, n=3.

132

Table A10: Effect of 24 h pretreatment with phyllanthin on the expression of cytoprotective proteins: SOD1 (Cu-ZnSOD), SOD2 (MnSOD), GR, GPx; and transcription factor Nrf2 in primary culture of rat hepatocytes.

Group	Nrf2 expression (% of control)	SOD1 expression (% of control)	SOD2 expression (% of control)	GR expression (% of control)	GPx expression (% of control)
Control	100.00	100.00	100.00	100.00	100.00
Phyllanthin 1 µg/ml	387.00 ± 37.02*	169.00 ± 12.50*	136.25 ± 4.17*	146.25 ± 5.54*	151.25 ± 3.97*
Phyllanthin 2 µg/ml	422.75 ± 15.99*	157.67 ± 14.44*	149.00 ± 9.28*	153.75 ± 5.41*	144.75 ± 5.65*
Phyllanthin 3 µg/ml	383.75 ± 36.20*	163.33 ± 8.09*	156.00 ± 8.03*	166.50 ± 4.79*	131.00 ± 2.55*
Phyllanthin 4 µg/ml	396.00 ± 24.67*	163.00 ± 7.00*	144.25 ± 9.81*	146.25 ± 2.95*	118.00 ± 4.78

Nrf2=nuclear factor-erythroid 2 p45-related factor 2; SOD1=copper-zinc superoxide dismutase; SOD2=manganese-superoxide dismutase; GR=glutathione reductase; GPx=glutathione peroxidase.

Values are Mean ± SEM, n=4.

*Significant difference at $p<0.05$ from control.

ศธ 0512.14 คสว 037/ 2552

Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 09-33-015	**Approval No. 09-33-015**
Protocol Title Protective activity of phyllanthin in ethanol-treated primary culture of rat hepatocytes	
Principal Investigator Pornpen Pramyothin, Ph.D.	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval June 29, 2009	**Date of Expiration** June 29, 2010
Applicant Faculty/Institution **Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Rd., Pathumwan BKK-THAILAND. 10330**	
Signature of Chairperson Withaya Janthasoot	**Signature of Authorized Official**
Name and Title **WITHAYA JANTHASOOT** **Chairman**	**Name and Title** **RUNGPETCH SAKULBUMRUNGSIL, Ph.D.** **Associate Dean (Research and Academic Service)**
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

VITAE

Miss Hemvala Chirdchupunseree was born on June 20, 1974 in Surat Thani province. She completed her B.N.S. from the Thai Red Cross College of Nursing in 1995. She received her M.Sc. in Pharmacology from Faculty of Graduate School, Chulalongkorn University in 2003.