ANTIOXIDATIVE ACTIVITY OF SEED EXTRACT FROM *SONNERATIA CASEOLARIS* AGAINST ETHANOL INDUCING HEPATOTOXICITY IN HEPG2 CELL

By

Nin Prapongsena

A Thesis Submitted in Partial Fulfillment of The Requirements for The Degree

MASTER OF PHARMACY

Program of Biopharmaceutical Sciences

Graduate School

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ฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากเมล็ดลำพูต่อความเป็นพิษของเซลล์ตับ HepG2 ที่ถูกเหนี่ยวนำด้วยเอทานอล

โดย

นายณิณ ประพงศ์เสนา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต

สาขาวิชาเภสัชศาสตร์ชีวภาพ

บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

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.....
(Assistant Professor Panjai Tantatsanawong , Ph D.)
Dean of Graduate School
24 / มิ.ย. / 54

The Thesis Advisor

Assistant Professor Juree Charoenteeraboon

The Thesis Examination Committee

Sunee Techaarpornkul ..... Chairman
(Assistant Professor Sunee Techaarpornkul, Ph D.)
23 / มิ.ย. / 54

Ariyaphong Wongnoppavich ..... Member
(Assistant Professor Ariyaphong Wongnoppavich, Ph D.)
23 / มิ.ย. / 54

Thawatchai Phaechamud ..... Member
(Associate Professor Thawatchai Phaechamud, Ph D.)
23 / มิ.ย. / 54

Penpun Wetwitayaklung ..... Member
(Associate Professor Penpun Wetwitayaklung, Ph D.)
23 / มิ.ย. / 54

Juree Charoenteeraboon ..... Member
(Assistant Professor Juree Charoenteeraboon, Ph.D.)
23 / มิ.ย. / 54

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Ethanol induces hepatotoxicity by generating free radicals that cause cell injury and cell death. Preliminary studies reported that several plant extracts can prevent free radicals causing cell damage. This study intended to investigate the hepatoprotective and the antioxidative activities of methanolic seed extract of *S. caseolaris* in human hepatocarcinoma (HepG2) cell culture that the cellular injury was induced by ethanol. The hepatoprotective effect of *S. caseolaris* seed extract on HepG2 cells treated with 1.7 M ethanol showed that the extract at the concentration of 10 to 30 µg/mL could incerase the number of cell survival measured by MTT assay and could reduce the number of cell injury measured by LDH leakage assay. Next, the antioxidative effects were analyzed against lipid peroxidation induced by 250 mM ethanol and hTNF-α secretion by 500 mM ethanol using TBARs assay and ELISA, respectively.. The results showed that treatment with 30 µg/mL of the extract significantly reduced the lipid peroxidation and the hTNF-α secretion. The hepatoprotective mechanisms of 10-30 µg/mL *S. caseolaris* extract against 500 mM ethanol were investigated by measuring activities of intracellular antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and intracellular GSH level using spectrophotometric methods. *S. caseolaris* seed extract at the concentration of 20 and 30 µg/mL significantly activated CAT and GPx activities and at the concentration of 30 µg/mL significantly increased intracellular GSH level while slightly decreased the SOD activity. These findings indicated that the methanolic seed extract of *S. caseolaris* effectively prevents ethanol-induced oxidative damage in HepG2 cells. The hepatoprotective mechanism is owing to the activation of CAT and GPx activities as well as the prevention of intracellular GSH depletion.

Program of Biopharmaceutical Sciences Graduate School, Silpakorn University Academic Year 2010

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Thesis Advisor's signature .....

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ณิณ ประพงศ์เสนา : ฤทธิ์ต้านอนุมูลอิสระของสารสกัดจากเมล็ดลำพูต่อความเป็นพิษของเซลล์ตับ HepG2 ที่ถูกเหนี่ยวนำด้วยเอทานอล. อาจารย์ที่ปรึกษาวิทยานิพนธ์: ผศ. ดร. จุรีย์ เจริญธีรบูรณ์. 115 หน้า.

เอทานอลเหนี่ยวนำความเป็นพิษต่อเซลล์ตับโดยสร้างอนุมูลอิสระที่ส่งผลให้เซลล์บาดเจ็บและตาย รายงานเบื้องต้นพบว่าสารสกัดจากพืชหลายชนิดสามารถป้องกันความเสียหายจากอนุมูลอิสระ งานวิจัยนี้มุ่งตรวจสอบฤทธิ์ปกป้องเซลล์ตับและฤทธิ์ต้านอนุมูลอิสระของสารสกัดเมทานอลจากเมล็ดลำพูต่อเซลล์มะเร็งตับมนุษย์เพาะเลี้ยง (HepG2) เมื่อถูกเหนี่ยวนำการบาดเจ็บด้วยเอทานอล ผลทดสอบฤทธิ์ปกป้องเซลล์ตับที่ถูกเหนี่ยวนำด้วยเอทานอล 1.70 โมลาร์ พบว่าสารสกัดที่ความเข้มข้น 10 ถึง 30 ไมโครกรัมต่อมิลลิลิตร เพิ่มปริมาณเซลล์รอดชีวิต ซึ่งวัดด้วยวิธี MTT assay และลดปริมาณเซลล์บาดเจ็บโดยลดการรั่วของเอนไซม์ LDH ออกจากเซลล์ จากนั้นทำการวิเคราะห์ฤทธิ์ต้านอนุมูลอิสระต่อการเกิดลิพิด เพอร์ออกซิเดชัน ซึ่งเหนี่ยวนำด้วยเอทานอล 250 มิลลิโมลาร์ และต่อการหลั่ง hTNF-α ด้วยเอทานอล 500 มิลลิโมลาร์โดยใช้วิธี TBARs assay และ ELISA ตามลำดับ ผลการทดลองพบว่าการเติมสารสกัดที่ความเข้มข้น 30 ไมโครกรัมต่อมิลลิลิตร สามารถลดการเกิดลิพิด เพอร์ออกซิเดชันและลดการหลั่ง hTNF-α อย่างมีนัยสำคัญ กลไกปกป้องเซลล์ตับของสารสกัดลำพู 10 ถึง 30 ไมโครกรัมต่อมิลลิลิตรต่อเอทานอล 500 มิลลิโมลาร์ วิเคราะห์โดยวัดกัมมันตภาพของเอนไซม์ต้านอนุมูลอิสระ ได้แก่ ซูเปอร์ออกไซด์ ดิสมิวเตส (SOD) คะตะเลส (CAT) และกลูตาไทโอน เพอร์ออกซิเดส (GPx) และวัดระดับกลูตาไทโอน (GSH) ภายในเซลล์ด้วยวิธีทางสเปคโทรโฟโทเมทรี สารสกัดเมล็ดลำพูที่ความเข้มข้น 20 และ 30 ไมโครกรัมต่อมิลลิลิตร กระตุ้นกัมมันตภาพของ CAT และ GPx อย่างมีนัยสำคัญ และที่ความเข้มข้น 30 ไมโครกรัมต่อมิลลิลิตรเพิ่มระดับของ GSH อย่างมีนัยสำคัญในขณะที่ลดกัมมันตภาพของ SOD ลงเพียงเล็กน้อย ข้อมูลดังกล่าวนี้แสดงให้เห็นว่าสารสกัดเมทานอลจากเมล็ดลำพูปกป้องเซลล์ตับ HepG2 ต่อความเสียหายที่ถูกเหนี่ยวนำด้วยเอทานอลได้อย่างมีประสิทธิภาพ กลไกปกป้องเซลล์ตับนี้มาจากการกระตุ้นกัมมันตภาพของ CAT และ GPx ตลอดจนการป้องกันการขาด GSH ภายในเซลล์

สาขาวิชาเภสัชศาสตร์ชีวภาพ บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร ปีการศึกษา 2553

ลายมือชื่อนักศึกษา .....Nin.....

ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ .....Jvree Chaowuteerabon.....

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CHAPTER 1

Introduction

Liver has been well recognized as the major organ in human body because of its functional diversity. Many substances can be synthesized, metabolized and detoxified by liver. Metabolism is the process for converting food into energy and detoxifying toxic substances. Detoxifying metabolism can be classified into phase I and II. Metabolism phase I involves oxidation, dealkylation, deamination, desulfation or dechlorination that convert substances to be more polar and more excrete. In this step, some free radicals are produced (Watkins, 1990). Metabolism phase II is the conjugation process with an endogenous substance, glutathione or glucuronate, which can be further excreted (William, 1971). If the liver function is impaired, some toxic substances will not be excreted from human body. The liver would become damaged.

Liver injury is classified into immunologically mediated and hepatotoxin induced liver injuries. The immunologically mediated liver injury is subdivided into hypersensivity and pathogen induced hepatic injury. The immune system responds to self-antigens or pathogens by activating immune cells to destroy self-antigens, pathogens and altered cells. Hepatotoxins induced liver injury is subdivided into direct hepatotoxins and indirect cytotoxic hepatotoxins that are classified by their mechanisms to induce liver injury. Direct hepatotoxins (e.g. carbon tetrachloride, chloroform, ethanol, etc.) damage the

hepatocyte membrane directly by generating the free radicals. Indirect hepatotoxins (such as aflatoxin B_1, acetaminophen, etc.) induce the hepatic injury by producing the selective biochemicals or physiologic lesions to disrupt the metabolism or integrity of cell (Auerbach, 1968).

Ethanol is the main factor contributing premature death and having the impact on public health. In 2007, population of alcoholic liver disease in Thailand has increased from 9,132 to 24,193 (National Statistical Office of Thailand, 2007). Ethanol induces the hepatic injury via its metabolic products such as acetaldehyde and reactive species (RS) which cause the oxidative stress (Puntarulo *et al.*, 1989; Shaw *et al.*, 1990; Garcia-Ruiz *et al.*, 1994; Lieber, 1997; Barnes *et al.*, 2004). Ethanol is the major toxic substance-induced hepatic injury in human. Therefore, ethanol is suitable hepatotoxic substance for searching of hepatoprotective agents.

Sonneratia caseolaris, common name is crabapple mangrove or firefly mangrove which is belong to *Sonneratiaceae*. This plant grows in flood area. It has been reported that *S. caseolaris* fruit extract is used as sprain poultices, in arresting hemorrhages and treating piles (Bose *et al.*, 1992). In recent year, *S. caseolaris* methanolic extract has been isolated and identified its chemical constituents that are composed of steroids, triterpenoids (e.g. betulin, lupenol, oleanolic, ursolic acid, maslinic acid, etc.) benzene carboxylic derivatives (e.g. 3,3'-di-*O*-methyl ether-ellagic acid, methyl gallate, etc.) and flavonoids such as dihydrokaemferol, quercetin, luteolin and luteolin-7-*O*-β-glycoside, etc. (Sadhu *et al.*, 2006; Tian *et al.*, 2009). Most of flavonoids, benzene carboxylic derivatives

and some triterpenoids in other plant extracts have been reported their antioxidative effects and hepatoprotective activity. Moreover, the extract of *S. caseolaris* has high yield from the seed part and can protect cell death against chloroform in HepG2 cell (Charoenteeraboon *et al.*, 2007). Therefore, this research work aimed to characterize hepatoprotective activity of *S. caseolaris* methanolic seed extract.

One of liver protective systems in human body is the balance between protection of the total antioxidative status and damage of the oxidative stress. The antioxidative systems in cell involve with enzymatic and nonenzymatic antioxidants. The example of nonenzymatic antioxidants are endogenous substances in metabolism phase II (e.g. glutathione, glucuronate, vitamin E, vitamin C, etc). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are enzymatic antioxidants that possess protective effect against oxidative damage. Superoxide dismutase is the initiation step to convert the superoxide radicals (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2), CAT converts hydrogen peroxide to water (H_2O) and oxygen. Glutathione peroxidase converts reduced glutathione (GSH) and lipids hydroperoxide to oxidized glutathione (GSSG) and lipids alcohol, respectively. Hepatoprotective systems protect hepatocytes by scavenging free radicals and neutralizing hepatotoxins (Sies, 1997).

The purpose of this research was to investigate the hepatoprotective activity and the antioxidative effects of *S. caseolaris* extract against ethanol in

HepG2 cells. Biological and biochemical properties of HepG2 cells are similar to those of human hepatocytes. Therefore, HepG2 cell is a suitable model for studying hepatoprotective activity. In this research work, the hepatoprotective activity was determined by MTT assay and LDH leakage assay. Evaluation of the antioxidative effects were investigated by measuring the lipid peroxidation and the human TNF-α (hTNF-α) secretion by TBARs assay, and hTNF-α ELISA, respectively. Moreover, the hepatoprotective mechanisms were explored by determining the effect of *S. caseolaris* extract on the activities of antioxidant enzymes (SOD, CAT, GPx) and the intracellular level of glutathione.

CHAPTER 2

Review literature

1. Liver

1.1 Morphology of liver

The liver is a necessary complex organ in human body. Its position is upper right quadrant of abdominal. This organ is divided into right and left lobe by falciforum ligaments and further subdivided into four lobes by their blood supply. In each lobe consists of the functional unit such as portal vein, hepatic artery and bile duct encompassing with hepatocytes. The liver of a 70 kg man contains 250 billion hepatocytes. These cells have diameter in the range of 20-30 μm and they have diversity and complexity functions.

1.2 Function of the liver

The liver functions are described as follow:

1. Liver secretes bile salts into intestine for emulsification and promotion the absorption of dietary fat.

2. Liver storages carbohydrates, proteins, vitamins and some lipids.

3. Liver synthesizes albumin, other plasma proteins, glucose, fatty acids, cholesterol and phospholipids.

4. Liver plays a major role in intestinal immune system.

5. Liver metabolizes, detoxifies, and inactivates exogenous componds e.g. drugs, insecticides and endogenous componds such as steroids

, and other hormones (Rappaport, 1969).

Detoxifying metabolism plays a major role in removing the toxic substances. Hepatotoxins are agents that can damage the liver of most recipients in a variety of species. They are referred to the true, predictable or intrinsic hepatotoxins (Rouiller, 1964). The principle of detoxifying metabolism is the change of non-polar to polar compounds because non-polar compounds are potentially toxic and able to reabsorb by the renal tubules. The liver will become an intolerable chemical burden whether non-polar compounds accumulate in the body (Remmer, 1970).

Detoxifying metabolism can be found in intestine, lung, kidney, nasal mucosa, skin and bacteria in intestinal lumen but mostly occurs in liver (Parkinson, 1996). This metabolism is classified into phase I and phase II as shown in Figure 1. Metabolism phase I prepares compound for conjugation in phase II by providing polar groups or allowing polar compounds into phase II. Metabolism phase II detoxifies metabolic products by conjugation with an endogenous substance to stable complex substances which can be excreted (William, 1971).

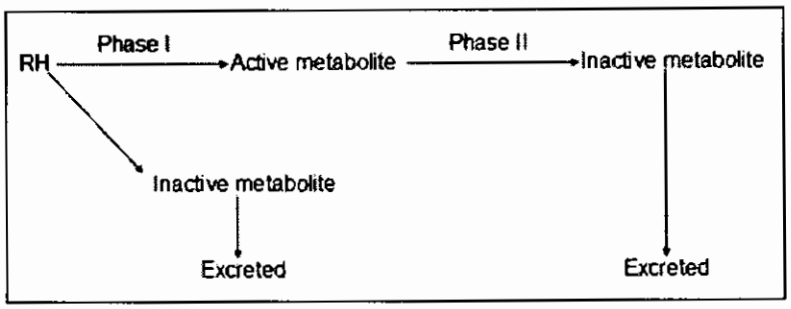

Figure 1 Schematic relation of phase I and phase II of drug (RH) metabolisms (Zimmerman, 1999).

Metabolism phase I is the toxifying phase which composes of oxidation, reduction, and hydrolysis reactions as shown in Table 1. These reactions provide the polar groups to original compound and generate reactive species (RS). Next, RS react to the macromolecules of hepatocytes and leading hepatic injury. Most reactions in metabolism phase I are oxidations that are catalysed by mixed function oxidase (MFO) system. The MFO system cosists of drugs, oxygen (the oxidant), NADPH (the reductant) and cytochrome P450 (substrate and oxygen binding enzymes) as shown in Figure 2 (Gibson *et al.*, 1986).

$$RH + NADPH + O_2 + 2H^+ \xrightarrow{MFO} ROH + NADP^+ + H_2O$$

RH = Drug, ROH = Oxidation product

Figure 2 The reaction of mixed function oxidase (MFO) (Zimmerman, 1999).

Table 1 Biotransformation phase I reactions (Zimmerman, 1999).

Oxidations	Reductions	Hydrolysis
Mixed function oxidase (MFO) dependent	Azo reductions	Esters
Aromatic hydroxylations	Nitro reductions	Amides
Aliphatic hydroxylations	Carbonyl reductions	
Oxidative dealkylation		
N-Oxidation		
S-Oxidation		
Deamination		
Desulfuration		
Amine oxidase		
Dehydrogenation		
Alcohols and aldehydes		

Cytochrome P450 (CYP450) has more than 500 isoforms which are found in animals, plants and eukaryotes. In human, CYP450 has been informed of 28 isoforms and at least 15 isoforms have been found in the liver (Plaa *et al.*, 1977). Each isoform of cytchrome P450 is able to metabolize different substrates but there are some overlaps of substrate specificity as shown in Table 2 (Guengerich, 1994).

Table 2 Isoforms of cytochrome P450 found in human and some of their substrates (Zimmerman, 1999).

Isoform	Drugs metabolized
CYP1A2	Acetaminophen, Theophylline, Warfarin
CYP1B1	Aromatic amines, Estradiol
CYP2A6	Aflatoxin B1, Coumarin, Valproic acid, Zidovudine
CYP2B6	Cyclophosphamide, Ifosphamide
CYP2C	Phenylbutazone, Retinol
CYP2C8	Carbamazepine, Taxol
CYP2C9	Diclofenac, Phenytoin, Tolbutamide, Piroxicam
CYP2C18	Cyclophosphamide, Omeprazole, Phenytoin
CYP2C19	Diazepam, Lansoprazole, Omeprazole, Propanolol
CYP2D6	Captopril, Fluoxetine, Cinnarizine
CYP2E1	Ethanol and other alcohols, Carbontetrachloride
CYP3A	Acetaminophen, Dapsone, Digitoxin
CYP4	Midazolam, Leukotrienes

In metabolism phase II, the metabolic products from metabolism phase I (unstable compounds) are captured by conjugating with endogenous compounds into the stable compounds which are allowed to excrete. Nevertheless, the conjugation of some compounds may provide more electrophilic and more toxic than the original compound (Farber, 1985). Selective induction of the isoenzymes are relied on induction of the P450 isoforms (Bock *et al.*, 1987). The conjugation reactions are shown in Table 3.

Table 3 Biotransformation phase II reactions (Zimmerman, 1999).

Type of conjugation	**Endogenous substance**	**Transfering enzyme**	**Type of xenobiotics and metabolics conjugated**
Glucurinidation	UDP-glucuronic acid	UDGP transferase	Alcohols, Phenols
Dihydrodiol	Water	Epoxide hydrolase	Aliphatic epoxides, arene oxides
Amino acid conjugation	Glycine, Glutamine, Ornithine, Arginine, Taurine	Acyl CoA-glycine transferase	CoA derivatives, Carboxylic acid
Sulfate conjugation	PAP-sulfate	Sulfotransferase	Phenols, Alcohols
Methylation	S-adenosyl-methionine	Transmethylase	Phenols, Amines
Glutathione conjugation	Glutathione	Glutathione S-transferase	Acetaminophen, Aflatoxin
Acetylation	Acetyl-CoA	N-acetyltransferase or deacetylase	Hydralazine, Arylamines

1.3 Liver injury

Liver disease is classified into acute hepatic injury and chronic hepatic injury. The chronic hepatic injury occurs with inflammation at least 6 months. The mechanism of liver disease is divided into immunological mediated hepatic injury and hepatotoxin-induced hepatic injury.

1.3.1 Immunological mediated hepatic injury

The purpose of immune system is differentiation and activation of immune cells e.g. T and B lymphocytes to defense against pathogenic microorganisms and tumor. Activated T cells or B cells mobilize and damage the antibody-coated target cells (ADCC) that induce the secretion of inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, etc. These cytokines stimulate and introduce macrophages, neutrophils, eosinophils and related cells to the inflammatory area. ADCC can activate complement system. Complement system is a cascade of proteins that produce inflammatory cytokines and lead to lysis of antigen-bearing cells, infected cells or tumor cells (Paul, 1989).

Immunological mediated injury is subdivided into viral hepatitis and autoimmune hepatitis that have immune response against viral antigen e.g. hepatitis A, B, C, D viral, cytomegalovirus, etc. (Field *et al.*, 1990) and self antigens, respectively. Autoimmune hepatitis is an idiosyncratic hepatic injury, in which the immune system losses its tolerance ability to self-antigens such as primary biliary cirrhosis, sclerosing cholangitis (Paul, 1989).

1.3.2 Hepatotoxin-induced hepatic injury

Hepatotoxins induce hepatic injury by their cytotoxicity. Thereafter, they cause the physiological and biochemical lesions. The two main forms of acute cytotoxic injury are steatosis and cell death development. Steatosis is caused by abnormal disposal of triglyceride (TG) in liver and oxidation of fatty acids (FA) in mitochondria that describe in hepatotoxic effects of ethanol (Farber, 1975). Chronic hepatic injury is caused by receiving hepatotoxins in long term that lead to generate the auto-antigen or produce the neoantigen as shown in Figure 3. These antigens stimulate immune response continuously (Hypersensivity Type II) that causes steatosis as same as acute cytotoxic injury, cirrhosis, phospholipidosis, cholestatic lesions, vascular lesions, and carcinogenesis.

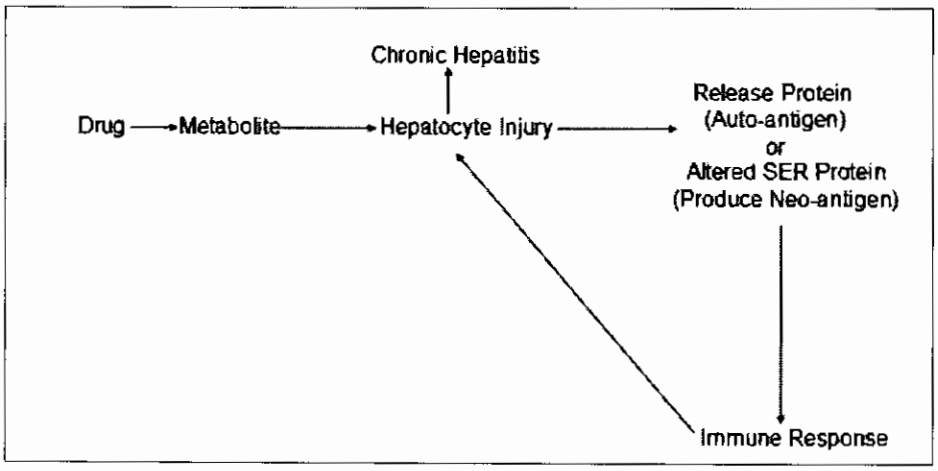

Figure 3 Hypothetical mechanisms by which prolonged administration of a drug can lead to chronic hepatitis of the autoimmune type. (Zimmerman, 1999).

Cirrhosis is the diffuse lesion caused by deposition of collagen and development of nodules of regenerating hepatocytes (Friedman, 1996). Phospholipidosis is an accumulation of phospholipid in lysosomes, hepatocytes, kupffer cells, etc. by which the lesions are occured (Lewis *et al.*, 1989). Cholestatic lesions are also caused by bile duct inflammation or duct destruction (Ishak *et al.*, 1972). Vascular lesions can be produced by hepatotoxins that involve blockage of efferent blood flow (Conn *et al.*, 1993). The carcinogenesis is induced by the reactive metabolics of hepatotoxins providing akyl-, acyl group or heterocyclic radicals to DNA. These reactions cause DNA mutation and initiate carcinogenesis (Pilot, 1994).

2. Hepatotoxic agents (Hepatotoxins)

Hepatotoxins are subdivided into direct hepatotoxins and indirect cytotoxic hepatotoxins that are classified by their mechanisms of liver injury induction.

2.1 Direct hepatotoxins

Direct hepatotoxins are toxic agents which their metabolic products damage the plasma membrane, endoplasmic reticulum, and other organelles of the hepatocytes directly. The metabolic products also include free radicals that have injurious effects on physiochemical of membrane as the result of lipid peroxidation, protein denaturation or destructive chemical changes, leading to

membrane destruction (Recknagel *et al.*, 1973). Examples of direct hepatotoxins are chloroform, carbon tetrachloride, ethanol, etc.

2.2 Indirect hepatotoxins

Indirect hepatotoxins induce hepatic injury by their metabolic products that disrupt specific metabolic pathway, and selective injury to a cell component or hepatic function by forming covalent bond with individual molecules of the cell (Zimmerman, 1968). Indirect hepatotoxins are subdivided into cytotoxic indirect hepatotoxins, cholestatic indirect hepatotoxins and carcinogenic indirect hepatotoxins.

Cytotoxic indirect hepatotoxins produce selective biochemical or physiological lesions such as aflatoxins B_1, galactosamine, etc. (Lieber, 1994). Cholestatic indirect hepatotoxins impair the liver function by selective interference bile flow. Carcinogenic indirect hepatotoxins have been reported that they could produce the hepatic carcinoma by changing macromolecules such as DNA or by selective lesions of cell growth and development (Miller *et al.*, 1976).

3. Hepatotoxic effects of ethanol

Ethanol has been reported as main factor contributing death and major problem on public health. Recently, World Health Organization has been reported that alcohol is estimated to cause about 3.7% of global deaths and 4.4% of the global burden of disease (World Health Organization, 2007). In 2007, population of alcoholic liver disease in Thailand has increased from 9,132 to 24,193 (National

Statistical Office of Thailand, 2007). Ethanol is mostly oxidized by aldehyde dehydrogenase (ADH) and CYP2E1 as following the equation.

$$\underset{\text{(Ethanol)}}{CH_3CH_2OH} \xrightarrow{\text{ADH or CYP 2E1}} \underset{\text{(Acetaldehyde)}}{CH_3CHO}$$

CYP2E1 is more important in ethanol oxidation at high ethanol concentrations and chronic consumption (Lieber, 1997). The metabolic products of ethanol are acetaldehyde and reactive oxygen species (ROS) (Garcia-Ruiz *et al.*, 1994).

3.1 Ethanol metabolic products

3.1.1 Acetaldehyde

Acetaldehyde can form covalent bond to and interfere the structure and function of several proteins of hepatocytes such as tubulin, actin, calmodulin, etc. and it also serve as neoantigen (Tuma *et al.*, 1995). Acetaldehyde initiates peroxidation by aldehyde oxidase (Shaw *et al.*, 1990) and xanthine oxidase (Puntarulo *et al.*, 1989). that generates free radicals and reveals iron mobilization from ferritin. Acetaldehyde also induces neo-antigen synthesis that instigaes immune cells to release proinflammatory cytokines (e.g. IL-1, TNF-α, etc.). These proinflammatory cytokines promote ROS levels, cause inflammation and cell death as shown in Figure 6. Interleukin-1 (IL-1) is one of the major cytokines of injury response and infection response that cause oxidative damage and inflammation. TNF-α is an activator of NF-κβ gene in many cell types that involves in inflammatory cytokines expression. TNF-α also involves in priming

phagocyte ROS production by increasing in iNOS levels. Therefore, TNF-α increases oxidative stress in its target cells and surrounding cells (Halliwell *et al.*, 2007).

3.1.2 Reactive oxygen species (ROS)

Reactive oxygen species are classified into superoxide radical such as superoxide ($O_2^{\bullet-}$), hydroxyl (e.g. hydroxyl ($OH^{\bullet}$)) and peroxyl (e.g. hydroperoxyl ($HO_2^{\bullet}$)) radicals. These radicals are resulted from the imbalance between oxidants and antioxidants leading to the damage in term of "oxidative stress" (Sies, 1985). Oxidative stress damages many cellular targets such as DNA, RNA, lipids, proteins and membranes. Its damages are divided by their cellular targets which are described as following.

Oxidative stress damages DNA structure (e.g. purine, pyrimidine base, and ribose sugar) by an activation of Ca^{2+} dependent endonuclease (von Sonntag, 2006). The oxidative stress causes the promotion of intracellular Ca^{2+} ions that change ions movement by regulating the expression or function of ion channel proteins, and changing cellular imbalance or membrane potential (Matalon *et al.* 2003). The Oxidative stress damages DNA directly by accelerating strand breakage or chemical modification of DNA, RNA base and ribose sugar (Barnes *et al.*, 2004). These effects may increase the mutation rate (Neeley *et al.*, 2006).

For lipids, lipoproteins or membranes damage is owing to lipid peroxidation. Lipid peroxidation is defined by Tappel A.L. as the oxidative

deterioration of polyunsaturated lipids (Park *et al.*, 1991). ROS initiate peroxidation of fatty acids, lipoproteins or membranes leading to form new carbon radicals. These radicals can react with O_2 to form new peroxyl radicals and induce the chain reaction of lipid peroxidation as shown in Figure 4.

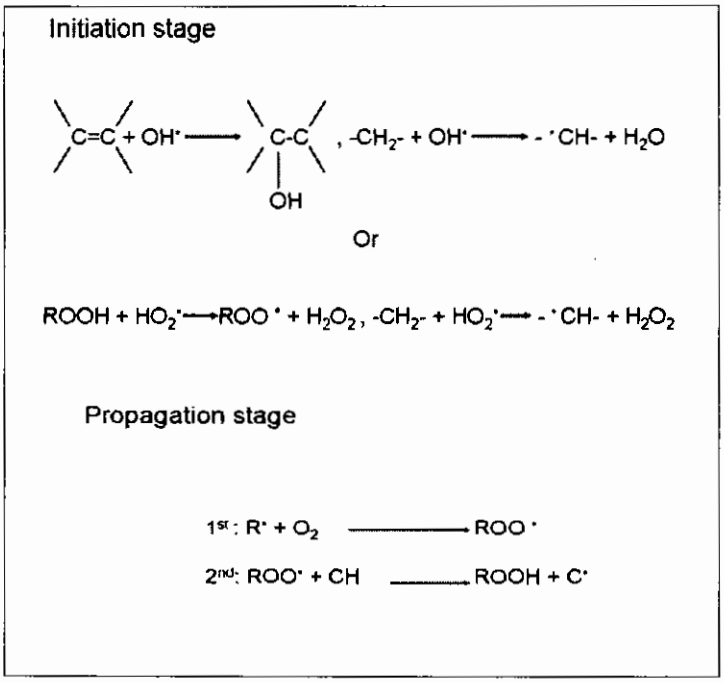

Figure 4 Initiation and propagation stage of lipid peroxidation. (Halliwell *et al.*, 2007)

Lipid peroxidation products such as lipid hydroperoxide (ROOH), malondialdehyde (MDA), 4-hydroxy-2-trans-noetal (HNE), etc. can reduce the membrane fluidity, lose the regulation of membrane permeability,

damage the membrane proteins and interlope the functions of enzymes or ion channels. These effects lead to lose membrane integrity and damage endoplasmic reticulum (ER), golgi- apparatus which affect to protein synthesis and transport. However, some lipid peroxidation products have more specific effects such as lipid hydroperoxide (ROOH) which is able to damage the immune system in rat (Hsieh *et al.*, 2005). Low level of ROOH can activate the proliferation but that of the high level inhibits the proliferation and lead to cell death (Tsunada *et al.*, 2003). MDA can damage proteins and react with DNA base leading to mutagenic lesions (Esterbauer *et al.*, 1991). High level of 4-HNE and other unsaturated aldehydes can damage mitochondria and trigger cell death. Whereas those in the moderate level inhibit DNA or protein synthesis and cellular proliferation. The low level of 4-HNE activate the fibrogenesis by increasing collagen biosynthesis, profibrogenesis and proinflammatory cytokines (Parola *et al.*, 1999).

ROS and lipid peroxidation products damage the protein by changing the chemical structure of proteins (Davies, 2005). For example, $H^{\cdot}$ is abstracted by $OH^{\cdot}$ which can damage peptide bonds and specific amino acid residues that impair the functions of antibody receptor, signal transduction, protein transporter, enzymes, and cause cell death.

3.2 Pathophysiological effect of ethanol

3.2.1 Hepatic steatosis

Hepatic steatosis can induce the physiologic lesions at multiple sites of lipid metabolism. Theoretically, steatosis is divided into impaired egression of lipid, impaired mitochondrial oxidation and elevation of lipid disposition.

Impaired egresssion of lipid is subdivided into impaired synthesis of apolipoprotein, impaired assembly of the triglyceride-apolipoprotein complex and impaired transport across the plasma membrane. Impaired synthesis of apolipoprotein and impaired assembly of the triglyceride-apolipoprotein complex are induced by ethanol, acetaldehyde and ROS. These impairments damage rough endoplasmic reticulum (RER) and its ribosome that lead to defective synthesis of the apolipoprotein. Apolipoprotein such as very-low density lipoprotein (VLDL) consists of glycoprotein, hexose components and glycosyl group that serve as triglyceride's transporter (Farber, 1975). While impaired transport across the plasma membrane is caused by ethanol and its metabolic products which damage the plasma membrane and then disturb movement of the VLDL (Dianzani, 1991).

Impaired mitochondrial oxidation is induced by ethanol and its metabolic products which damage the mitochondria. Impairment of mitochondria activity causes depletion of NAD and bioenergetic adenosine triphosphate (Grimbert *et al.*, 1993). The increasing of lipid disposition is induced

by acetaldehyde that raises fatty acid mobilization from adipose tissues (Lieber, 1978). Acetaldehyde promote nicotinamide adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH) production. The promotion of NADH and NADPH can enhance the lipogenesis (Lieber, 1997) as shown in Figure 5.

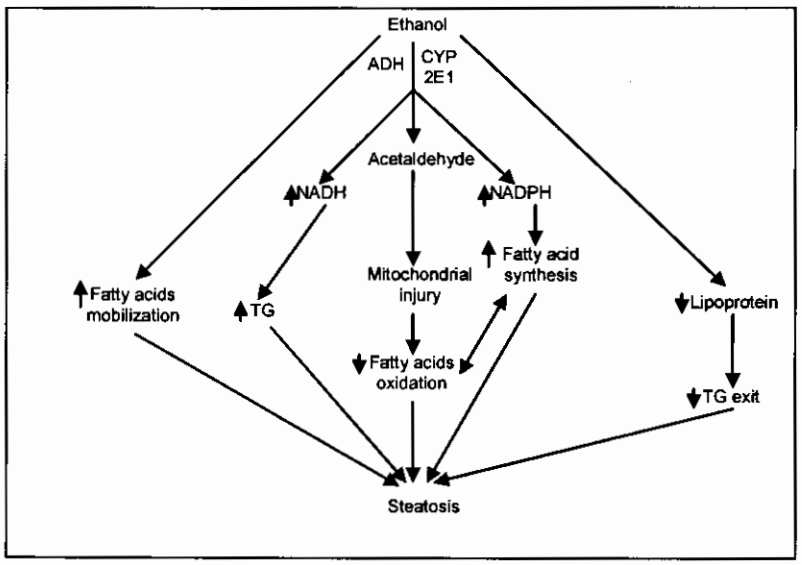

Figure 5 The mechanism of ethanol that lead to steatosis. (ADH, aldehyde dehydrogenase; TG, Triglycerides) (Zimmerman, 1999).

3.2.2 Necrosis

Necrosis is initiated by acetaldehyde inducing the injury to hepatocyte organelles and membranes by covalent binding to cell membrane (Tuma *et al.*, 1995) or initiating the peroxidative injury (Tsukamato *et al.*, 1996).

Organelles injury and mitochondria damage cause depletion of ATP and loss of the normal selective permeability of the mitochondrial membrane (Rosser *et al.*, 1995). RER injury conduces to the impairment of protein synthesis and the cellular lesions (Rouiller, 1964). SER injury induces alteration of neo-antigen. Neo-antigen has been suggested to exihibit a role in idiosyncratic damage (Homburg *et al.*, 1985). Lysosome injury causes the secretion of destructive and proteolytic enzymes which can damage other organelles (Rosser *et al.*, 1995). Plasma membranes damage lead to the loss of intracellular electrolytes (e.g. K^+), enzymes, and coenzymes and to entry of extracellular electrolytes, especially Ca^{2+} ions (Farber, 1975). These organelle injuries contribute to necrosis.

Peroxidative injury includes the peroxidative action of acetaldehyde, the production of ROS and iron-stimulated peroxidation to microsomal that are induced by ethanol. Ethanol is a CYP2E1 inducer resulting in the elevation of acetaldehyde, ROS and other biotoxic agents. Ethanol also disturbs glutathione synthesis resulting in glutathione depletion that leads to hypoxia and necrosis (Israel *et al.*, 1975; Lieber, 1997).

3.3 Cirrhosis

Cirrhosis is the deposition of collagen and development of nodules of regenerating hepatocytes that are induced by steatosis and necrosis as shown in Figure 6. Both necosis and steatosis stimulate connective tissues synthesis, increase collagen synthesis, and reduce collagen metabolism (Rojkind, 1985).

Acetaldehyde induces the conversion of stellate cells or fat storage cells to myofibroblast and stimulated fibrogenesis.

Cirrhosis disturbs the pattern of blood flow through the liver that conduces to the impairment of perfusion of hepatic lobules. Thereafter the impairment of protein synthesis, and drugs metabolism are occured (Friedman, 1996).

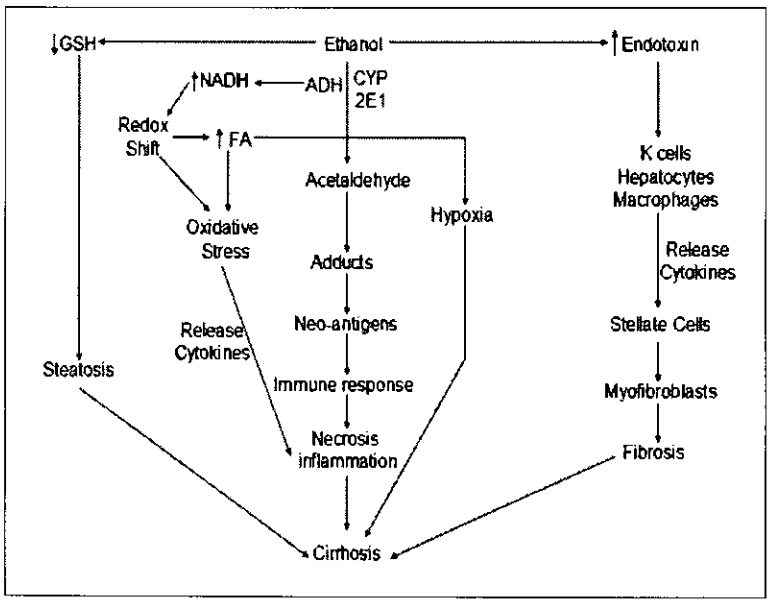

Figure 6 Simplified mechanisms of ethanol that lead to necrosis and cirrhosis (Zimmerman, 1999).

4. Defense mechanism of oxidative stress in cells

Antioxidant is substance that significantly delays, prevents or removes

the oxidative damage of the target molecules. These substances are divided into enzymatic antioxidant and non-enzymatic antioxidant.

4.1 Enzymatic antioxidant

Enzymatic antioxidant has three major antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).

4.1.1 Superoxide dismutase (SOD)

SODs catalyse the dismutation of superoxide ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) as the following equation.

$$\text{Enzyme-ion}^{2+} + O_2^{\bullet-} \longrightarrow \text{Enzyme-ion}^{+} + O_2$$

$$\text{Enzyme-ion}^{+} + O_2^{\bullet-} + 2H^{+} \longrightarrow \text{Enzyme-ion}^{+} + H_2O_2$$

$$\underline{\text{Net reaction}}: 2O_2^{\bullet-} + 2H^{+} \xrightarrow{\text{SODs}} H_2O_2 + O_2$$

SODs are subdivided into copper-Zinc SOD (CuZnSOD), manganese SOD (MnSOD), iron SOD (FeSOD) and nickle SOD (NiSOD) based on their ion containing in active site. CuZnSOD and MnSOD can be found in animals whereas FeSOD and NiSOD present in bacterial.

CuZnSOD is a stable enzyme which endures heating, proteinase and denaturation. This enzyme locates at cytosol, lysosomes, nucleus, space between inner-outer mitochondrial membranes and peroxisomes (Okado-Matsumoto *et al.*, 2001). CuZnSOD contains two subunits, and each subunit consists of one copper and one zinc ion at active site (Fridovich, 1995). Copper ion (Cu^{2+}) accelerates dismutaion of $O_2^{\bullet-}$ whereas zinc ion (Zn^{2+}) stabilizes the enzyme.

MnSOD is less stable than CuZnSOD which is found in mitochondria. Amount of MnSOD and CuZnSOD rely on tissues and species. These enzymes co-operate to maintain total cellular SOD activity. In some condition, when cells have copper supply restricted, more MnSOD is synthesized and transferred to cytosol for removing $O_2^{\bullet-}$ (Shatzman *et al.*, 1979). Therefore, MnSOD is another SOD that is necessary as CuZnSOD.

4.1.2 Catalase (CAT)

Catalase catalyses the decomposition of hydrogen peroxide directly. This enzyme can be found in all animal organs, especially liver and locates at peroxisomes, mitochondria and endoplasmic reticulum. Catalase composes of four subunits, and each subunit consists of Fe(III)-haem at active site that reduces H_2O_2 to water and oxygen molecule (Lardionis, 1995) as the following equation.

$$\text{Catalase-Fe(III)} + H_2O_2 \longrightarrow \text{Compound I} + H_2O$$

$$\text{Compound I} + H_2O_2 \longrightarrow \text{Catalase-Fe(III)} + H_2O + O_2$$

$$\underline{\text{Net reaction}}: 2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

4.1.3 Glutathione peroxidase (GPx) family

Glutathione peroxidase involves in the removal of H_2O_2, lipid hydroperoxide (ROOH) and other hydroperoxides by conjugating with reduced glutathione (GSH). GPx is subdivided into cytosolic GPx (GPx1), intestinal GPx (GPx2), mammalian plasma GPx (GPx3) and phospholipid hydroperoxide GPx (GPx4). GPx1 and GPx2 do not hydrolyze cholesterol hydroperoxides. GPx3

slowly hydrolyzes cholesterol hydroperoxides, whereas GPx4 is able to oxidize the fatty acid residues within membranes and lipoproteins (Brigelius-Flohé, 1999). Gpx1, Gpx2, and GPx3 composes of four protein subunits, while GPx4 is monomer. Each subunit of GPx contains one selenium atom at active site that reacts with peroxides as the following equation.

$$\text{GPx-Se}^- + \text{ROOH} + \text{H}^+ \longrightarrow \text{ROH} + \text{GPx-SeOH}$$

$$\text{GSH binding:}\quad \text{GPx-SeOH} + \text{GSH} \longrightarrow \text{GPx-Se-SG} + \text{H}_2\text{O}$$

$$\text{GSH binding:}\quad \text{GPx-Se-GSSG} + \text{GSH} \longrightarrow \text{GPx-Se}^- + \text{H}^+ + \text{GSSG}$$

GPx co-operate with glutathione-*S*-transferase (GST) and glutathione reductase (GSR) to reduce the oxidative damages. GST accelerates the conjugation of metabolic products of xenobiotics with GSH (Hayes *et al.*, 2005) as the following equation.

$$\text{RX} + \text{GSH} \xrightarrow{\text{GST}} \text{R-GSSG} + \text{HX}$$

GSR involves in the maintenance level of GSH by stimulating the conversion of GSSG to GSH as the following equation.

$$\text{H}^+ + \text{GSSG} + \text{NADPH} \xrightarrow{\text{GSR}} 2\text{GSH} + \text{NADP}^+$$

4.2 Non-enzymatic antioxidant

The example of non-enzymatic antioxidants are glutathione, transferrins, albumins, vitamin A/C/E and the other compounds derived from diet or herb (e.g. plant phenol, carotenoid, etc.).

Glutathione is a tripeptide which is composed of glutamate, cysteine and glycine as shown in Figure 7. The cysteine contains thiol group (-SH)

which provides the reactive portion. Glutathione is involved in metabolism and detoxification of metabolic products as described above. Glutathione scavenges RS and chelates copper ions that diminish their ability to generate hydroxyl radicals ($OH^{\bullet}$) from hydrogen peroxide. However, thiol of cysteine prones easy to oxidize and generate ROS, while glutamate regulates the reactivity of the thiols. Therefore, glutathione is able to maintain in cells without ROS generation (Hanna *et al.*, 1992).

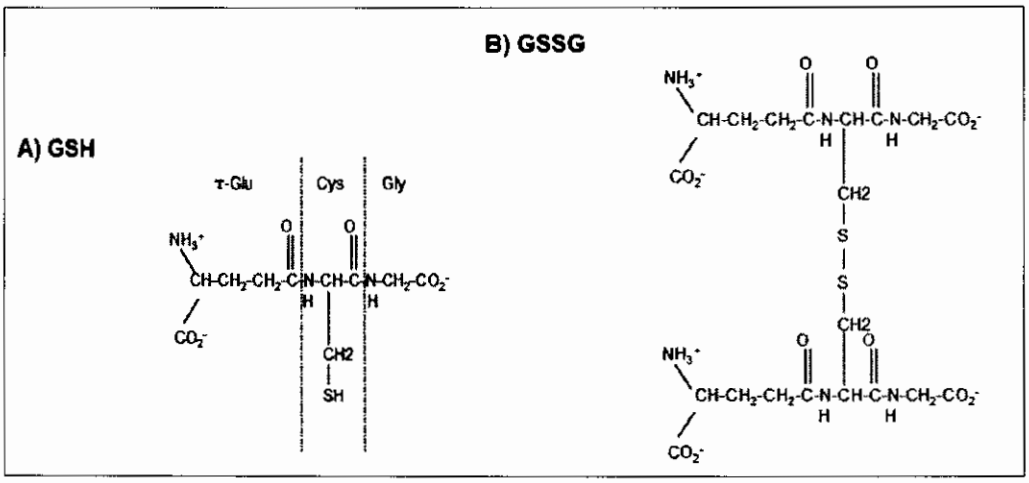

Figure 7 Structure of reduced glutathione (GSH) and oxidized glutathione (GSSG) (Halliwell *et al.*, 2007).

Transferrin binds iron and inhibits its pro-oxidant activity (Hentze *et al.*, 2004). Albumins chelate copper and iron, which can scavenge hypochlorous acid (HOCl) and peroxynitrous acid (ONOOH) (Halliwell *et al.*, 1990).

In animal, ascorbic acid or vitamin C can interact metal ions and then induce the hydrogen peroxide production. Therefore it can become to the potential pro-oxidant and induce the oxidative stress. Ascorbic acid also serves as antioxidant by scavenging RS to protect the plasma lipids, membranes and lipoproteins against oxidative damage (Padayatty *et al.*, 2003).

Vitamin E refers to tocopherols and tocotrienols. Both tocopherols and tocotrienols are subdivided into α-, β-, γ-, δ- form. α-, β-, γ-, δ-tocoherols differ in their methyl groups (phytyl tail) on the chromanol ring. Tocopherols can be found in sunflower, peanut, sesame and olive oil. In contrast, α-, β-, γ-, δ-tocotrienols differ in their unsaturated tail. The major sources of tocotrienols are palm oil, rice bran, coconut oil, cocoa butter, soybean, barley and wheat gram (Sheppard *et al.*, 1993). Vitamin E exerts antioxidative effect that inhibits lipid peroxidation by scavenging the chain-propagation peroxyl radical and the quenching singlet oxygen (Burton *et al.*, 1981).

Plants phenols (Rice-Evans *et al.*, 1996) are compounds that contain hydroxyl (-OH) group attaching to a benzene ring. Most phenols exert antioxidative effects that prevent lipid peroxidation by acting as inhibitors of peroxyl radical and RS scavengers. Phenols can chelate metal ions to poorly active form that can not promote the free radical reactions. The example of plant phenols are flavonoids, hydroxycinnamic acids, hydroxybenzoic acids and condensed tannins as shown in table 4.

Table 4 Some dietary sources of plant phenols (Halliwell *et al.*, 2007).

Compound	Sources
Flavanols	
Epicathechin	Green teas
Catechin	Red wine
Epigallocatechin	Cocoa, chocolate
Epicatechin gallate	
Epigallocatechin gallate	
Flavonones	
Naringin	Citrus fruits
Taxifolin	
Flavonols	
Kaempferol	Endive, Leek, broccoli, radish, grapefruit, black tea
Quercetin	Onion, lettuce, broccoli, cranberry, apple skin, berries, olive, tea, red wine
Myricetin	Cranberry, grapes, red wine
Flavones	
Chrysin	Fruit skin
Apigenin	Celery, parsley
Anthocyanidins	
Malvidin	Red grapes, red wine
Cyanidin	Cherry, raspberry, strawberry, grapes
Apigenidin	Coloured fruits and peels
Pelargonidin	
Hydroxycinnamic acid derivatives	
Caffeic acid	White grapes, white wine, olives, olive oil, spinach, cabbage, asparagus,coffee
p-Coumaric acid	White grapes, white wine, tomatoes, spinach, cabbage, asparagus
Chlorogenic acid	Apples, pears, cherries, plums, peaches, apricots, blueberries, tomatoes, anise, coffee, antichoke, aubergine

Flavonoids are the polyphenolic compounds subdivided into flavonols, flavanols, flavones, flavanones, isoflavans and isoflavones. Polyphenols and flavonoids are the potential antioxidants that serve as reductant, hydrogen-donating antioxidant, and singlet oxygen quencher (Rice-Evans *et al.*, 1995) and some polyphenols also act as metal chelator (Brown *et al.*, 1998).

Carotenoids are the precursors of vitamin A which their major sources are fruits and vegetables. Carotenoids can scavenge free radical, especially singlet oxygen ($^1O_2$). Carotenoids also act as synergistically in antioxidant defense with tocopherol (Palozza *et al.*, 1992).

5. *Sonneratia caseolaris*

Sonneratia caseolaris has been well recognized as crabapple mangrove and firefly mangrove. Its Thai tradition name is Lumpoo which is classified into family of *Sonneratiaceae*. The plant height is mild to moderate (5-15 m) that grown in fresh or salt water in flood area as shown in Figure 8. Many parts of *S. caseolaris* have various usefulness. Pneumatophore is used in fishing floats or making corks. Fruits of *S. caseolaris* are used as food or transformed into wine or juice. Its extracts have been reported to be employed in curing bleeding, hemorrhages, piles and sprain poultices (Bose *et al.*, 1992).

5.1 The chemical constituents of *S. caseolaris*

Sonneratia caseolaris methanolic extracts was reported to contain steroids (e.g. 6-*O*-Acetyl-β-daucosterol, β-sitosterol, stigmasterol, β-sitosteryl palmitate, cholesterol, cholest-5-en-3β,-7α-diol, stamast-5-en-3β-*O*-(6-*O*-hexadecanoyl-β-D-glucopyranoside and daucosterol), triterpenoids (e.g. betulin, lupeol, lup-20(29)-en-3β,24-diol, 3β-*O*-(*E*)-cumaroyl-alpitholinic acid, 3β-*O*-acetyl-oleanolic acid, oleanolic acid, 3β,13β-dihydroxy-urs-11-en-28-oic acid-13-lactone, ursolic acid and 3β-hydroxy-20(29)-lupen-24-oic acid), benzene carboxylic derivatives (e.g. bis(2-ethylhexyl)benzene-1,2-dicarboxylate, 3,3'-di-*O*-methylether egallic acid, 3,3',4-*O*-tri-*O*-methylether egallic acid and methyl gallate) and flavonoids (e.g. (+)-dihydroxykaempferol, 3',4',5,7- tetrahydroxy flavone and quercetin-3-*O*- β-L-arabinopyranoside) (Tian *et al.*, 2009). However, *S. caseolaris* methanolic extract has also been reported that they contain lueolin and luteolin-7-*O*-β-glycoside (Sadhu *et al.*, 2006).

Figure 8 *Sonneratia caseolaris*. Source: ห้อง SAR โรงเรียนหาดใหญ่วิทยาลัย, หิ่งห้อยชนิดใหม่ ในไทย ลูซิโอลา อะควอติลิส [Online], accessed 19 November 2010. Available from http://202.129.48.218/sar/photo/lampoo.jpg

5.2 Medical uses

The chemical constituents of *S. caseolaris* extracts which are found in other plants have been reported as medical uses such as triterpenoids and flavonoids.

Triterpenoids are found in *S.caseolaris* methanolic extract such as lupeol, ursolic acid and oleanolic acid. These compounds in other plant extracts have been reported their pharmacological activities diversity such as anti-inflammatory, antitumor and hepatoprotective activities.

For anti-inflammatory activities, lupeol can inhibit inflammatory cytokines release such as IL4, IL-5, IL-6, IL-13, etc. (Bani *et al.*, 2006; Ding *et al.*, 2009), and inhibit protein kiase C (PKC) that restrain proinflammatory cytokines e.g. TNF-α, IL-1β expression (Huguet *et al.*, 2000). Ursolic acid and oleanolic acid are able to inhibit histamine releasing from mast cells (Balanehru *et* al., 1994), lipoxygenase and cyclooxygenase (COX) activity (Simon *et al.*, 1992), complement activity possibly by inhibiting C_3-convertase activity (Kapil *et al* ., 1994) and CYP450 activity (Kim *et al.*, 2004).

For antitumor activity, both betulin and lupeol can inhibit topoisomerase II (topo II) that conduce to inhibit the relaxation of DNA supercoiled and stop the DNA expression, respectively (Chaturvedula *et al.*, 2004), Betulin and lupeol can inactivate farnesyltransferase (FTase) that inhibit cell signaling from membrane receptor and also inhibit angiogenesis (Käβmeyer *et al.*, 2009). Oleanolic acid and ursolic acid have been reported to suppress the tumor promotion but their mechanisms are unknown (Liu, 1995).

For hepatoprotective, lupeol can restore serum liver enzymes, enzymatic antioxidants and nonenzymatic antioxidants (e.g. glutathione, vitamin C, vitamin E) and reduce ROS level in hepatocyte (Prasad *et al.*, 2007). Ursolic acid and oleanolic acid inhibit CYP450 activity resulting to inhibit the metabolic activation of hepatotoxins (Zhang *et al.*, 1992) and promote some nonenzymatic antioxidants such as glutathione, metallothionein, and zinc (Liu *et al.*, 1995).

Flavonoids are found in *S. caseolaris* methanolic extract such as luteolin, quercetin, luteolin-7-*O*-β glycoside and kaempferol in other plant extracts have been reported their hepatoprotective effects that they can reduce cell death and cell injury, inhibit lipid peroxidation and prevent glutathione depletion. While luteolin and quercetin decrease the DNA damage significantly (Lima *et al.*, 2006).

Sonneratia caseolaris methanolic extract is composed of triterpenoids and flavonoids (Sadhu *et al.*, 2006; Tian *et al.*, 2009) that have the hepatoprotective effects (Lima *et al.*, 2006; Domitrović *et al.*, 2009; Liu *et al.*, 2010). Therefore, this research work aimed to characterize hepatoprotective activity of *S. caseolaris* methanolic seed extract. If *S. caseolaris* extract has hepatoprotective activity, its extract will be developed as a new natural hepatoprotective drug for the prevention of alcoholic liver disease (ALD).

6. The *in vitro* hepatoprotective studies

In the *in vitro* hepatotoxicity evaluation of compounds, it is necessary to determine the biomarkers which represent the *in vivo* toxicity. Traditional approaches for *in vitro* cytoxicity testing are the assay of lactate dehydrogenase (LDH) leakage, an indicator of morphological changes. The assay of MTT conversion, an indicator of the toxicity on mitochondrial respiration (Horii *et al.*, 2007).

6.1 MTT assay

MTT assay is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. MTT enters the cells and localizes into the mitochondria. It reduces to insoluble formazan product (dark purple). Then formazan products are dissoloved with organic solvent e.g. dimethylsulfoxide (DMSO). Then solubilized formazan reagent absorbs visible light at the wavelength 550 nm. Since, the reduction of MTT can only occur in metabolically active cells, the level of activity is the measurement of the viability of the cells (Mosmann, 1983).

6.2 LDH leakage assay

LDH leakage assay is a colorimetric method to determine cytotoxicity quantitatively relied on the measurement of LDH released from damaged cells into the supernatant. LDH activity is determined in coupling enzymatic reaction as shown in Figure 9. NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate, whereas diaphorase catalyzes transfer hydrogen atom (H) from NADH to tetrazolium salt which is reduced to formazan. This formazan absorbs visible light at the wavelength 485 nm (Kondo *et al.*, 1993).

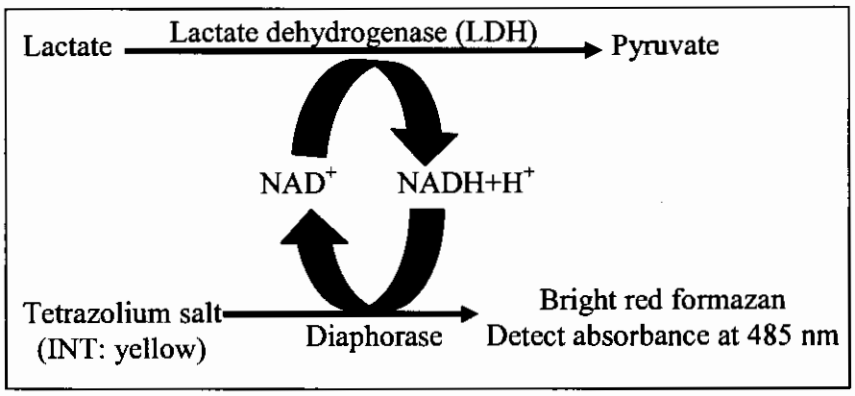

Figure 9 Principle of LDH leakage assay.

7. Antioxidative activity studies

7.1 Thiobarbituric acid reactive substance (TBARs) assay

TBARs assay is a method to determine the malondialdehyde (MDA) which is lipid peroxidation product. MDA forms a 1:2 adduct with thiobarbituric acid (TBA) which can be measured by fluorometry or spectrophotometry. The decrease of TBARs amount over time is found, upon the presence of antioxidants (Yagi, 1982).

7.2 Human TNF-α enzyme-linked immunosorbent assay (ELISA)

hTNF-α ELISA is a sandwich ELISA format to evaluate the hTNF-α secretion by measuring horseradish peroxidase (HRP) activity. HRP catalyzes the conversion of 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)-diammonium salt to chromogen. The level of colored product is relied on the bound enzyme-linked detection antibody that proportional to the quantity of released hTNF-α (Jordan, 2005) as shown in Figure 10.

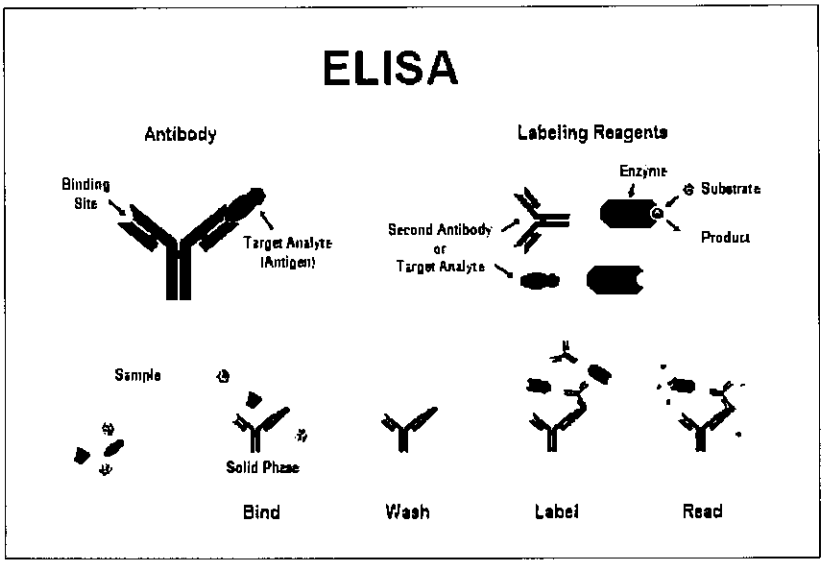

Figure 10 Principle of sandwich ELISA.

Source: Katarina Calibur, ISOTYPES [Online], accessed 10 March 2011. Available from http://64.202.120.86/upload/image/articles/2006/biopen/biopen-elisa-schematic.jpg

7.3 Superoxide dismutase (SOD) assay

SOD assay is an indirect method to measure the reduction of superoxide ions (O_2^-). Superoxide ions are generated by conversion of xanthine to uric acid and hydrogen peroxide using xanthine oxidase (XOD). Subsequently, O_2^- coupling reaction with water soluble tetrazolium salt (WST) to produce WST-diformazan, which absorbs visible light at the wavelength 550 nm. SOD catalyzes the dismutation of superoxide ions and thereby lowers the amount of WST-

diformazan formation. The reduction of WST-diformazan represents the SOD activity in an experimental sample (Ukeda *et al.*, 1999).

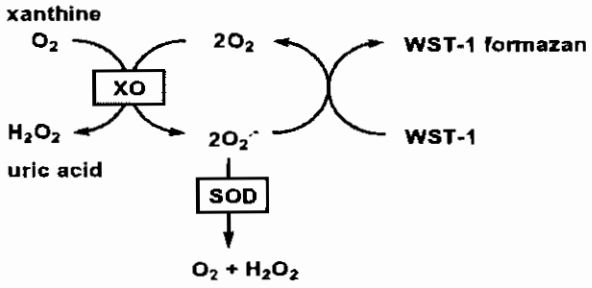

Figure 11 Principle of superoxide dismutase (SOD) assay (Indirect method).

Source: abcam®, Superoxide Dismutase Activity Colorimetric Assay Kit (ab65354) [Online], accessed 19 November 2010. Available from http:// www.abcam.com/ps/datasheet/Images/65/ab65354/ab65354-1.jpg

7.4 Catalase (CAT) assay

In CAT assay, catalase reacts with H_2O_2 to produce water and oxygen (O_2). Subsequently, horseradish peroxidase (HRP) catalyzes the resorufin production which is resulted from the raction of the rest H_2O_2 and Amplex Red reagent as shown in Figure 12. Resorufin is highly fluorescent and strongly absorbance oxidation product. It absorbs visible light at the wavelength 550 nm, Therefore, as catalase activity increases, the signal from resorufin decreases (Mohanty *et al.*, 1997)

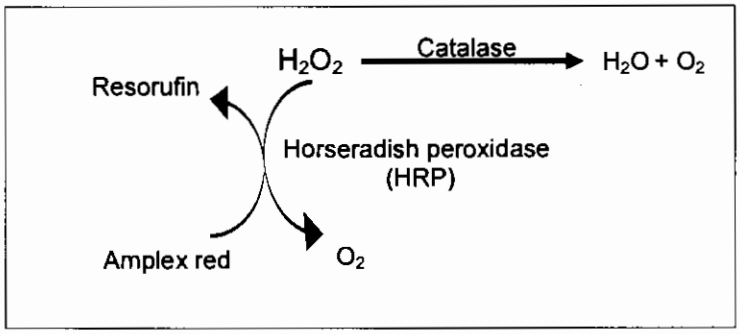

Figure 12 Principle of catalase (CAT) assay.

7.5 Glutathione peroxidase (GPx) assay

GPx assay measures GPx activity indirectly by coupling reaction with glutathione reductase (GSR). The GSSG is produced upon the reduction of hydroperoxide by GPx. Then, the GSSG is recycled to GSH by GSR and NADPH as shown in Figure 13. The oxidation of NADPH to NAD^+ is determined by a decrease of absorbance at the wavelength 340 nm. In this condition, GPx activity is rate limiting step, the rate of decrease absorbance directly represents the GPx activity in sample (Paglia *et al.*, 1967).

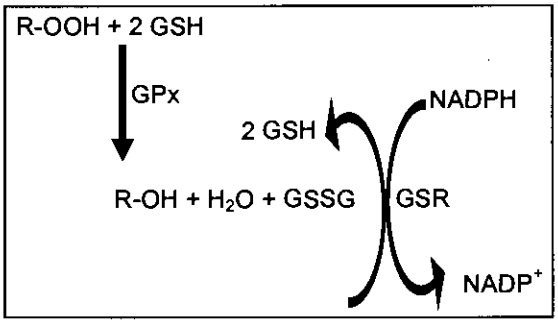

Figure 13 Principle of glutathione peroxidase (GPx) assay.

7.6 Reduced glutathione (GSH) assay

The reduced glutathione assay determines the GSH directly by the reaction of DTNB (5,5'-Dithiobis(2-nitrobenzoic acid)) and GSH that generates 2-nitro-5-thiobenzoic acid and GSSG as shown in Figure 14. This product is determined by measuring the absorbance at the wavelength 412 nm. The increasing absorbance is directly proportional to the quantity of GSH in sample (Tietze, 1969).

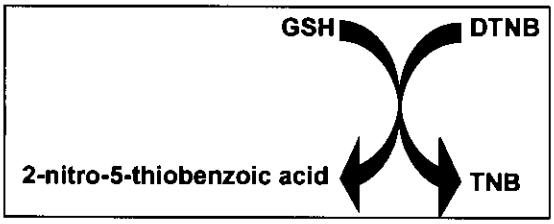

Figure 14 Principle of reduced glutathione (GSH) assay.

CHAPTER 3

Materials and methods

1. Materials

Minimum essential medium (Gibco™, Auckland, New Zealand)

Fetal bovine serum (Gibco™, Auckland, New Zealand)

Glutamine (Gibco™, Auckland, New Zealand)

Trypsin-EDTA (Gibco™, Auckland, New Zealand)

Absolute ethanol (Merck KGaA, Darmstadt, Germany)

Phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, USA)

Dimethylsulfoxide (Merck Schuchardt OHG, Hohenbrunn, Germany)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or MTT (MANN Research Labolatories, New York, USA)

TritonX-100 (Scharlau Chemie S.A., Barcelona, Spain)

Thiobarbituric acid (Merck KGaA, Dannstadt, Germany)

Malondialdehyde (Merck Schuchardt OHG, Hohenbrunn, Germany)

Rabbit anti-hTNF-α monoclonal antibody (Peprotech, Inc., Rocky Hill, NJ)

Biotinylated rabbit anti-hTNF-α monoclonal antibody (Peprotech, Inc., Rocky Hill, NJ)

Horseradish peroxidase conjugated avidin (Peprotech, Inc., Rocky Hill, NJ

2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid)- diammonium salt or ABTS substrate (Sigma-Aldrich Co., St. Louis, USA)

WST working solution (Sigma-Aldrich Chemie GmbH, Buschs, Switzerland)

Xanthine oxidase (Sigma-Aldrich Chemie GmbH, Buschs, Switzerland)

Superoxide dismutase from bovine erythrocytes (Sigma-Aldrich Chemie GmbH, Buschs, Switzerland)

10-acetyl-3,7-dihydroxyphenoxazine or Amplex red reagent (Molecular Probes, Inc., Eugene, Oregon)

Horseradish peroxidase (Molecular Probes, Inc., Eugene, Oregon)

Hydrogen peroxide (Molecular Probes, Inc., Eugene, Oregon)

Catalase (Molecular Probes, Inc., Eugene, Oregon)

Coomassie blue dye (Bio-Rad Laboratories, Singapore)

Glutathione peroxidase assay kit (QuantiChrom™ Hayward, USA)

Reduced glutathione assay (QuantiChrom™ Hayward, USA)

LDH cytotoxicity detection kit (TaKaRa Bio Inc., Otsu, Shiga, Japan)

2. Equipments

Microplate reader (Fusion™, A Packard Bioscience Company, USA)

Spectrofluorometry (RF-1501, Shimadzu, Japan)

Vibra-cell™ VCX130 (Sonics & Materials, Inc., NewTown, USA)

pH meter Professional Meter PP-15 (Sartorious, Goettingen, Germany)

Water bath (Julabo, Japan)

3. Methods

3.1 Plant materials

Sonneratia caseolaris seeds were dried at 55°C for 3 days and ground then sieved through 30 mesh sieve. The ground seeds were macerated with methanol (seed powder: methanol 1:4) for 3 days. The methanolic extract was filtered through Whatman filter paper no.1 and evaporated by rotary evaporator. The dried extract was kept in dessicator for 1 week and continuously storaged at 4°C.

3.2 Cell preparation methods

3.2.1 Maintainance of HepG2

Culture medium was removed from HepG2 75cm^2 culture flask and washed with phosphate buffered saline (PBS) pH 7.4. Cells were trypsinized with 1 mL of 0.25%v/v trypsin-EDTA and incubated at 37 °C for 10 min. Then, the reaction was stopped by adding with 3 mL complete MEM (MEM + 10 % FBS). The 1.5 mL suspension cell was kept and grown in new culture flask with 10 mL complete MEM. Cells were incubated in 5% CO_2 incubator at 37 °C for 3 days.

3.2.2 Seeding HepG2 cells

HepG2 cells were trypsinized as described above. Four milliliters of the cell suspension were transferred into 15 mL centrifuge tube and

centrifuged at 1,000 x g, 4 °C for 5 min. Supernatant was discarded then collected cell pellets and resuspended with 2 mL serum free medium. Cell suspension was counted by hemacytometer and diluted to propriate number for each assay. Cells were seeded in cell-culture plate or petridish and incubated in 5% CO_2 incubator at 37 °C.

3.2.3 Cell Treatment

The HepG2 cells were sustained in serum free medium that incubated in CO_2 incubator at 37 °C for 24-48 h. Culture mediums were discarded and refreshed the medium. Cells were treated with *S. caseolaris* methanolic seed extract at various concentrations for 2 h and followed with ethanol. The experiment for MTT assay was performed later after 30 min exposed to ethanol, whereas LDH leakage assay, TBARs assay, hTNF-α ELISA, SOD assay, CAT assay, GPx assay and reduced glutathione assay were done after 24 h ethanol exposure.

3.3 Experiments and protocols

3.3.1 The hepatoprotective activity study

Ethanol metabolic products have injurious effect on physiochemical of cell membrane that leading to LDH leakage and triggering cell death. Therefore, the hepatoprotective activity was investigated by determining cell death and cell injury. In this research work, cell viability was assessed by MTT assay and cell injury was evaluated by LDH leakage assay.

3.3.1.1 Determination of cell viability

HepG2 cells were seeded in 96-well flat bottom plate at $2x10^4$ cells/well and incubated in 5% CO_2 incubator at 37 °C. After 24 h incubation, cells treated with 10, 20 or 30 µg/mL *S. caseolaris* methanolic seed extract for 2 h and followed with 1.70 M ethanol. After exposed to ethanol for 30 min, cell viability was determined by MTT assay as previously method described by van Golen *et al.* (1996). Briefly, 20 µL of 5 mg/mL MTT solutions was added in each well. Mixtures were incubated at 37 °C for 2 h. Supernatant was discarded and 100 µL dimethylsulfoxide (DMSO) was added for dissolving formazan crystal. Cell viability was determined by measuring the absorbance at the wavelength 550 nm using microplate reader (Fusion™, A Packard Bioscience Company, USA). Experimental data were analyzed as percentage of cell viability and compared with that of cell control. The percentage of cell viability in the assay was calculated as follows:

$$\% \text{ cell viability} = \frac{(A_{550} \text{ sample} - A_{550} \text{ blank}) \times 100}{(A_{550} \text{ control} - A_{550} \text{ blank})}$$

3.3.1.2 Determination of cell injury

HepG2 cells were seeded in 96-well flat bottom plate at $2x10^4$ cells/well and incubated in 5% CO_2 incubator at 37 °C. After 24 h incubation, cells treated with 10, 20 or 30 µg/mL *S. caseolaris* methanolic seed extract for 2 h and followed with 100 mM ethanol for 24 h. Cell injury was estimated according to TAKARA's protocol. Plate was centrifuged at 250 x g for

10 min and then 100 µL supernatant was transferred into new 96-wells flat bottom plate. One hundred microliters of reaction mixtures was added into each well. The reaction mixtures contained NAD^+, and diaphorase enzyme. Reactions were incubated at room temperature for 20 min with light protection and stopped reaction by adding 50 µL of 1 N hydrochloric acid. The absorbance was measured at the wavelength 485 nm using microplate reader (Fusion™, A Packard Bioscience Company). Cell injury was analyzed as percentage of LDH leakage and compared with total LDH. The percent LDH leakage in the assay was calculated as follows:

$$\% \text{ LDH leakage} = \frac{(A_{485} \text{ sample} - A_{485} \text{ blank})}{(A_{485} \text{total LDH} - A_{485} \text{ blank})} \times 100$$

3.3.2 The antioxidative effects study

Ethanol induces hepatotoxicity by generating ROS. Excessive ROS can react to macromolecules directly or initiate lipid peroxidation. Lipid peroxidation products e.g. MDA, 4-HNE etc. are able to damage organelles and trigger cell death. While hTNF-α involves in promoting nitric oxide synthases (iNOS) enzyme that increases the phagocyte ROS production. Therefore, one of the possible hepatoprotective mechanisms against oxidative damage may conduce to the antioxidative effects. The antioxidative effects were investigated by determining lipid peroxidation and human TNF-α (hTNF-α) secretion. In this research work, lipid peroxidation was assessed by TBARs assay whereas hTNF-α secretion was measured by hTNF-α ELISA.

3.3.2.1 Determination of lipid peroxidation

HepG2 cells were seeded in 6-wells plate at 10^6 cells/well and incubation in 5% CO_2 incubator at 37 °C. After 48 h incubation, cells treated with 10 or 30 μg/mL *S. caseolaris* methanolic seed extract for 2 h and followed with 250 mM ethanol for 24 h. The medium was collected into 1.5 mL microcentrifuge tube whereas cells were lysed by adding 300 μL homogenizing medium that composed of 0.5 M Tris-HCL (pH 6.8) and 0.1 g sodium dodecyl sulfate (SDS) in reverse osmosis (RO) water. Cells were scraped and collected into new 1.5 mL centrifuge tube. Both lysate and culture medium were homogenized by Vibra-cell™ VCX130 (Sonics & Materials, Inc., NewTown, USA) 40% milliampere (mA), 3 s interval and pooled in new centrifuge tube. All processes were conducted at 4°C. Proteins were precipitated by adding 200 μL 10% v/v trichloroacetic acid (TCA) and incubated at 4°C for 15 min. Lysate was centrifuged at 2,200 x g for 15 min and then the supernatant was used for TBARs assay.

Lipid peroxidation product (malondialdehyde; MDA) was determined by TBARs assay as previously method described by Kaviarasan *et al.* (2006). Five hundred microliters of lysate or standard 0, 0.2, 0.4, 0.6, 0.8 or 1 μM were transferred to 1.5 mL microcentrifuge tubes and added with 1 mL thiobarbituric acid (ratio of MDA:TBA = 1:2). Reactions were stimulated by heating at 95 °C for 1 h and kept at 4 °C immediately. Mixtures were centrifuged at 12,000 x g at 4 °C for 5 min. MDA in supernatant was determined the

fluorescence intensity (FI) at excitation 520 nm emission 549 nm by using spectrofluorometry (RF-1501, Shimadzu, Japan). Experimental data were analyzed as MDA concentration and compared with standard as shown in Figure 27.

3.3.2.2 Determination of tumor necrosis factor (TNF)-α release

HepG2 cells were seeded in 6-wells plate at 10^6 cells/well and incubated in 5% CO_2 incubator at 37 °C for 48 h. After incubation, cells treated with 10 or 30 μg/mL *S. caseolaris* methanolic seed extract for 2 h and followed with 500 mM ethanol. After exposed to ethanol for 24 h, human TNF-α in culture medium was determined by human TNF-α enzyme-linked immunosorbent assay (ELISA) according to PEPROTECH's protocol. ELISA plate was coated by adding capture antibody (primary antibody) 100 μL/well and incubated at room temperature for 1 h. Plate was washed twice by adding 300 μL washing buffer to remove excessive capture antibody. Surface space of plate was blocked by blocking buffer (1% bovine serum albumin (BSA) in PBS) and incubated for at least 2 h. Plate was washed twice to remove excessive BSA.

Twenty five microliter samples or hTNF- α (0, 0.125, 0.25, 0.5, 1, 2 ng/mL) was added and incubated at room temperature for at least 2 h. Plate was washed twice to remove the uncaptured human TNF-α Human TNF-α was captured again by addition of 100 μL detection antibody (secondary antibody) and incubated at room

temperature for 2 h. Plate was washed twice to remove the free secondary antibodies. Then, 100 μL horseradish peroxidase conjugated avidin was added and incubated at room temperature for 30 min. Plate was washed twice to remove the excessive horseradish peroxidase conjugated avidin. The reaction was initiated by addition of 100 μL ABTS substrate into each well and incubated at room temperature for 15 min. The absorbance was measured at the wavelength 405 nm using microplate reader (Fusion^{TM}, A Packard Bioscience Company, USA). Human TNF-α secretion was analyzed as hTNF-α concentration and compared with standard as shown in Figure 28.

3.3.3 The hepatoprotective mechanism study

Normally, the defensive mechanism of cells against oxidative damage involves both enzymatic antioxidants (e.g. SOD, CAT and GPx) and non-enzymatic antioxidants such as glutathione whereas ROS induces a depletion or inactivation antioxidant enzymes and intracellular glutathione. Therefore, this research work investigated hepatoprotective mechanism by determining activity of enzymatic antioxidants and measuring the intracellular GSH level. Enzymatic antioxidants activities were assessed by SOD assay, CAT assay, GPx assay, respectively. The intracellular GSH level was determined by reduced GSH assay.

3.3.3.1 Cells lysate preparation

HepG2 cells were seeded in 6-wells plate at 10^6 cells/well with and incubation in 5% CO_2 incubator at 37 °C for 24 h. After incubation, cells treated with 10, 20 or 30 μg/mL *S. caseolaris* methanolic seed

extract for 2 h and followed with 500 mM ethanol for 24 h. Briefly, culture medium was discarded while cells were lysed by adding 300 µL homogenizing medium that containing PBS (pH 7.4), 0.1% TritonX-100 and 1M phenylmethylsulfonyl fluoride (PMSF). Cells were scraped and collected to 1.5 mL centrifuge tube. Cell suspension was homogenized by Vibra-cell™ VCX130 (Sonics & Materials, Inc., NewTown, USA) 40% mA, 5 s interval and centrifuged at 12,000 x g for 15 min. All processes were conducted at 4°C. Supernatant was used for SOD assay, CAT assay, GPx assay, reduced GSH assay and total protein assay or kept at -80 °C.

3.3.3.2 Superoxide dismutase (SOD)

SOD was analyzed with the previously method according to SIGMA ALDRICH's protocol. Cell lysate was used for SOD assay. Twenty microliters of sample or SOD was added into each well and followed with 200 µL (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST) working solution which consisting of WST and xanthine substrate. Next, 20 µL XOD was added into sample, standard, and blank1 well. The reaction was incubated at 37 °C for 30 minutes. SOD activity was determined by measuring the absorbance at the wavelength 450 nm using microplate reader (Fusion™, A Packard Bioscience Company, USA). Experimental data were analyzed as percentage inhibitory rate and compared with standard as shown in Figure 29. The percentage inhibitory rate in the assay was calculated as follows:

$$\% \text{ inhibitory rate} = \frac{[(A_{450}\text{blank1} - A_{450}\text{blank3}) - (A_{450}\text{sample} - A_{450}\text{blank2})] \times 100}{(A_{450}\text{blank1} - A_{450}\text{blank3})}$$

Note:

Blank 1: 20 μL water + 200 μL WST working solution + 20 μL XOD

Blank 2: 20 μL sample + 200 μL WST working solution + 20 μL dilution buffer

Blank 3: 20 μL water + 200 μL WST working solution + 20 μL dilution buffer

3.3.3.3 Catalase (CAT)

CAT was determined by Amplex® Red Catalase Assay according to Invitrogen's protocol. Cell lysate was used for CAT assay. Twenty five microliters of sample or catalase (0, 1, 2, 3 or 4 U/mL) was added into 96-well falt bottom plate and followed with 25 μL of 40 μM hydrogen peroxide solution. Mixtures were mixed gently and incubated at room temperature for 30 minutes. Fifty microliters of working solution which consisting of 100 μM Amplex Red reagent and 0.4 U/mL horseredish-peroxidase (HRP) in assay buffer was added into each well. Reactions were incubated at 37 °C for 30 minutes with light protection. CAT activity was determined by measuring the absorbance at the wavelength 550 nm using microplate reader (Fusion™, A Packard Bioscience Company, USA). Experimental data were analyzed as CAT activity and compared with standard as shown in Figure 30.

3.3.3.4 Glutathione Peroxidase (GPx)

GPx activity was determined with the previously method according to Cayman's protocol. Twenty microliters of samples was added into

96-well plate. Subsequently, added 100 µL assay buffer that containing 50 mM Tris-HCl, pH 7.6 and 5 mM EDTA. Thereafter, 50 µL co-substrate mixture which consisting of NADPH, glutathione and glutathione reductase was added in each well. Reactions were initiated by adding 20 µL cumene hydroperoxide in each well and mixed gently. GPx activity was determined at the wavelenght 340 nm. NADPH level is measured before and after 4 min incubation with cumene hydroperoxide using microplate reader (Fusion™, A Packard Bioscience Company, USA). Experimental data were analyzed as GPx activity. GPx activity in this assay was calculated as follows:

$$\text{GPx activity (nmol/min/mL)} = \frac{(A_{340}\ \text{time 1} - A_{340}\ \text{time 2}) \times 9.5\ \text{ml}}{(\text{time 2} - \text{time 1})} \times \frac{1}{0.00373\mu M^{-1}}$$

3.3.3.5 Reduced glutathione (GSH)

GSH was estimated according to Quantichrom™'s protocol. One hundred microliters of tartazine was diluted by 200 µL distilled water which its absorbance is equivalent to 100 µM glutathione (positive control). One hundred and twenty microliters of sample was added into 1.5 mL centrifuge tube and followed with 120 µL working reagent containing 5% trichloroacetic acid (TCA) and 5,5'-dithiobis(2-dinitro benzoic acid (DTNB)). Mixtures were centrifuged at 12,000 x g, 4°C for 5 min. Two-hundred microliters of supernatant was added and followed with 100 µL 35 mM NADPH substrate. Reactions were incubated at room temperature for 25 min. GSH level was measured at the wavelength 412 nm using microplate reader (Fusion™, A Packard Bioscience

Company, USA). Experimental data were analyzed as GSH concentration and compared with positive control. GSH concentration in the assay was calculated as follows:

$$\text{GSH concentration } (\mu M) = \frac{(A_{412}\,\text{sample} - A_{412}\,\text{blank})}{(A_{412}\,\text{calibrator} - A_{412}\,\text{blank})} \times 100 \times n$$

Note: n. is the sample dilution factor.

3.3.3.6 Determination of total protein

Total protein of homogenate was determined by Bio-Rad protein assay based on the method of Bradford as described by Bradford (1976). Ten microliters of sample or standard was added into 96-well round bottom plate and followed with 200 μL of 20% v/v Bradford reagent (coomassie blue dye). Mixtures were mixed gently and incubated at room temperature for 20 min. The absorbance was measured at the wavelength 550 nm using microplate reader (Fusion™, A Packard Bioscience Company, USA).

4. Statisticals

All data presented as mean ± standard error (X ± SE). Differences between groups were analyzed by using the one-way analysis of variance (one way ANOVA, Turkey equal variances assumed). Difference was considered to be statistically significant if the probability value was less than 0.01 ($P<0.01$).

CHAPTER 4

Results and discussion

1. Study of ethanol inducing hepatotoxicity in HepG2 cell.

Ethanol is well known as hepatotoxicant contributing to hepatic injury and cell death. Typically, ethanol exhibits the different pathological effect to HepG2 cells. Therefore, the ethanol toxicity was conducted in the HepG2 cells that are treated with ethanol at various concentrations. The ethanol toxicity was evaluated for cell death and cell injury by MTT assay and LDH leakage assay, respectively. HepG2 cells were killed by ethanol in concentration dependent manner. Ethanol at the concentration of 1.70 M caused 50% cell death (Figure 15). Cells were damaged by ethanol in concentration dependent manner but ethanol at higher concentrations than 100 mM interfered the assay of LDH activity. One hundred millimolars ethanol induced cell injury without interfering the assay of LDH activity (Figure 16), this concentration was used to cell injury study. Oxidative damage in HepG2 cells was evaluated by the occurance of lipid peroxidation and the secretion of human tumor necrosis factor (hTNF)-α. The lipid peroxidation was measured by TBARs assay. The human TNF-α secretion was analyzed by ELISA technique. Effect of ethanol toxicity at concentration 100, 250 and 500 mM on the lipid peroxidation and the human TNF-α secretion were investigated. The results showed that 250 mM ethanol generated the highest

amount of lipid peroxidation in cell (Figure 17), whereas ethanol at the concentration of 500 mM induced the highest secretion of human TNF-α level (Figure 18). All these data suggested that different ethanol concentration exhibited different pathological achievement in HepG2 cells. The suitable ethanol concentrations were used for further studies.

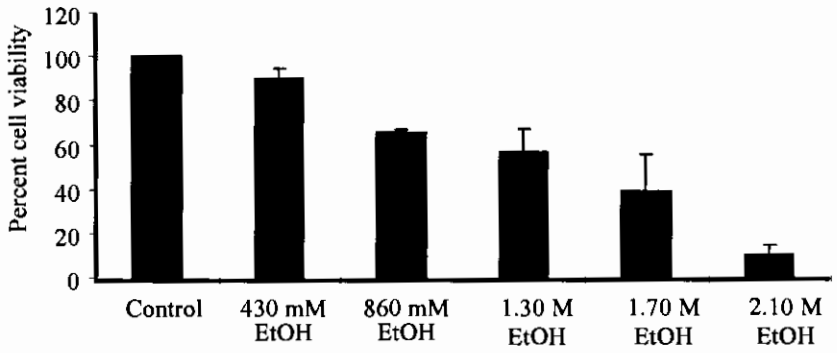

Figure 15 Ethanol toxicity analysis using MTT assay.

The percentages of HepG2 cell viability after treatment with various ethanol concentrations. Values are mean ± SE of two independent experiments (n=3).

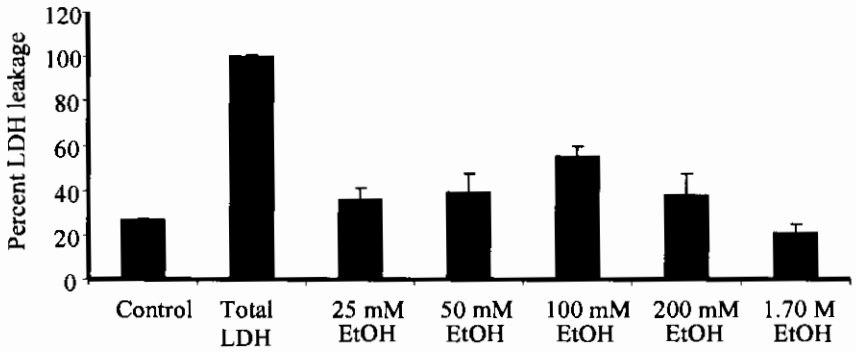

Figure 16 Ethanol toxicity analysis using LDH leakage assay.

The injury of HepG2 cells after treatment with various ethanol concentrations. Values are mean ± SE of three individual samples.

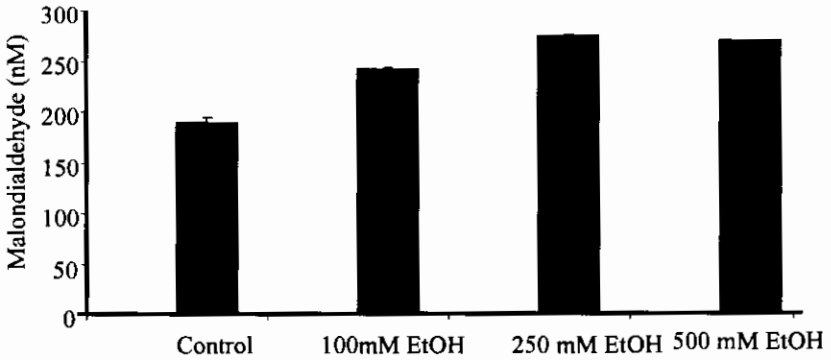

Figure 17 Ethanol toxicity analysis using TBARs assay.

The amount of lipid peroxidation of HepG2 cells after treatment with various ethanol concentrations. Values are mean ± SE of two individual samples.

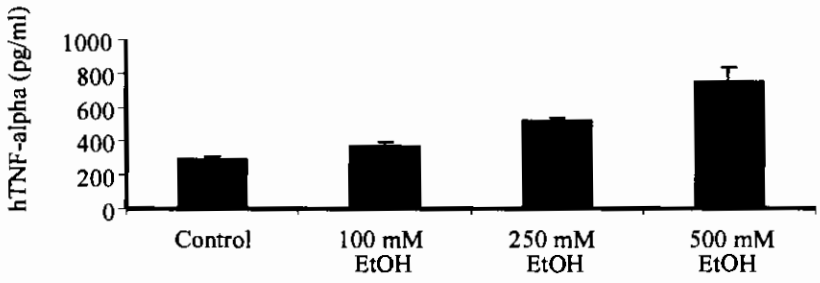

Figure 18 Ethanol toxicity analysis using hTNF-α ELISA.

The amount of hTNF-α secretion of HepG2 cells after ethanol treatment at various ethanol concentrations. Values are mean ± SE of two independent experiments (n=3).

2. Hepatoprotective activity of seed extract from *Sonneratia caseolaris* against ethanol inducing hepatotoxicity in HepG2 cell.

Ethanol is classified as a direct hepatotoxin that its metabolic products have injurious effect on physiochemical of cell membrane. Cell membranes are damaged leading to LDH leakage and triggering cell death (Recknagel *et al.*, 1973). In this study, the hepatoprotective activity of *S. caseolaris* seed extract was investigated. The cell viability was determined by MTT assay and cell injury was evaluated by LDH leakage assay.

The viability of control cells is expressed as 100 %. Cell viability after incubation with 10, 20 and 30 μg/mL *S. caseolaris* extract alone were 100.00 ± 1.85, 100.00 ± 1.37, and 100.00 ± 1.38 %, respectively when compared to that of control cells (Figure 19A). Cell viability was 55.58 ± 3.00% in 1.70 M ethanol treatment alone. While cell viability after treatment with 10, 20 and 30 μg/mL *S. caseolaris* extract and followed with 1.70 M ethanol were 96.26 ± 4.20, 91.10 ± 2.00 and 89.88 ± 0.57 %, respectively when compared to that of control cells as shown in Figure 19B. *Sonneratia caseolaris* extract at concentration 10, 20, and 30 μg/mL alone did not caused cell death indicated that *S. caseolaris* extract at concentration lower than 30 μg/mL was not hepatotoxic. Significantly, *S. caseolaris* extract at concentration 10, 20 and 30μg/mL could raise cell viability against ethanol ($P<0.01$) when compared to ethanol-treated cells. For cell injury, cells exposed to Triton X-100 or total LDH in cell is expressed as 100%.

LDH leakage level of control cells and 100 mM ethanol-treated cells were 27.06 ± 0.21 and 55.48 ± 2.87 % when compared to total LDH in cell. Whereas LDH leakage after exposed to 10, 20, and 30 µg/mL *S. caseolaris* extract alone were 12.97 ± 0.47, 12.23 ± 0.29 and 18.01 ± 1.79%, respectively when compared to that of total LDH in cell (Figure 20A). Cells after treatment with 10, 20, and 30 µg/mL *S. caseolaris* extract and followed with 100 mM ethanol were 14.62 ± 0.65, 12.58 ± 0.54 and 12.38 ± 0.64 %, respectively when compared to that of total LDH in cell (Figure 20B). *Sonneratia caseolaris* extract at concentration 10, 20, and 30 µg/mL was able to reduce LDH leakage significantly ($P<0.01$) when compared to that of ethanol-treated cells. Moreover, hepatotoxic effect of *S. caseolaris* extract was lower than that of cells control. The results suggested that *S. caseolaris* extract treatment could reduce the oxidative damage in normal condition.

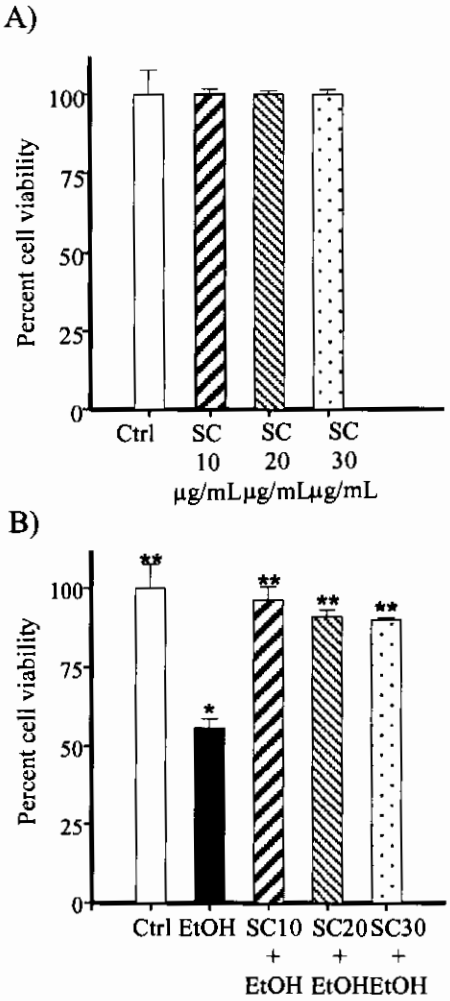

Figure 19 Protective cell death analysis using MTT assay.

A) Cell viability of HepG2 cells after treatment with 10, 20, and 30 µg/mL *S. caseolaris* extract alone. B) Cell viability of HepG2 cells after treatment with *S. caseolaris* extract against 1.70 M ethanol. Viability of unexposed cells is expressed as 100%. Values are mean ± SE of three individual samples.

* $P < 0.01$ significant difference when compared with cells exposed to ethanol.

**$P < 0.01$ significant difference when compared with control cells.

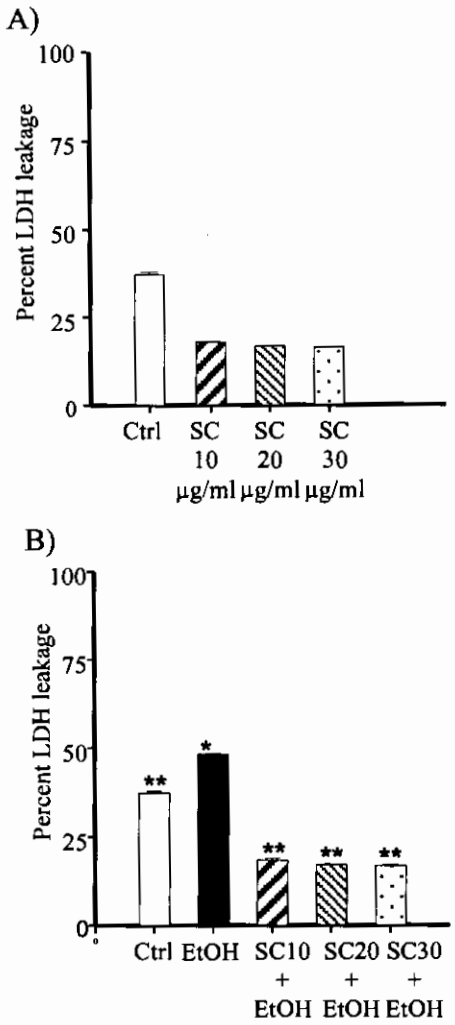

Figure 20 Protective cell injury analysis using LDH leakage assay.

A) LDH leakaged from HepG2 cells after treatment with 10, 20, and 30 µg/mL *S. caseolaris* extract alone. B) LDH leakaged from HepG2 cells after treatment with *S. caseolaris* extract against 100 mM ethanol. Cells exposed to TritonX-100 are expressed as total LDH. Values are mean ± SE of three individual samples.

*$P < 0.01$ significant difference when compared with control cells.

**$P < 0.01$ significant difference when compared with cells exposed to ethanol.

Cell viability after exposure to *S. caseolaris* extract increased significantly over the cell treated with ethanol implying that *S. caseolaris* extract exhibited the hepatoprotective effects against ethanol. Since some flavonoids, luteolin and quercetin have been reported to disrupt MTT assay (Peng *et al.*, 2005). The treatment alone with 10, 20, and 30 μg/mL *S. caseolaris* extract in HepG2 cells were performed and there was no significantly increased formazan production over the cells control. This result was similar to previous study that luteolin and quercetin did not increase MTT reduction at concentrations less than 50 μg/mL (Peng *et al.*, 2005). Moreover, hepatic damage was reduced significantly confirming that *S. caseolaris* extract effectively protected the hepatocyte against ethanol exposure. The results from MTT assay and LDH leakage assay were consistent with the previous study that phenolic compounds including luteolin, luteolin-7-*O*-β glycoside and quercetin could protect cell death against oxidative damage (Lima *et al.*, 2006; Chadan *et al.*, 2008).

The hepatoprotective activity of *S. caseolaris* extract might be due to the action of its chemical constituents. *Sonneratia caseolaris* extract has been reported that it consists of triterpenoids (e.g. betulin, lupeol, oleanolic acid, ursolic acid) and flavonoids such as kaempferol, quercetin, luteolin and luteolin-7-*O*-β-glycoside (Sadhu *et al.*, 2006; Tian *et al.*, 2009). Some triterpenoids found in other plant extracts have shown their cell protective effect against carbon tetrachloride (CCl_4) (Hunan Med. Inst., 1975; Shukla *et al.*, 1992) in rats or parenchymal cell

(Ma *et al.*, 1982). Flavonoids found in other plants phenol have been reported for their cell protective effect against TBHP (Lima *et al.*, 2006) or ethanol (You *et al.*, 2010).

3. Antioxidative activity of seed extract from *S. caseolaris* against ethanol inducing hepatotoxicity in HepG2 cell.

Oxidative stress could be induced by ROS generation when ethanol is metabolized. ROS has been suggested as one of the major mechanisms of ethanol-induced hepatic damages (Cederbaum, 2001). It has been reported that excessive ROS can damage DNA and RNA structure directly (Matalon *et al.*, 2003; von Sonntag, 2006; Barnes *et al.*, 2004) and can initiate the lipid peroxidation. Lipid hydroperoxide (ROOH), malondialdehyde (MDA), 4-hydroxy-2-trans-noetal (HNE), etc. are lipid peroxidation products which damage endoplasmic reticulum (ER), golgi apparatus, immune system (Hsieh *et al.*, 2005), mitochondria and trigger cell death (Parola *et al.*, 1999). These lipid peroxidation products represent the amount of lipid peroxidation. TNF-α involves in promoting the increase of nitric oxide synthases (iNOS) enzyme that increases the phagocyte ROS production. Therefore, one of the possible hepatoprotective activity of *S. caseolaris* extract might be owing to the antioxidative activity. The effects of *S. caseolaris* extract against ethanol toxicity on the amount of lipid peroxidation and hTNF-α secretion were investigated. The amount of lipid peroxidation was assessed by TBARs assay whereas hTNF-α secretion was determined by ELISA technique.

The amount of lipid peroxidation of cells after exposed to 10 and 30 μg/mL *S. caseolaris* extract was 121.88 ± 0.11 and 99.38 ± 0.17 nM, respectively (Figure 21A). Control cells and ethanol-treated cells had 215.08 ± 0.68 and

250.95 ± 0.55 nM of lipid peroxidation, respectively. The amount of lipid peroxidation of cells after incubation with 10 and 30 μg/mL of *S. caseolaris* extract against 250 mM ethanol were 127.27 ± 0.10 and 112.82 ± 0.04 nM (Figure 21B). *Sonneratia caseolaris* extract at concentration 10 and 30 μg/mL could decrease the amount of lipid peroxidation significantly ($P<0.01$) when compared to that of ethanol-treated cells. The amount of hTNF-α secretion of cells after treated with 10 and 30 μg/mL *S. caseolaris* extract alone were 1,920.57 ± 126.09 and 549.04 ± 44.54 pg/mL. The amount of hTNF-α secretion of control cells and ethanol-treated cells were 1,217.45 ± 146.05 and 2,881.22 ± 70.89 pg/mL (Figure 22A). The amount of hTNF-α secretion of cells treated with 10 and 30 μg/mL *S. caseolaris* extract and followed with 500 mM ethanol were 2,390.77 ± 172.02 and 1,298.47 ± 55.88 pg/mL (Figure 22B). *Sonneratia caseolaris* extract especially at the concentration of 30 μg/mL could reduce the hTNF-α secretion significantly ($P<0.01$). Surprisingly, lipid peroxidation and hTNF-α secretion of *S. caseolaris* extract treated cells were lower than that of control cells. This suggested that *S. caseolaris* treatment might reduce the oxidative stress in normal condition.

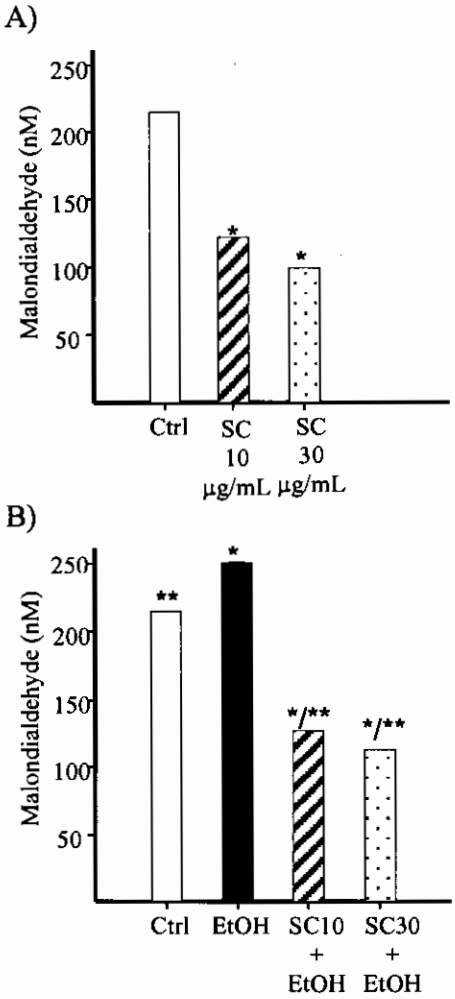

Figure 21 Lipid peroxidation analysis using TBARs assay.

A) The amount of malondialdehyde of HepG2 cells after treatment with 10 and 30 µg/mL *S. caseolaris* extract alone. B) The amount of malondialdehyde of HepG2 cells after treatment with *S. caseolaris* extract against 250 mM ethanol. Values are mean ± SE of three individual samples.

*$P < 0.01$ significant difference when compared with control cells.

**$P < 0.01$ significant difference when compared with cells exposed to ethanol.

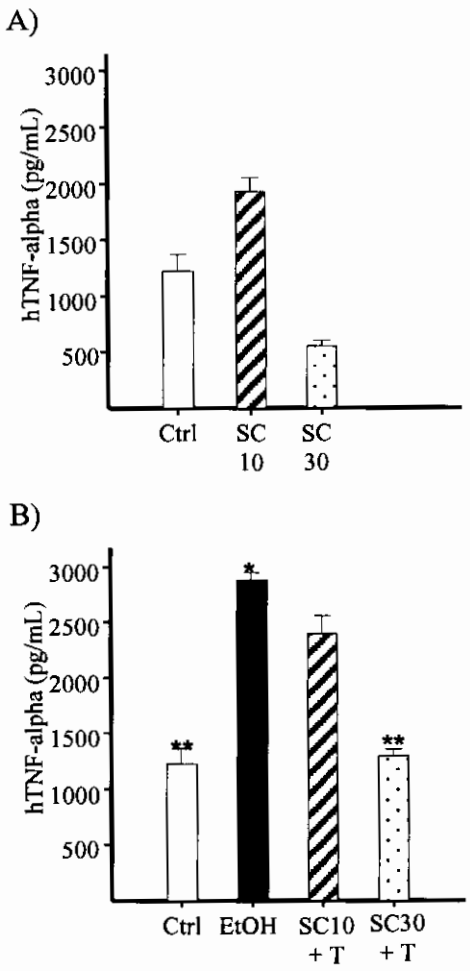

Figure 22 hTNF-α secretion analysis using TBARs assay.

A) The amount of hTNF-α secretion from HepG2 cells after treatment with 10 and 30 μg/mL *S. caseolaris* extract alone. B) The amount of hTNF-α secretion from HepG2 cells after treatment with *S. caseolaris* extract against 500 mM ethanol. Values are mean ± SE of three individuals samples.

*$P < 0.01$ significant difference when compared with control cells.

**$P < 0.01$ significant difference when compared with cells exposed to ethanol.

The amount of lipid peroxidation of cells treated with *S. caseolaris* extract were less than ethanol treated cells, suggesting that *S. caseolaris* extract exerted antioxidative activity by inhibiting the lipid peroxidation. This data is similar to previous study that several phenolic compounds were able to reduce production of MDA (Chandan *et al.*, 2008; Hsu *et al.*, 2008). However, several compounds other than MDA could react with TBA and cause false chromogens. Measurement of $(TBA)_2$-MDA in this study evaluated by measuring fluorescence intensity that could distinguish other products from MDA complex as decribed by Gutteridge and Quinlan (1983). While the amount of hTNF-α secretion of cells treated with *S. caseolaris* was reduced significantly, assuring that *S. caseolaris* extract possessed the antioxidative effect. This result is consistency with the previous study that quercetin could inhibit the TNF-α production at the concentrations of 125, 250, and 500 μM (Wang *et al.*, 2002). Since *S. caseolaris* extract composed of flavonoids and triterpenoids (Sadhu *et al.*, 2006; Tian *et al.*, 2009).

Flavonoids are well recognized as phenolic compounds that inhibited the lipid peroxidation, chelated metal ions (Rice-Evans *et al.*, 1996; Lima *et al.*, 2006) or inhibited hTNF-α secretion (Amat *et al.*, 2010). Some triterpenoids such as ursolic acid and oleanolic acid have been reported that they inhibited CYP450 activities (Kim *et al.*, 2004). This findinding supported that antioxidative effect was one of hepatoprotective mechanisms of *S. caseolaris* extract against ethanol toxicity.

4. Effects of *Sonneratia caseolaris* on the liver enzymatic antioxidants and the reduced glutathione levels in ethanol-induced acute hepatic injury in HepG2 cells.

Normally, the defensive mechanism of cells against oxidative damage involves both enzymatic antioxidants e.g. SOD, CAT and GPx and non-enzymatic antioxidants such as glutathione (Sies, 1997) whereas ROS induces a depletion or inactivation antioxidant enzymes and intracellular GSH (Lieber, 1997). From our previous results indicated that hepatoprotective activity of *S. caseolaris* might be owing to antioxidative effects of *S. caseolaris* extract against ethanol toxicity. Therefore, this research work investigated hepatoprotective mechanisms of *S. caseolaris* extract by determining activities of enzymatic antioxidants and intracellular GSH level. Superoxide dismutase, catalase, glutathione peroxidase specific activities and intracellular reduced glutathione level were evaluated by spectophotometric methods.

Superoxide dismutase activities of cells after exposed to 10, 20, and 30 μg/mL *S. caseolaris* extract alone were 98.93 ± 0.44, 94.50 ± 3.88, and 88.90 ± 4.79 U/mL/mg protein, respectively. SOD activity of control cells was 111.49 ± 1.69 U/mL/mg protein and ethanol-treated cells was 96.11 ± 2.76 U/mL/mg protein (Figure 23A). Ethanol at the concentration of 500 mM partially inactivated SOD activity when compared with that of cells control. The activities of SOD of cells treated with 10, 20, and 30 μg/mL of *S. caseolaris* extract and followed with

500 mM ethanol were 118.06 ± 6.94, 111.66 ± 2.28 and 73.97 ± 1.09 U/mL/mg protein, respectively (Figure 23B). *Sonneratia caseolaris* extract at the concentration of 30 μg/mL partially inactivated SOD activity when compared to that of ethanol-treated cells.

Catalase activities of cells after exposed to 10, 20, and 30 μg/mL *S. caseolaris* extract alone were 1,211.75 ± 59.02, 1,343.50 ± 48.04 and 1,497.34 ± 10.20 μmol/min/mL/mg protein, respectively. CAT activities of control cells and ethanol-treated cells were 1,027.05 ± 19.40 and 1,179.78 ± 51.85 μmol/min/mL/mg protein, respectively (Figure 24A). Ethanol at the concentration of 500 mM activated CAT activity nonsignificantly when compared to that of cells control. CAT activities of cells treated with 10, 20, and 30 μg/mL *S. caseolaris* extract and followed with 500 mM ethanol were 1,134.07 ± 0.00, 1,477.81 ± 7.91 and 1,780.68 ± 13.21 μmol/min/mL/mg protein, repectively (Figure 24B). *Sonneratia caseolaris* extract especially at the concentration of 20 and 30 μg/mL elevated CAT activity significantly ($P<0.01$) when compared to that of ethanol-treated cells.

Glutathione peroxidase activities of HepG2 cells after exposed to 10, 20, and 30 μg/mL *S. caseolaris* extract alone were 1242.69 ± 16.57, 850.02 ± 27.42 and 944.97 ± 37.80 pmol/min/mL/mg protein, respectively. The level of glutathione peroxidase in control cells and ethanol-treated group were 266.62 ± 0.00 and 448.95 ± 16.63 pmol/min/mL/mg protein (Figure 25A). It seem that ethanol at the concentration of 500 mM slightly inactivated GPx specific activity

compared to control cells. The GPx activities of 10, 20, and 30 μg/mL *S. caseolaris* extract treated cells against 500 mM ethanol were 732.68 ± 35.74, 1107.39 ± 48.15 and 2266.62 ± 26.06 pmol/min/mL/mg protein, repectively (Figure 25B). *Sonneratia caseolaris* extract treatment at the concentration of 10, 20, and 30 μg/mL are able to increase GPx activity in HepG2 cells significantly ($P<0.01$) when compared to that of ethanol-treated cells.

The intracellular GSH level after incubation with 10, 20, and 30 μg/mL *S. caseolaris* extract alone were 129.71 ± 14.93, 146.63 ± 25.06 and 85.31 ± 4.11 μM/mg protein, respectively. The amount of GSH in control cells and ethanol-treated cells were 94.10 ± 0.91 and 84.87 ± 5.05 μM/mg protein (Figure 26A). Ethanol at the concentration of 500 mM did not induce intracellular GSH depletion when compared with that of cells control. The intracellular GSH level of cells after exposed to 10, 20, and 30 μg/mL *S. caseolaris* extract and followed with 500 mM ethanol were 98.15 ± 7.47, 124.62 ± 11.20 and 183.91 ± 3.31 μM/mg protein, respectively (Figure 26B). *Sonneratia caseolaris* extract treatment at the concentration of 30 μg/mL significantly increased the intracellular GSH level ($P<0.01$) when compared to that of ethanol-treated cells. Moreover, CAT and GPx activities and intracellular GSH level of *S. caseolaris* extract treated cell were higher than that of cells control. These results are consistent with our previous results that *S. caseolaris* extract treatment reduced the oxidative damage in normal condition.

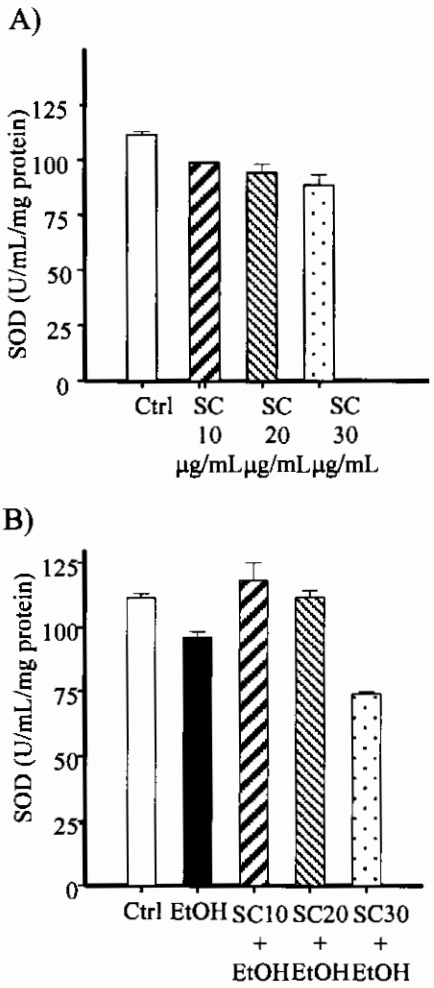

Figure 23 Superoxide dismutase activity analysis using SOD assay.

A) Superoxide dismutase activity of HepG2 cells after treatment with 10, 20, and 30 μg/mL *S. caseolaris* extract alone. B) Superoxide dismutase activity of HepG2 cells after treatment with *S. caseolaris* extract against 500 mM ethanol. Values are mean ± SE of two individuals samples.

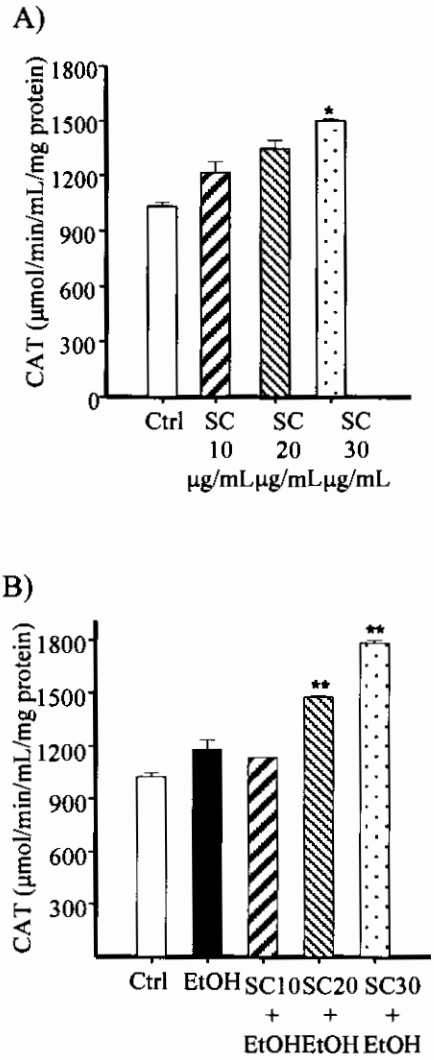

Figure 24 Catalase activity analysis using CAT assay.

A) Catalase activity of HepG2 cells after treatment with 10, 20, and 30 µg/mL *S. caseolaris* extract alone. B) Catalase activity of *S. caseolaris* extract against 500 mM ethanol. Catalase activity was assessed by CAT assay. Values are mean ± SE of two individuals samples.

*$P < 0.01$ significant difference when compared with cells control.

**$P < 0.01$ significant difference when compared with cells exposed to ethanol.

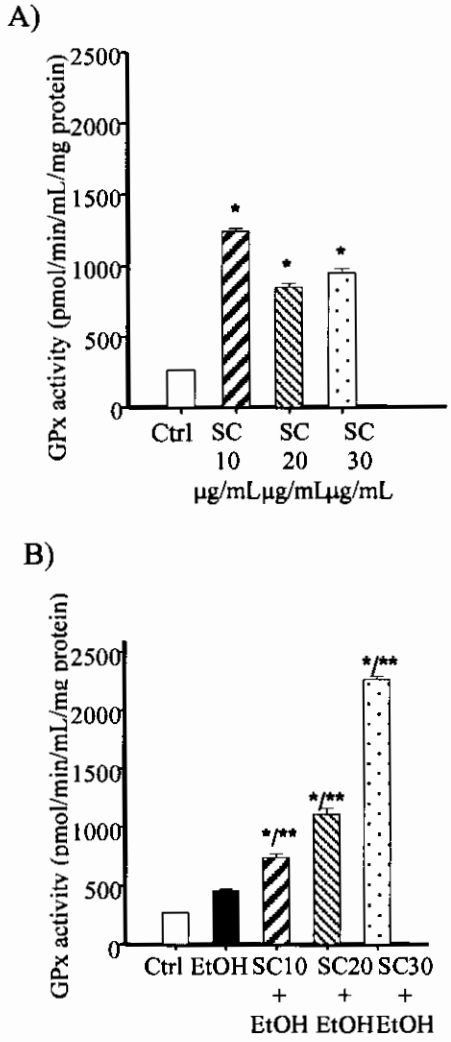

Figure 25 Glutathione peroxidase activity analysis using GPx assay.

A) Glutathione peroxidase activity of HepG2 cells after treatment with 10, 20, and 30 µg/mL *S. caseolaris* extract alone. B) Glutathione peroxidase activity of *S. caseolaris* extract against 500 mM ethanol. Values are mean ± SE of two individuals samples.

*P < 0.01 significant difference when compared with cells control.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

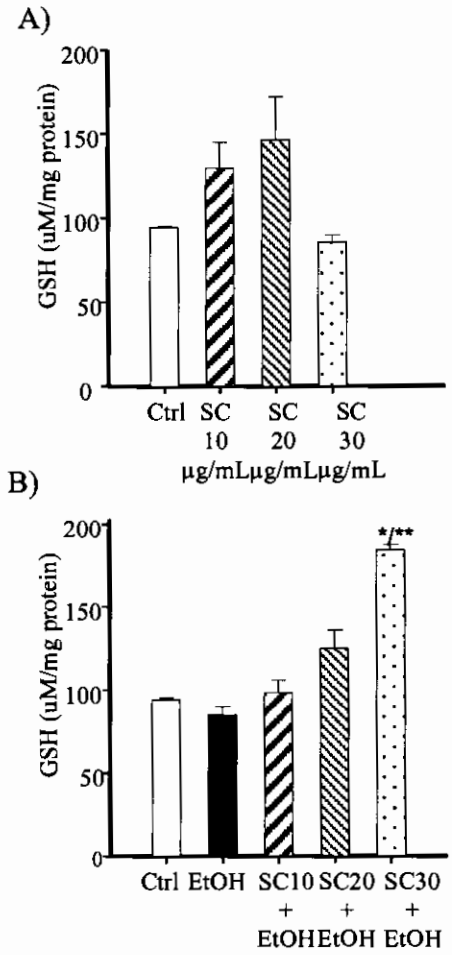

Figure 26 Quantitative of reduced glutathione analysis using GSH assay.

A) The intracellular GSH level of HepG2 cells after treatment with 10, 20, and 30 µg/mL *S. caseolaris* extract alone. B) The intracellular GSH level of *S. caseolaris* extract against 500 mM ethanol. Values are mean ± SE of two individuals samples.

*P < 0.01 significant difference when compared with cells control.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Ethanol partially inactivated SOD activity and caused GSH depletion but slightly activated CAT and GPx activity. These results did not correlate to previous studies that ethanol inactivated CAT and GPx activities (Balasubramaniyan *et al.*, 2003; You *et al.*, 2010). The activation of the antioxidant enzymes might be occurred in an oxidative stress condition that cells defensed against oxidative damage (Shankar *et al.*, 2008). The activites of CAT and GPx and intracellular GSH level after treatment with *S. caseolaris* extract were promoted higher than cell treated with ethanol indicated that some hepatoprotective mechanisms of *S. caseolaris* treatment activated activities of some antioxidant enzymes and prevented the intracellular GSH depletion. These results are similar to the previous study which has been claimed that some phenolic compounds are able to activate CAT activity and GPx activity (Shankar *et al.*, 2008), and to raise the intracellular GSH level against oxidative damage (Lima *et al.*, 2006).

Our observation demonstrated that *S. caseolaris* extract treatment improved the impaired the antioxidant enzymes and intracellular GSH level against ethanol in HepG2 cells, as shown by promotion of some antioxidant enzymes activities and GSH level. This effect of *S. caseolaris* extract might be owing to the action of flavonoids such as kaempferol, quercetin, luteolin, luteolin-7-*O*-β-glycoside and triterpenoids (e.g. betulin, lupeol, oleanolic acid, ursolic acid) (Sadhu *et al.*, 2006; Tian *et al.*, 2009). The protective effects of some flavonoids

against oxidative stress have been reported. For example quercetin, luteolin and luteolin-7-*O*-β-glycoside served as ROS scavenger (Rodriguez-Fragoso *et al.*, 2008) and prevented the GSH depletion caused by TBHP-induced liver damage (Lima *et al.*, 2006). While the hepatoprotective effects of triterpenoids against hepatotoxic agents were evident. For example ursolic acid and oleanolic acid acted as CYP450 activities inhibition (Kim *et al.*, 2004) and lupeol was able to restore the enzymatic antioxidants and promote the non-enzymatic antioxidants (Prasad *et al.*, 2007).

CHAPTER 5

Conclusions

The purpose of this research work is to study the hepatoprotective effects and antioxidative activity of *S. caseolaris* seed extract. The hepatoprotective activity was determined by MTT assay and LDH leakage assay. The MTT results from MTT assay indicated that *S. caseolaris* exract could protect the cell death whereas the LDH leakage results confirmed that the effective protection against the hepatic injury caused by ethanol.

Since *S. caseolaris* extract is composed of flavonoids and triterpenoids which have been reported their antioxidative effects and hepatoprotective activity. Therefore, some hepatoprotective activity of *S. caseolaris* extract might be contributed to the antioxidative effects. The antioxidative activities were investigated by evaluating the lipid peroxidation determination and measuring the hTNF-α secretion. The lipid peroxidation and the hTNF-α secretion were reduced after treatment with *S. caseolaris* extract. Therefore one hepatoprotective mechanism of *S. caseolaris* extract against ethanol might be owing to antioxidative effect.

The defensive mechanism of cell against the oxidative stress involves enzymatic antioxidants (e.g. SOD, CAT, GPx) and non-enzymatic antioxidants such as GSH. Consequently, this research work investigated hepatoprotective mechanisms of *S. caseolaris* methanolic seed extract by determining

activities of enzymatic antioxidants and intracellular GSH level. *Sonneratia caseolaris* extract promoted CAT, GPx activities and raised the intracellular GSH level. These data suggested hepatoprotective mechanism of *S. caseolaris* extract were activated the activities of CAT and GPx and prevented the intracellular GSH depletion.

Moreover, cell damage, the amount of lipid peroxidation in cell and the hTNF-α secretion after treatment with *S. caseolaris* extract alone were reduced whereas CAT and GPx activities and intracellular GSH level were promoted. These data indicated that *S. caseolaris* extract could reduce the oxidative stress in normal condition.

In this study, *S. caseolaris* extract exhibited the hepatoprotective activity against ethanol by inhibiting lipid peroxidation, inhibiting hTNF-α production, activating some antioxidant enzymes and preventing GSH depletion. For further study, *S. caseolaris* extract should be investigated the other hepatoprotective mechanisms and its toxicity before further development to protect or cure the liver disease.

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Appendix

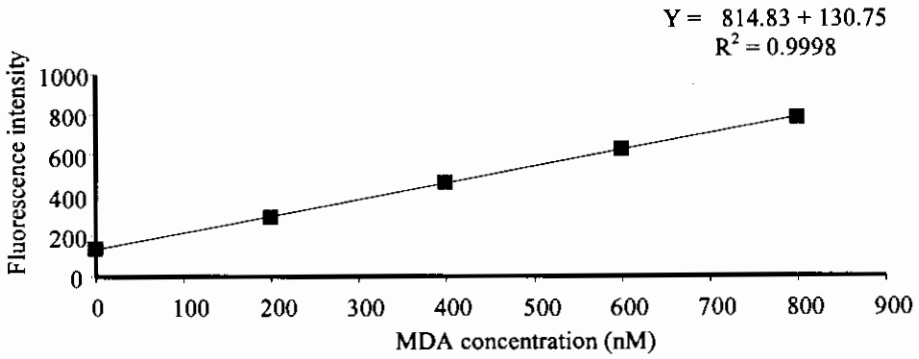

Figure 27 Standard curve of MDA (n=3).

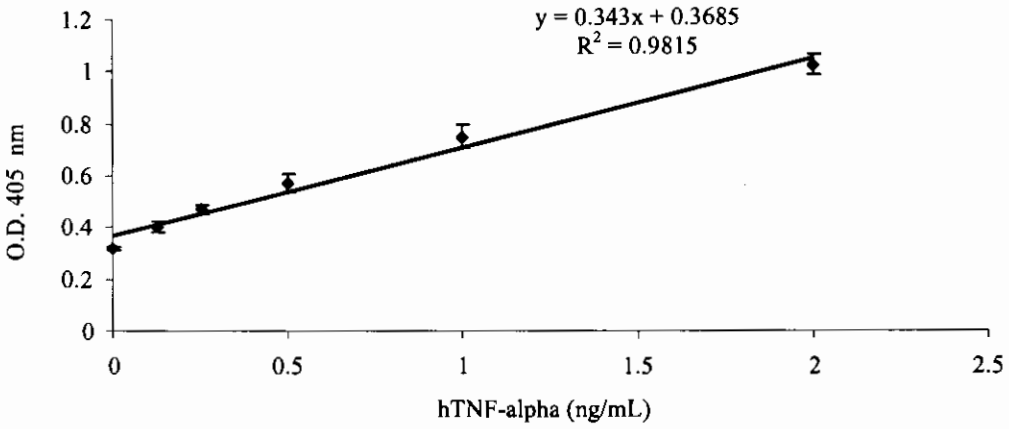

Figure 28 Standard curve of hTNF-α (n=3).

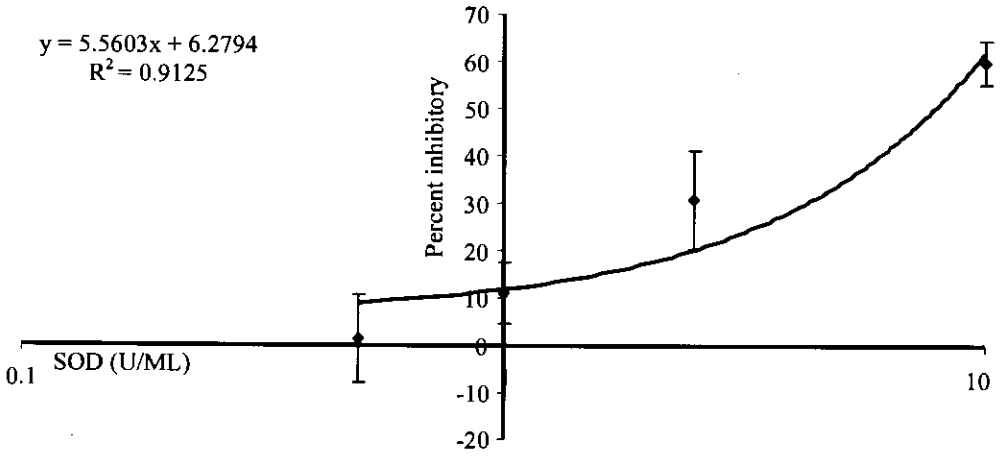

Figure 29 Standard curve of superoxide dismutase activity (n=3).

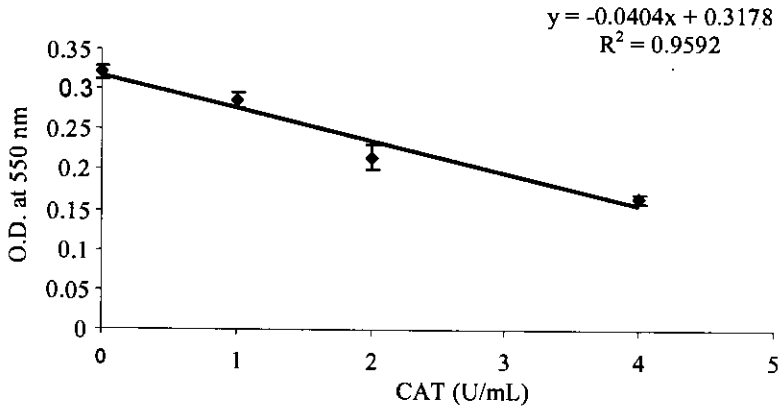

Figure 30 Standard curve of catalase (n=3).

Table 5 Protective cell cell death analysis using MTT assay

Cell viability of HepG2 after treated with *S. caseolaris* methanolic seed extract and followed with 1.7 M ethanol. Cell viability was determined by MTT assay.

	Percent cell viability (Mean ± SE)		
	1	**2**	**3**
Control (unexposed cells)	100.00 ± 7.72**	100 ± 9.00**	100 ± 8.10**
1.70 M Ethanol	46.57 ± 4.22*	53.86 ± 0.57*	57.86 ± 1.54*
S. caseolaris 10 µg/mL	100.00 ± 1.85	100.00 ± 1.71	100.00 ± 4.10
S. caseolaris 20 µg/mL	100.00 ± 1.37	100.00 ± 1.80	100.00 ± 4.92
S. caseolaris 30 µg/mL	100.00 ± 1.38	100.00 ± 2.11	100.00 ± 0.98
S. caseolaris 10 µg/mL + 1.70 M Ethanol	96.26 ± 4.20**	87.47 ± 1.98**	98.18 ± 2.90**
S. caseolaris 20 µg/mL + 1.70 M Ethanol	91.10 ± 2.00**	97.92 ± 2.92**	95.12 ± 9.63**
S. caseolaris 30 µg/mL + 1.70 M Ethanol	89.88 ± 0.57**	87.71 ± 3.11**	89.58 ± 4.95**

*P < 0.01 significant difference when compared with unexposed cell.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Table 6 Protective cell injury using LDH leakage assay.

Cell injury of HepG2 after treated with *S. caseolaris* methanolic seed extract and followed with 100 mM ethanol. Cell injury was evaluated by LDH leakage assay. Cells exposed to TritonX-100 are expressed as 100% (total LDH).

	Percent LDH leakage (Mean ± SE)		
	1	**2**	**3**
Control (unexposed cells)	27.06 ± 0.21**	37.35 ± 0.60**	20.33 ± 0.07**
100 mM Ethanol	55.48 ± 2.87*	48.33 ± 0.28*	47.88 ± 3.06*
S. caseolaris 10 µg/mL	12.97 ± 0.47	18.25 ± 0.63	11.39 ± 0.16
S. caseolaris 20 µg/mL	12.23 ± 0.29	17.14 ± 0.16	10.29 ± 0.12
S. caseolaris 30 µg/mL	18.01 ± 1.79	16.82 ± 0.26	11.77 ± 0.38
S. caseolaris 10 µg/mL + 100 mM Ethanol	14.62 ± 0.65**	18.25 ± 0.63**	20.12 ± 0.29**
S. caseolaris 20 µg/mL + 100 mM Ethanol	12.58 ± 0.54**	17.14 ± 0.16**	21.22 ± 0.66**
S. caseolaris 30 µg/mL + 100 mM Ethanol	12.38 ± 0.64**	16.82 ± 0.26**	25.17 ± 0.65**

*P < 0.01 significant difference when compared with unexposed cell.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Table 7 Lipid peroxidation analysis using TBARs assay.

Lipid peroxidation of HepG2 after treated with *S. caseolaris* methanolic seed extract and followed with 250 mM ethanol. Lipid peroxidation was assessed by TBARs assay.

	Malondialdehyde nM (Mean ± SE)		
	1	**2**	**3**
Control (unexposed cells)	215.08 ± 0.68**	215.64 ± 4.97**	631.14 ± 0.03**
250 mM Ethanol	250.95 ± 0.55*	248.92 ± 1.79*	806.22 ± 0.31*
S. caseolaris 10 µg/mL	121.88 ± 0.11*	139.34 ± 1.74*	269.14 ± 0.13*
S. caseolaris 30 µg/mL	99.38 ± 0.17*	135.82 ± 0.76*	224.42 ± 0.07*
S. caseolaris 10 µg/mL + 250 mM Ethanol	127.27 ± 0.10*,**	145.03 ± 4.31*,**	238.77 ± 0.70*,**
S. caseolaris 30 µg/mL + 250 mM Ethanol	112.82 ± 0.04*,**	135.81 ± 2.13*,**	238.14 ± 0.19*,**

*P < 0.01 significant difference when compared with unexposed cell.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Table 8 hTNF- α secretion analysis using hTNF- α ELISA.

hTNF-α release of HepG2 after treated with *S. caseolaris* methanolic seed extract and followed with 500 mM ethanol. hTNF-α release was determined by ELISA.

	hTNF-α release pg/mL (Mean ± SE)		
	1	**2**	**3**
Control	1217.45 ± 146.05**	1963.29 ± 84.13**	1889.23 ± 132.26**
500 mM Ethanol	2881.22 ± 70.89*	2806.95 ± 6.67*	3502.92 ± 25.14*
S. caseolaris 10 µg/mL	1920.57 ± 126.09	2116.26 ± 17.08	2617.59 ± 47.74*
S. caseolaris 30 µg/mL	549.04 ± 44.55	867.22 ± 25.08*	959.67 ± 18.54*
S. caseolaris 10 µg/mL + 500 mM Ethanol	2390.77 ± 172.02	2039.01 ± 56.07**	2630.22 ± 82.40**
S. caseolaris 30 µg/mL + 500 mM Ethanol	1298.47 ± 55.88**	921.52 ± 28.75**	1259.96 ± 40.41**

*P < 0.01 significant difference when compared with unexposed cell.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Table 9 Superoxide dismutase activity analysis using SOD assay.

SOD activity of HepG2 after treated with *S. caseolaris* methanolic seed extract and followed with 500 mM ethanol. SOD activity was determined by SODs assay.

	SOD U/mL/mg protein (Mean ± SE)		
	1	**2**	**3**
Control (unexposed cells)	111.49 ± 1.69	92.07± 2.72	64.39± 0.60
500 mM Ethanol	96.11 ± 2.76	87.68 ± 8.09	63.55 ± 1.11
S. caseolaris 10 µg/mL	98.93 ± 0.44	103.73 ± 5.57	69.05 ± 0.67
S. caseolaris 20 µg/mL	94.50 ± 3.88	99.85 ± 1.72	71.41 ± 0.36
S. caseolaris 30 µg/mL	88.90 ± 4.79	85.60 ± 0.13	58.37 ± 0.39
S. caseolaris 10 µg/mL + 500 mM Ethanol	118.06 ± 6.94	100.35 ± 8.26	56.15 ± 1.21
S. caseolaris 20 µg/mL + 500 mM Ethanol	111.66 ± 2.28	106.74 ± 15.54	49.76 ± 4.37
S. caseolaris 30 µg/mL + 500 mM Ethanol	73.97 ± 1.09	80.13 ± 1.09	44.82 ± 3.01**

*P < 0.01 significant difference when compared with unexposed cell.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Table 10 Catalase activity analysis using CAT assay.

CAT activity of HepG2 after treated with *S. caseolaris* methanolic seed extract and followed with 500 mM ethanol. CAT activity was determined by CAT assay.

	CAT µmol/min/mL/mg protein (Mean ± SE)		
	1	**2**	**3**
Control (unexposed cells)	1027.05 ± 19.40	801.26 ± 4.94	1156.06 ± 16.18
500 mM Ethanol	1179.78 ± 51.85	749.87 ± 14.70	1191.12 ± 5.36
S. caseolaris 10 µg/mL	1211.75 ± 59.02	747.12 ± 38.35	1081.31 ± 2.61
S. caseolaris 20 µg/mL	1343.50 ± 48.04	498.97 ± 29.05*	1228.10 ± 15.37*
S. caseolaris 30 µg/mL	1497.34 ± 10.20	516.09 ± 19.14*	1212.01 ± 40.11*
S. caseolaris 10 µg/mL + 500 mM Ethanol	1134.07 ± 0.00	810.92 ± 5.45	912.18 ± 35.24
S. caseolaris 20 µg/mL + 500 mM Ethanol	1477.81 ± 7.91**	863.00 ± 5.83	1329.67 ± 0.00
S. caseolaris 30 µg/mL + 500 mM Ethanol	1780.68 ± 13.21**	1065.45 ± 18.00**	1555.66 ± 12.36**

*P < 0.01 significant difference when compared with unexposed cell.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Table 11 Glutathione peroxidase activity analysis using GPx assay.

GPx activity of HepG2 after treated with *S. caseolaris* methanolic seed extract and followed with 500 mM ethanol. GPx activity was determined by GPx assay.

	GPx pmol/min/mL/mg protein (Mean ± SE)		
	1	**2**	**3**
Control (unexposed cells)	266.62 ± 0.00	266.35 ± 14.80	206.09 ± 27.22
500 mM Ethanol	448.95 ± 16.63	613.78 ± 33.18	326.02 ± 12.08
S. caseolaris 10 µg/mL	1242.69 ±16.57*	1259.86 ± 41.72*	366.03 ± 38.53*
S. caseolaris 20 µg/mL	850.02 ± 27.42*	977.62 ± 54.31*	262.37 ± 0.00
S. caseolaris 30 µg/mL	944.97 ± 37.80*	857.37 ± 19.06*	655.83 ± 4.41*
S. caseolaris 10 µg/mL + 500 mM Ethanol	732.68 ± 35.74**	749.21 ± 9.03	284.66 ± 24.40
S. caseolaris 20 µg/mL + 500 mM Ethanol	1107.39 ± 48.15**	1062.53 ± 49.04**	360.97 ± 8.40
S. caseolaris 30 µg/mL + 500 mM Ethanol	2266.62 ± 26.06**	2216.36 ± 119.45**	852.24 ± 17.94**

*P < 0.01 significant difference when compared with unexposed cell.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Table 12 Quantitative of reduced glutathione analysis using GSH assay.

The intracellular GSH of HepG2 after treated with *S. caseolaris* methanolic seed extract and followed with 500 mM ethanol. GSH was determined by GSH assay.

	GSH µM/mg protein (Mean ± SE)		
	1	**2**	**3**
Control (unexposed cells)	94.10 ± 0.91	117.90 ± 6.74*	263.33 ± 1.92**
500 mM Ethanol	84.87 ± 5.05	96.81 ± 0.66**	168.59 ± 2.42*
S. caseolaris 10 µg/mL	129.71 ± 14.93	147.08 ± 2.62	176.53 ± 9.18*
S. caseolaris 20 µg/mL	146.63 ± 25.06	131.26 ± 0.00	192.03 ± 5.31*
S. caseolaris 30 µg/mL	85.31 ± 4.11	137.42 ± 5.06	254.23 ± 3.24
S. caseolaris 10 µg/mL + 500 mM Ethanol	98.15 ± 7.47	118.12 ± 3.0*	252.78 ± 1.85*
S. caseolaris 20 µg/mL + 500 mM Ethanol	124.62 ± 11.20**	128.65 ± 0.80**	245.84 ± 0.00*
S. caseolaris 30 µg/mL + 500 mM Ethanol	183.91 ± 3.31**	173.47 ± 6.31**	355.31 ± 2.95*

*P < 0.01 significant difference when compared with unexposed cell.

**P < 0.01 significant difference when compared with cells exposed to ethanol.

Minimum Essential Medium (MEM)

Dissolve 9.5000 g of MEM and 1.5000 g of sodium bicarbonate ($NaHCO_3$) in 1 L of distilled water (DW). Adjust pH to 7.4 with 1 N sodium hydroxide (NaOH) or concentrate hydrochloric acid (HCl). Add 2 mM of glutamine and sterile by filter through 0.22 μm membrane filter. Store at 4 °C.

Phosphate Buffer Solution (PBS)

Dissolve 8.0000 g of sodium chloride (NaCl), 0.2030 g of potassium chloride (KCl), 0.6140 g of sodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) and 0.1900 g of potassium dihydrogen phosphate in 1 L of DW. Adjust pH to 7.4 with 1 N NaOH or concentrate HCl and sterile at 121°C for 20 minutes. Store at room temperature.

MTT solution

Dissolve 0.2500 g of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in 50 mL of PBS and sterile by filter through 0.22 μm membrane filter. Store at -20 °C.

0.5 M Tris-HCl buffer

Dissolve 12.1100 g of Tris-base in 200 mL of DW. Adjust pH to 6.8 with 1 N NaOH or concentrate HCl and sterile at 121°C for 20 minutes. Store at room temperature.

6X SDS Lysis Buffer

Dissolve 0.6000 g of sodium dodecyl sulfate (SDS) in 3.6 mL of 0.5 M Tris-HCl. Adjust volume to 1 mL with reverse osmosis (RO) water.

Thiobarbituric acid reagents (TBA)

Dissolve 0.0670 g of thiobarbituric acid in 10 mL of DW.

Blocking buffer (for ELISA)

Dissolve 1.0000 g of bovine serum albumin (BSA) in 100 mL of PBS and sterile by filter through 0.22 μm membrane filter.

Dilution buffer (for ELISA)

Dissolve 0.1000 g of BSA in 150 mL PBS and add 75 μL of polysorbate-20. sterile diluent buffer by filter through 0.22 μm membrane filter.

Washing buffer (for ELISA)

Add 375 μL of polysorbate-20 in PBS and adjust volume to 750 mL.

10X Phenylmethylsulfonyl fluoride (PMSF) solution

Dissolve 0.1740 g of PMSF in 10 mL of isopropanolol.

List of abbreviations

A/OD	absorbance/ optical density
ABTS	2,2'-azino-di-[3-ethylbenzthiazoline -6-sulfonic acid]-diammonium salt
ADCC	antibody-dependent cell-mediated cytotoxicity
ALD	aldehyde dehydrogenase
Ald	alcoholic liver disease
Amplex red	10-acetyl-3,7-dihydroxyphenoxazine
AOX	aldehyde oxidase
ATP	adenosine triphosphate
°C	celcius
CAT	catalase
COX	cycloxygenase
CYP	cytochrome P
DMSO	dimethyl sulfoxide
DNA	deoxyribose nucleic acid
DTNB	(5,5'-dithiobis(2-nitrobenzoic acid))
EDTA	ethylenediaminetetraacetic acid
ELISA	enyme-linked immunosorbent assay
EtOH	ethanol
FA	fatty acids

FBS	fetal bovine serum
FI	fluorescence intensity
FTase	farnesyltransferase
g	gravity
GPx	glutathione peroxidase
GSH	reduced glutathione
GSR	glutathione reductase
GSSG	oxidized glutathione
GST	glutathione-s-transferase
HepG2	human hepatocellular carcinoma
4-HNE	4-hydroxy-2-trans-noetal
HRP	horseradish peroxidase
hTNF-α	human tumor necrosis factor- α
IL	interleukin
iNOS	nitric oxide synthase
Kg	kilograms
LDH	lactate dehydrogenase
m	meter
M	molar
mA	milliampere
MDA	malondialdehyde
MEM	minimum essential medium

MFO	mixed function oxidase
mg	milligram
min	minute
mL	milliliter
mM	millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	normality
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nm	nanometer
nmol	nanomol
PAP-sulfate	phosphoadenosine phosphosulfate
PBS	phosphate buffered saline
pg	picogram
PKC	protein kinase C
pmol	picomole
PMSF	phenylmethyl sulfonyl fluoride
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RO	reverse osmosis
ROS	reactive oxygen species
RS	reactive species

s	second
SDS	sodium dodecyl sulfate
SER	smooth endoplasmic reticulum
SOD	superoxide dismutase
TBARs	thiobarbituric acid assay
TBA	thiobarbituric acid
TBHP	tert-butyl hydroperoxide
TCA	trichloroacetic acid
TG	triglycerides
Topo II	topoisomerase II
UDP	uridine diphosphate-linked
UDGP	uridine diphosphate glucuronosyl
VLDL	very-low density lipoprotein
v/v	volume by volume
WST	water soluble tetrazolium
XO	xanthine oxidase
µg	microgram
µL	microliter
µm	micrometer
µM	micromolar
µmol	micromole
%	percentage

BIOGRAPHY

NAME	Nin Prapongsena, Mr
BIRTH DATE	5 April 1985
HOME PLACE	1044/14 Sukumvit Rd., Banjak, Prakhanong, Bangkok 10260
WORK PLACE	Faculty of Pharmacy, Huachiew Chalermprakiet University, 18/18 Bangna-Trad Rd., Bangchalong, Bang Pli, Samut Prakarn, 10540

INSTITUTION ATTENDED

2006	Silpakorn University, Bachelor of Pharmacy (Pharmaceutical Science)
2010	Studying at Silpakorn University, Master of Biopharmaceutical Science

WORK ATTENDED

2011-Now	Lecturer, Faculty of Pharmacy, Huachiew Chalermprakiet University