Study on Antioxidant Activity and Protective Effect of Benjakul Against Carbon Tetrachloride-Induced Hepatotoxicity in Rats.

Akkawit Jieokok

A Thesis Submitted in Partial Fulfillment of the Requirements

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Author	Mr. Akkawit Jieokok
Major Program	Pharmacology

Major Advisor :

……
(Assoc. Prof. Wibool Ridtitid, M.D.)

Co-advisors :

……
(Asst. Prof. Werawath Mahatthanatrakul, M.D.)

……
(Assoc. Prof. Malinee Wongnawa)

Examining Committee :

…….Chairperson
(Asst. Prof. Dr. Tawat Taesotikul)

…….....
(Assoc. Prof. Wibool Ridtitid,M.D.)

…….……...
(Asst. Prof. Werawath Mahatthanatrakul, M.D.)

…….…….....
(Assoc. Prof. Malinee Wongnawa)

……
(Assoc. Prof. Dr. Payom Wongpoowarak)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Pharmacology.

……
(Assoc. Prof. Dr. Krerkchai Thongnoo)
Dean of Graduate School

ชื่อวิทยานิพนธ์ การศึกษาฤทธิ์ของเบญจกูลในการต้านออกซิเดชันและป้องกันการเกิดพิษของคาร์บอนเตตระคลอไรด์ต่อตับในหนูขาวใหญ่

ผู้เขียน นายเอกวิทย์ เจียวก๊ก

สาขาวิชา เภสัชวิทยา

ปีการศึกษา 2552

บทคัดย่อ

เบญจกูล เป็นตำรับยาแผนโบราณที่ประกอบด้วยตัวยาแห้ง 5 ชนิด ได้แก่ ดอกดีปลี รากช้าพลู เถาสะค้าน รากเจตมูลเพลิง และเหง้าขิงแห้ง ใช้เพื่อปรับธาตุทั้งห้าของร่างกายให้สมดุลซึ่งจะช่วยให้ร่ายกายแข็งแรง ปราศจากโรคต่างๆ การศึกษานี้จึงมีวัตถุประสงค์ศึกษาว่าน้ำต้มเบญจกูล จะสามารถป้องกันการเกิดพิษต่อตับของหนูขาวที่ถูกเหนี่ยวนำด้วยคาร์บอนเตตร้าคลอไรด์ได้หรือไม่ โดยให้เบญจกูล ซึ่งประกอบด้วยสมุนไพรทั้งห้าชนิดในอัตราส่วนเท่าๆกัน ในขนาด 62.5, 125, 250, 500 และ 1000 มิลลิกรัมต่อกิโลกรัม ทางปาก วันละ 2 ครั้ง เช้า-เย็น เป็นเวลา 2 และ 7 วัน และป้อนคาร์บอนเตตร้าคลอไรด์ (CCl_4) ในขนาด 1.5 มิลลิลิตรต่อกิโลกรัม (เตรียม 1:1 ในน้ำมันข้าวโพด) ในตอนเช้าของวันที่ 2 และ 7 ตามลำดับ เก็บเลือดและตับหลังจากได้รับ CCl_4 24 ชั่วโมง พบว่า หนูที่ได้รับเบญจกูลในขนาดสูง (1000 มิลลิกรัมต่อกิโลกรัม) เป็นเวลานานทั้งแบบ 2 วัน และ 7 วัน มีระดับของเอนไซม์ AST และ ALT ในซีรัมลดลงประมาณร้อยละ 50 แต่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติเฉพาะแบบ 2 วัน เมื่อเทียบกับกลุ่มที่ได้รับ CCl_4 อย่างเดียว (ค่า AST และ ALT คิดเป็นร้อยละ 50 และ 49 ของกลุ่ม CCl_4 ในแบบ 2 วัน, ร้อยละ 55 และ 57 ของกลุ่ม CCl_4 ในแบบ 7 วัน) ปริมาณ GSH ในตับ ที่ลดลงในกลุ่มที่ได้รับ CCl_4 อย่างเดียว (ร้อยละ 73 และ 85 ของกลุ่มควบคุม) กลับเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ คิดเป็นร้อยละ 110 และ 138 ของกลุ่มควบคุม (หรือร้อยละ 150 และ 162 ของกลุ่มที่ได้รับ CCl_4) ในกลุ่มที่ได้รับเบญจกูล 2 และ 7 วัน ตามลำดับ สำหรับหนูที่ได้รับเบญจกูลในขนาดต่ำ (62.5 มิลลิกรัมต่อกิโลกรัม) พบว่า กลุ่มที่ได้รับเป็นเวลา 2 วัน มีเฉพาะระดับของเอนไซม์ ALT ในซีรัมลดลง อย่างมีนัยสำคัญทางสถิติ (คิดเป็น 43 ของกลุ่ม CCl_4) และปริมาณ GSH ในตับ เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อเทียบกับกลุ่มที่ได้รับ CCl_4 อย่างเดียว (ร้อยละ 155 ของกลุ่มที่ได้รับ CCl_4 หรือร้อยละ 114 ของกลุ่มควบคุม) ในขณะที่กลุ่มที่ได้รับเบญจกูลเป็นเวลา 7 วัน กลับมีระดับของเอนไซม์ AST และ ALT ในซีรัมเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ คิดเป็นร้อยละ 168 และ 183 ของกลุ่ม CCl_4 และปริมาณ GSH ในตับไม่เพิ่มขึ้น (คิดเป็นร้อยละ 94 ของกลุ่มควบคุม หรือ ร้อยละ 110 ของกลุ่มที่ได้รับ CCl_4)

ปริมาณ MDA ในตับของหนูที่ได้รับ CCl_4 อย่างเดียว เพิ่มขึ้นคิดเป็นร้อยละ

113-123 ของกลุ่มควบคุม และของหนูที่ได้รับเบญจกูลทุกกลุ่ม ไม่แตกต่างจากกลุ่มที่ได้รับ CCl_4 อย่างเดียว เบญจกูลในขนาดอื่นๆ (125, 250 และ 500 มิลลิกรัมต่อกิโลกรัม) ให้ผลไม่แตกต่างจากกลุ่มที่ได้รับ CCl_4 อย่างเดียว สรุปว่าเบญจกูล อาจมีผลป้องกันหรือเสริมฤทธิ์การเกิดพิษต่อตับของหนูขาวที่ถูกเหนี่ยวนำด้วยคาร์บอนเตตร้าคลอไรด์ โดยขึ้นกับขนาดและระยะเวลาที่ได้รับเบญจกูล ซึ่งผลดังกล่าวอาจเกิดจากปฏิกิริยาต่อกันระหว่างคาร์บอนเตตร้าคลอไรด์ และสารออกฤทธิ์หลายชนิดจากสมุนไพรทั้งห้าชนิดที่เป็นองค์ประกอบของเบญจกูล โดยสารออกฤทธิ์อาจมีผลเสริมฤทธิ์ หรือหักล้างฤทธิ์กันเอง

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ABSTRACT

Benjakul (BJK), a traditional Thai medicine, is composed of five dried medicinal plants, *Piper chaba* (Piperaceae) fruit, *Piper sarmentosum* (Piperaceae) root, *Piper sp.* (Piperaceae) stem (that locally called Sa-karn), *Plumbago indica* (Plumbaginaceae) root and *Zingiber officinale* (Zingiberaceae) rhizome. It has been used since ancient time to keep balance of physiological function of the human body which is the key to a long life free from disease. This study aims to investigate whether BJK have protective effect against carbon tetrachloride-induced hepatotoxicity in rats. Rats were pretreated with BJK, in equal amount of each, (62.5, 125, 250, 500 and 1000 mg/kg BW) twice daily for either 2 or 7 days. A single dose of carbon tetrachloride (1.5 ml/kg, CCl_4 1:1 in corn oil, orally) was administered on the second or seventh day after BJK, respectively. Blood samples and liver tissues were collected after 24 h of CCl_4 administration for assaying biochemical parameters. The results showed that the BJK at high dose (1000 mg/kg) lowered the serum AST and ALT about 50% in both 2 and 7 days pretreatment, even significant only in 2 days pretreatment (50 and 49 % of CCl_4 in 2 days, 55 and 57 % of CCl_4 in 7 days pretreatment), when compared to CCl_4-treated group. The hepatic GSH that were reduced by CCl_4 to 73-85 % of control were brought to control level and significantly higher than that of CCl_4-treated group in both treatment of BJK (110 % of control in 2 days and 138 % of control in 7 days pretreatment). For the pretreatment of BJK at low dose (62.5 mg/kg) for 2 days also significantly decreased the serum ALT (43 % of CCl_4), but not AST, and the hepatic GSH was significantly increased when compared to CCl_4 –treated rats (114 % of control or 155 % of CCl_4). On the

contrary, the pretreatment for 7 days resulted significant increase in the serum AST and ALT (168 and 183 % of CCl_4) with no increase in the hepatic GSH (94% of control or 110 % of CCl_4). The hepatic MDA of CCl_4-treated rats was increased to 113-123% of the control rats. The hepatic MDA of all treatment of BJK were not different from the CCl_4-treated rats. BJK at other doses of treatment showed on changes in effects when compared to the CCl_4-treated rats. In conclusion, this study might show that BJK either protected or potentiated against CCl_4-induced hepatotoxicity in rats, depending on dose and time of pretreatment of BJK. These complexity of this herb–toxicant interactions may involved the interaction of various effects of numerous constituents in different herbs in BJK.

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Akkawit Jieokok

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LIST OF ABBREVIATIONS AND SYMBOLS

ALT	=	alanine aminotransferase
AST	=	aspartate aminotransferase
DTNB	=	5,5/ dithio-bis (2-nitrobenzoic acid)
GPx	=	glutathione peroxidase
GSH	=	reduced glutathione
GSSG	=	glutathione disulfide
GST	=	glutathione S-transferase
H_2O_2	=	hydrogen peroxide
$HO^{\bullet}$	=	hydroxyl radical
i.p.	=	intraperitoneal
I.U.	=	international unit
$LO^{\bullet}$	=	lipidalkoxyl radical
$LOO^{\bullet}$	=	lipidperoxyl radical
LOOH	=	lipidhydroperoxide
LPO	=	lipid peroxidation
M	=	molar
MDA	=	malondialdehyde
Min	=	minute
mM	=	milimolar
nm	=	nanometer
nmole	=	nanomole
$NO^{\bullet}$	=	nitric oxide
$ONOO^{-}$	=	peroxynitrite
$O2^{\bullet-}$	=	superoxide anion
P	=	P value
p.o.	=	per os

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

PSC	=	prostatic cancer
rpm	=	round per minute
ROS	=	reactive oxygen species
S.E.M	=	standard error of mean
SOD	=	superoxide dismutase
TCA	=	thiazolidine-4-carboxylic acid
TBA	=	thiobarbituric acid
TMP	=	1,1,3,3 tetramethoxypropane
µl	=	microlitre
µM	=	micromolar
µmole	=	micromole
vs	=	versus

CHAPTER 1

INTRODUCTION

Liver damage has been increased as a result of exposure to some drugs and various environmental toxins such as paracetamol, carbon tetrachloride (CCl_4), nitrosamines, and polycyclic aromatic hydrocarbons. Liver injuries induced by CCl_4 are the best-characterized system of the chemical-induced hepatotoxicity and is a commonly used model for the screening of the anti-hepatotoxic /hepatoprotective effect of drugs in experimental animals (Brattin *et al*., 1985; Recknagel *et al*., 1989; Recknagel *et al*., 1991; Williams and Burk, 1990; Brent and Rumack, 1993). CCl_4 requires biotransformation by the hepatic microsomal cytochrome P450 system, particularly CYP 2E1, in liver to produce highly reactive metabolites, trichloromethyl radical ($CCl_3^{\bullet}$) and proxy trichloromethyl radical ($OOCCl_3^{\bullet}$) (Gonzalez, 1988.; Koop, 1992; Zangar *et al*., 2000; Aeschbach *et al*., 1994). These reactive free radicals react with cellular proteins and are considered as the initial reactant in the chain reaction of oxidation which may attack lipids on the membrane, finally resulting in cell necrosis, cell membrane disruption and consequent cell death (Brattin *et al*., 1985; Recknagel *et al*., 1989; Recknagel *et al*., 1991; Williams and Burk, 1990; Brent and Rumack, 1993).

Benjakul (BJK), a traditional Thai medicine, is composed of five dried medicinal plants, *Piper chaba* (Piperaceae) fruit, *Piper sarmentosum* (Piperaceae) root, *Piper sp*. (Piperaceae) stem that locally called Sa-karn, *Plumbago indica* (Plumbaginaceae) root and *Zingiber officinale* (Zingiberaceae) rhizome. BJK exhibits a number of beneficial effects against various types of diseases in humans. It was an ancient philosophical concept used to normalize the five primary elements (earth, water, air, fire and space) that regulate physiological function within the human body in relationship to the natural environment. It has been used at various different ratios based on traditional examination of patients to restore balance. The traditional Thai medicine holds that specific disease conditions are symptoms of an underlying

imbalance and thus living in balance is the key to a long life free from disease. However, there is no scientific report support its uses. Benjakul contains several bioactive constituents such as alkaloids, flavonoids, lignins, phenylpropanoids, terpenes and etc. Among these, (6)-gingerol, a major pungent ingredient of ginger, has been reported having great potent antioxidant activity (Aeschbach *et al*., 1994; Shukla and Singh, 2007).

Hence, we hypothesized that BJK would be useful in the prevention of liver injuries induced by hepatotoxins. This study was undertaken to evaluate the protective effects of BJK on CCl_4-induced liver damage in rats.

CHAPTER 2

LITERATURE REVIEWS

2.1 Carbon tetrachloride-induced hepatotoxicity

2.1.1 Carbon tetrachloride metabolism

Carbon tetrachloride (CCl_4) is an extensively used industrial solvent, and it is the best-characterized animal model of xenobiotic-induced free radical-mediated hepatotoxicity (Recknagel and Glende, 1973).

CCl_4 is metabolized by cytochrome P450 system of phase I in liver and yields the reactive metabolic trichloromethyl radical ($CCl_3^{\bullet}$). These trichloromethyl free radicals then readily interacts with oxygen to form highly reactive trichloromethylperoxy radical ($^{\bullet}OOCCl_3$) (Brattin *et al*., 1985; Recknagel *et al*., 1991; Williams and Burk, 1990; Brent and Rumack, 1993).

The trichloromethylperoxy radical ($^{\bullet}OOCCl_3$) is then detoxicified by phase II enzyme, using GSH, to from phosgene ($COCl_2$) and consequently digluta thionyl dithiocarbonate and other inactive metabolites.These free radicals can bind with biomolecules such as protein, DNA, lipid, cause damage in cell membrane, change enzyme activity and finally induce hepatic injury or necrosis (Weber et al., 2003).

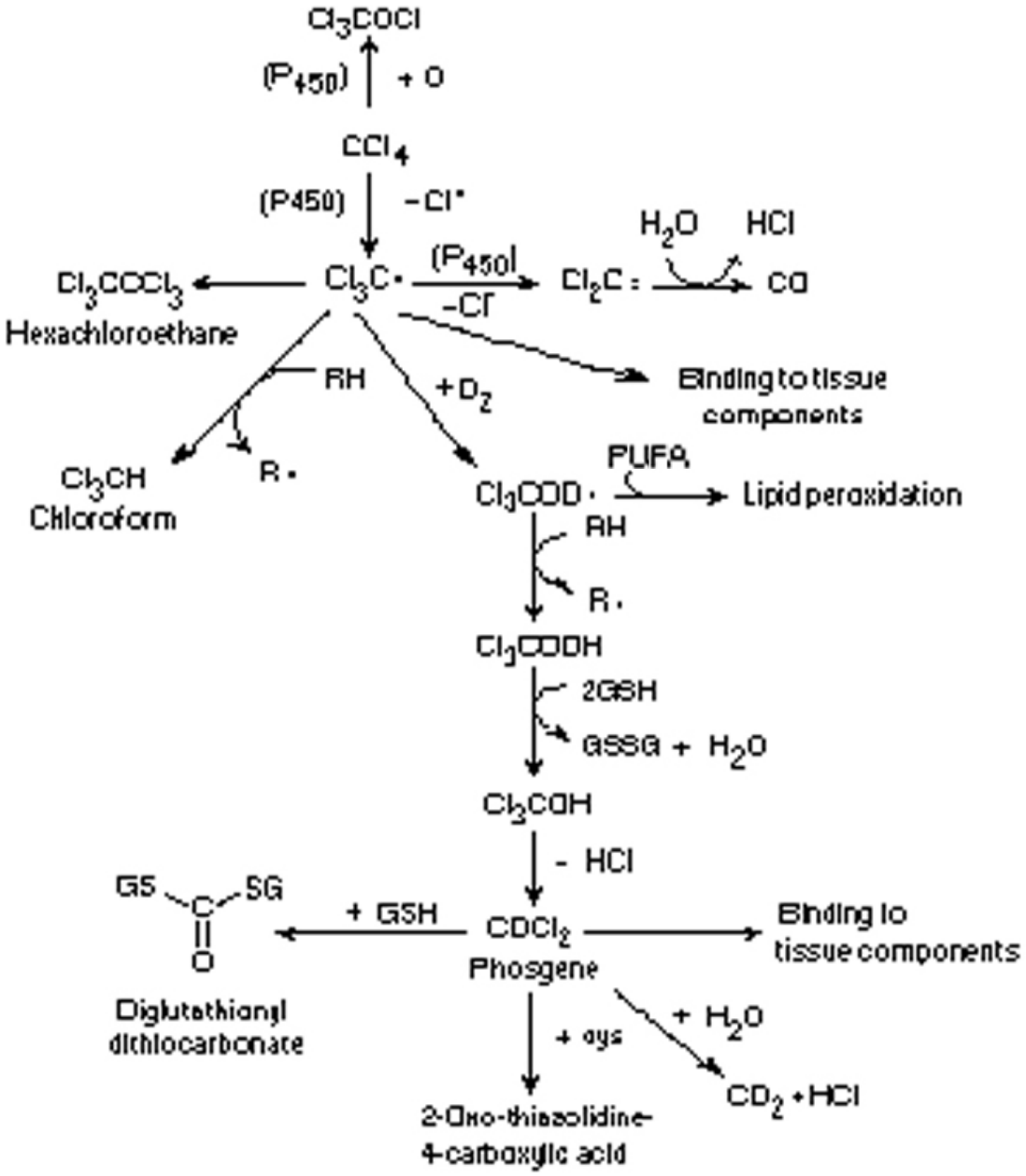


Figure 1. Biotransformation of carbon tetrachloride (From Harris & Anders, 1981; Anders & Jakobson, 1985; McGregor & Lang, 1996)

2.1.2 Mechanism of CCl_4-included hepatotoxicity

The mechanism of CCl_4-included hepatic injury has interested many investigators (Kalf *et al.,* 1987), and the compound has become the reference substance for all hepatotoxic compounds. Injury produced by CCl_4 seem to be mediated by a reactive metabolite-trichloromethyl free reacical ($CCl_3^{\bullet}$)-formed by the hemolytic cleavage of CCl_4, or by an even more reactive species-trichloromethylperoxy free reacical ($Cl_3COO^{\bullet}$)-formed by the reaction of $CCl_3^{\bullet}$ with O_2 (Slater, 1982). This biotransformation is catalyzed by a cytochrome P450-dependent monoxygenases, mainly cytochrome P450 2E1. Thus, agents such as DDT and phenobarbital, which induce such enzymes, strikingly enhance the hepatotoxic effects of CCl_4, conversely, agents that inhibit the drug-metabolizing activity diminish the hepatotoxicity of CCl_4.

The toxicity produced by CCl_4 is thought to be due to the reaction of free radicals ($CCl_3^{\bullet}$ or $Cl_3COO^{\bullet}$) with lipid and proteins; however, the relative importance of interactions with various tissue constituents in producing injury is controversial. The free radical causes the peroxidation of the polyenoic lipids of the endoplasmic reticulum and the generation of secondary free radicals derived from these lipids-a chain reaction. This destructive lipid peroxidation leads to breakdown of membrane structure and function, and, if a sufficient quantity of CCl_4 has been consumed, the intracellular cytoplasmic Ca^{2+} increases, resulting in cell death (Plaa, 1991; Kalf *et al*., 1987; Recknagel *et al*., 1989).

2.1.3 Lipid peroxidation

Lipids in biological systems can undergo oxidation, leading to deterioration. In foods, these reactions can lead to rancidity, loss of nutritional value from the destruction of vitamins (A, D, and E) and essential fatty acids, and the possible formation of toxic compounds and colored products.

Lipid peroxidation is a chain reaction process that involves the participation and the production of free radical species. Free radicals can cause cellular injury when produced in sufficient amounts to overcome the normally efficient protective mechanism. Lipid peroxidation is a free radical mediated chain

reaction which is enhanced as a consequence of oxidative stress (a general term used to describe a state of damage caused by reactive oxygen species (ROS), and it results in an oxidative deterioration of membrane polyunsaturated fatty acids). It is a continuous physiological process occurring in cell membranes (Loeckie *et al*., 1999), which defined as the oxidative deterioration of polyunsaturated fats. The important lipids involved in oxidation are the unsaturated fatty acid moieties, oleic, linoleic, and linolenic. The rate of oxidation of these fatty acids increases with the degree of unsaturation. The membrane of mammalian cells contains large amount of polyunsaturated fatty acids which are especially susceptible to lipid peroxidation due to allylic sites. Lipid peroxidation of cell membranes results in decreased membrane fluidity, inability to maintain ionic gradients, cellular swelling, and tissue inflammation. It involved the reaction of oxygen and polyunsaturated lipids to form lipid free radicals and semistable hydroperoxides, which in turn promote free radicals chain reaction as show in (Figure 2).

The overall mechanism of lipid oxidation consists of three phases: (1) initiation, the formation of free radicals; (2) propagation, the free-radical chain reactions; and (3) termination, the formation of nonradical products (Noguchi and Niki, 1999).

Initiation	:	$RH + O_2$	$\rightarrow$	$R^{\bullet} + OH^{\bullet}$
Propagation	:	$R^{\bullet} + O_2$	$\rightarrow$	$^{\bullet} + ROO$
		$ROO^{\bullet} + RH$	$\rightarrow$	$R^{\bullet} + ROOH$
		$ROOH$	$\rightarrow$	$RO^{\bullet} + HO$
Termination	:	$R^{\bullet} + R^{\bullet}$	$\rightarrow$	RR
		$R^{\bullet} + ROO^{\bullet}$	$\rightarrow$	$ROOR$
		$ROO^{\bullet} + ROO^{\bullet}$	$\rightarrow$	$ROOR + O_2$

Where RH is any unsaturated fatty acid; $R^{\bullet}$ is a free radical formed by removing a labile hydrogen from a carbon atom adjacent to a double bond; and ROOH is a hydroperoxide.

The initial step of lipid peroxidation occurs when a radical species, such as the hydroxyl radical ($OH^{\bullet}$), removes an allylic hydrogen from a polyunsaturated fatty acid (PUFA). Initiation can also be stimulated by using the ions

or chelates of transition metals, like iron, which exacerbate the toxicity of H_2O_2 by promoting the formation of the hydroxyl radical. The removal of the allylic hydrogen from a polyunsaturated fatty acid forms a lipid redical ($L^{\bullet}$). An immediate rearrangement occurs, forming a more stable lipid radical, whose dienes are conjugated. In an aerobic conditions this radical reacts with oxygen, giving rise to a lipid peroxyl radical ($LOO^{\bullet}$).

Propagation reactions can continue this process at this point by the lipid peroxyl radical abstracting an allylic hydrogen atom from another adjacent PUFA, result in a lipid hydroperoxide (LOOH) and a second lipid radical ($L^{\bullet}$). This second lipid radical can proceed through the same reactions as the first, generating additional lipid hydroperoxides (Halliwell and Gutteridge, 1998C). These hydroperoxides can undergo decomposition to numerous aldehyde products of different chain lengths. The malonyldialdehyde (MDA) is a frequently used as an indicator of lipid peroxidation in biological tissue (Young and McEneny, 2001).

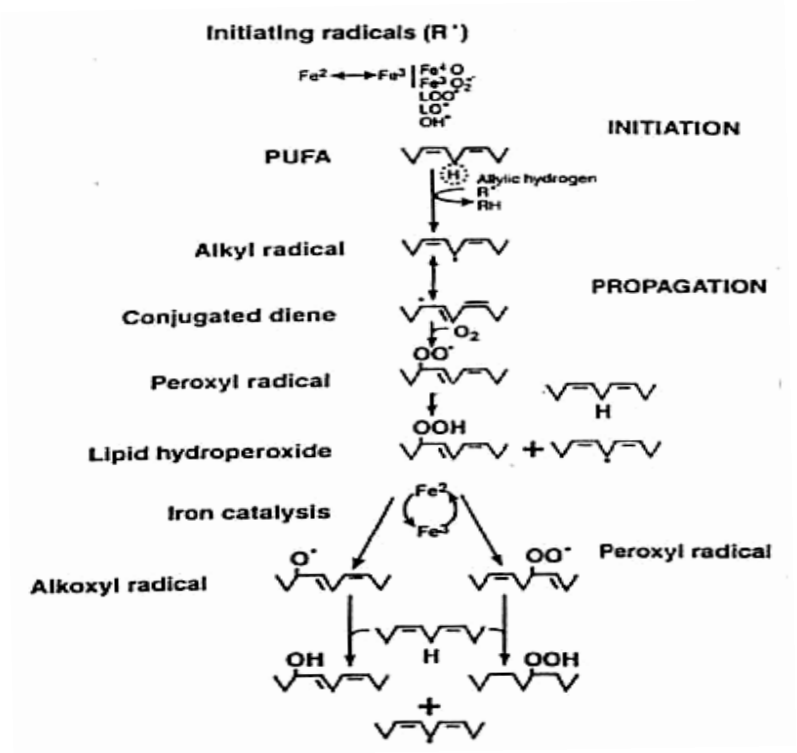


Figure 2. Schematic proceed of lipid peroxidation, chain reactions resulting in the formation of many lipid peroxide radicals (Halliwell and Gutteridge,1998C)

The chain propagation does not continue forever, but the chain oxidation is terminated when lipid radical or lipid peroxyl radical is scavenged by an antioxidant such as vitamin E and C or when two lipid peroxyl radicals react to give non radical products such as ketones and alcohols.

If an attempt is made to evoke a causative role for peroxidation, it must be shown that the peroxidation precedes or accompanies the cell damages and that prevention of peroxidation by antioxidants prevents the cell damages. Measurement of lipid peroxidation may therefore be an excellent marker of tissue damages (Gutteridge, 1995).

2.1.3 Parameters of hepatotoxicity

Liver function tests represent a broad range of normal functions performed by the liver. The diagnosis of liver disease depends upon a complete history, complete physical examination, and evaluation of liver function tests and further invasive and noninvasive tests.

The hepatobiliary tree represents hepatic cells and biliary tract cells. Inflammation of the hepatic cells results in elevation in the alanine aminotransferase (ALT), aspartate aminotransferase (AST) and possibly the bilirubin. Inflammation of the biliary tract cells results predominantly in an elevation of the alkaline phosphatase. In liver disease there are crossovers between purely biliary disease and hepatocellular disease. To interpret these, the physician will look at the entire picture of the hepatocellular disease and biliary tract disease to determine which is the primary abnormality.

Alanine Aminotransferase (ALT):

ALT is the enzyme produced within the cells of the liver. The level of ALT abnormality is increased in conditions where cells of the liver have been inflamed or undergone cell death. As the cells are damaged, the ALT leaks into the bloodstream leading to a rise in the serum levels. Any form of hepatic cell damage can result in an elevation in the ALT. The ALT level may or may not correlate with the degree of cell death or inflammation. ALT is the most sensitive marker for liver cell damage.

Aspartate Aminotransferase (AST):

This enzyme also reflects damage to the hepatic cell. It is less specific for liver disease. It may be elevated and other conditions such as a myocardial infarct. Although AST is not a specific for liver as the ALT, ratios between ALT and AST are useful to physicians in assessing the etiology of liver enzyme abnormalities.

Alkaline Phosphatase (ALP):

Alkaline phosphatase is an enzyme, which is associated with the biliary tract. It is not specific to the biliary tract. It is also found in bone and the placenta. Renal or intestinal damage can also cause the alkaline phosphatase to rise. If the alkaline phosphatase is elevated, biliary tract damage and inflammation should be considered. However, considering the above other etiologies must also be entertained. One way to assess the etiology of the alkaline phosphatase is to perform a serologic evaluation called isoenzymes. Another more common method to asses the etiology of the elevated alkaline phosphatase is to determine whether the GGT is elevated or whether other function tests are abnormal (such as bilirubin).

Alkaline phosphatase may be elevated in primary biliary cirrhosis, alcoholic hepatitis, prostatic cancer (PSC), gallstones in choledocholithiasis.

Gamma Glutamic Transpeptidase (GGT):

This enzyme is also produced by the bile ducts. However, it is not very specific to the liver or bile ducts. It is used often times to confirm that the alkaline phosphatase is of the hepatic etiology. Certain GGT levels, as an isolated finding, reflect rare forms of liver disease. Medications commonly cause GGT to be elevated. Liver toxins such as alcohol can cause increases in the GGT.

Bilirubin:

Bilirubin is a major breakdown product of hemoglobin. Hemoglobin is derived from red cells that have outlived their natural life and subsequently have been removed by the spleen. During splenic degradation of red blood cells, hemoglobin (the part of the red blood cell that carries oxygen to the tissues) is separated out from

iron and cell membrane components. Hemoglobin is transferred to the liver where it undergoes further metabolism in a process called conjugation. Conjugation allows hemoglobin to become more water-soluble. The water solubility of bilirubin allows the bilirubin to be excreted into bile. Bile then is used to digest food.

As the liver becomes irritated, the total bilirubin may rise. Total bilirubin has undergone conjugation and direct bilirubin (at portion of bilirubin) has not been metabolized. When this fraction is elevated, the cause of elevated bilirubin (hyperbilirubinemia) is usually outside the liver. These types of causes are typically gallstones. This type of abnormality is usually treated with surgery (such as a gallbladder removal or choleycystectomy).

If the direct bilirubin is low, while the total bilirubin is high, this reflects liver cell damage or bile duct damage within the liver itself.

Albumin:

Albumin is the major protein present within the blood. Albumin is synthesized by the liver. As such, it represents a major synthetic protein and is a marker for the ability of the liver to synthesize proteins. It is only one of many proteins that are synthesized by the liver. However, since it is easy to measure, it represents a reliable and inexpensive laboratory test for physicians to assess the degree of liver damage present in the in any particular patient. When the liver has been chronically damaged, the albumin may be low. This would indicate that the synthetic function of the liver has been markedly diminished. Such findings suggest a diagnosis of cirrhosis. Malnutrition can also cause low albumin (hypoalbuminemia) with no associated liver disease.

Prothrombin time (PT):

Another measure of hepatic synthetic function is the prothrombin time. Prothrombin time is affected by proteins synthesized by the liver. Particularly, these proteins are associated with the incorporation of vitamin K metabolites into a protein. This allows normal coagulation (clotting of blood). Thus, in patients who have prolonged prothrombin times, liver disease may be present. Since a prolonged PT is not a specific test for liver disease, confirmation of other abnormal liver tests is

essential. This may include reviewing other liver function tests or radiology studies of the liver. Diseases such as malnutrition, in which decreased vitamin K ingestion is present, may result in a prolonged PT time. An indirect test of hepatic synthetic function includes administration of vitamin K (10mg) subcutaneously over three days. Several days later, the prothrombin time may be measured. If the prothrombin time becomes normal, then hepatic synthetic function is intact. This test does not indicate that there is no liver disease, but is suggestive that malnutrition may coexist with (or without) liver disease.

Platelet count:

Platelets are cells that form the primary mechanism in blood clots. They are also the smallest of blood cells. They derived from the bone marrow from the larger cells known as megakaryocytes. Individuals with liver disease develop a large spleen. As this process occurs platelets are trapped with in the sinusoids (small pathways within the spleen) of the spleen. While the trapping of platelets is a normal function for the spleen, in liver disease it becomes exaggerated because of the enlarged spleen (splenomegaly). Subsequently, the platelet count may become diminished.

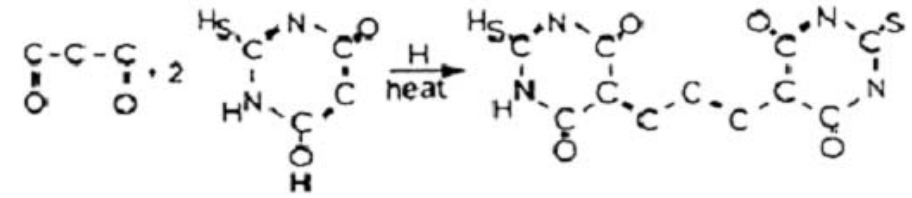
Peroxide value

Peroxides are the main initial products of autoxidation. They can be measured by techniques based on their ability to liberate iodine from potassium iodide, or to oxidize ferrous to ferric ions. Their content is usually expressed in terms of milliequivalents of oxygen per kilogram of fat. Although the peroxide value is applicable for following peroxide formation at the early stages of oxidation, it is, nevertheless, highly empirical. The accuracy is questionable, the results vary with details of the procedure used, and the test is extremely sensitive to temperature changes. During the course of oxidation, peroxide values reach a peak and then decline.

Thiobarbituric acid (TBA)

TBA is the most widely used test for measuring the extent of lipid

peroxidation in foods due to its simplicity and because its results are highly correlated with sensory evaluation scores. The basic principle of the method is the reaction of one molecule of malonaldehyde and two molecules of TBA to form a red malonaldehyde-TBA complex, which can be quantitated spectrophotometrically (530nm). However, this method has been criticized as being nonspecific and insensitive for the detection of low levels of malonaldehyde. Other TBA-reactive substances (TBARS) including sugars and other aldehydes could interfere with the malonaldehyde-TBA reaction. Abnormally low values may result if some of the malonaldehyde reacts with proteins in an oxidizing system. In many cases, however, the TBA test is applicable for comparing samples of a single material at different states of oxidation.



Malonaldehyde **TBA** **TBA chromagen**

Figure 3. Proposed TBA reaction

2.2 Oxidants

2.2.1 Free radicals

A free radical is any chemical species that has an odd number of electrons, because it contains one or more unpaired electron(s), that is an electron that occupies an atomic or molecular orbital by itself (Halliwell and Gutteridge, 1998a). A radical will thus be indicated, as recommended, by a superscripted dot at the right side of the formula in parenthesis so as to show that no judgment as to the location of the unpaired electron is made. The symbol $R^{\bullet}$ will be used throughout to represent an unspecified radical which may be positively charged (cation radical, $R^{+\bullet}$), negatively charged (anion radical, $R^{-\bullet}$) or neutral (neutral radical, $R^{\bullet}$). Radical can be formed by the less of a single electron from a non-radical, or by the gain of a single electron by the non-radical. They can easily be formed when a covalent bond is broken if one

electron from each of the pair shared remains with each atom, a process known as hemolytic fission.

$$A : B \rightarrow A^{\bullet} + B^{\bullet}$$

2.2.2 Biological Radicals

Radical reactions are generally chain reactions. The radicals are generated in a step or steps call "initiation", they participate in a sequence of "propagation" reactions in which their number is conserved, finally they are destroyed in a "termination" process or processes (Roberfroid and Colderon, 1995). Initiation of radical chain reaction, oxygen (O_2), hydrogen peroxide (H_2O_2), water (H_2O) and polyunsaturated fatty acids appear to play an essential role as the major substrate for these events.

O_2 does so because it serves as an acceptor for single electrons arising in cells either via the one-electron enzymatic reduction of endogenous and exogenous organic molecules or as a side product of incomplete electron transport in mitochondria, endoplasmic reticulum or nuclear membranes. Monoelectronic reduction of O_2 produces superoxide anion ($O_2^{\bullet -}$). H_2O_2, the product of $O_2^{\bullet -}$ dismutation, plays a role mainly because it is the substrate of mental in catalyzed decomposition to yield the hydroxyl radical ($OH^{\bullet}$). Polyunsaturated fatty acids occupy a key position in radical chain reaction, not because they are direct substrates of major initiation processes, but because they can easily be transformed in alkyl hydroperoxides (LOOH). The alkyl hydroperoxides are transformed to either alkoxyl ($LO^{\bullet}$) or alkylperoxyl ($LOO^{\bullet}$) radical as a result of a molecule assisted homolysis.

Propagation of radical chain reaction: the hydroxyl radical ($OH^{\bullet}$) plays a major role, but hydroperoxide (LOOH) as well as alkoxyl or alkylperoxyl ($LO^{\bullet}$, $LOO^{\bullet}$) radicals of polyunsaturated fatty acids are still key targets and/or key intermediates. H-atom transfer, electron transfer, as well as addition are the major events in biologically relevant radical propagation.

Termination of radical chain reaction: Both dismutation and reduction involve electron transfer, either by electron exchange between identical

molecular entities or by electron capture in a classic oxidoreduction process. Scavenging involves the formation of a radical that is less likely to propagate the radical but, rather, is likely to homolink. The most important homolinking reaction in biological system is that involving 2 glutathione radicals ($GS^{\bullet}$) to form the oxidized glutathione (GSSG).

Superoxide radical ($O_2^{\bullet-}$) is derived from molecular oxygen by the addition of a single electron (Yu, 1994). Reactions that produce superoxide radicals occur in many organelles of cell. For example, oxyhemoglobin in cytosol of erythrocytes can decompose and release $O_2^{\bullet-}$ and methemogolbin. Some phagocytes such as neutrophils and macrophages possess a reduced Micotinamide adeninedinucleotide phosphate oxidase (NADPH oxidase) that produce $O_2^{\bullet-}$ when the cells are activated by a component of the bactericidal armamentarium (Babior, 1978).

Superoxide dismutase enzyme is responsible for its detoxification. The fundamental reaction of producing $O_2^{\bullet-}$ is as follow

$$X + O_2 \rightarrow X^+ + O_2^{\bullet-}$$

An electron is transported from a donor, X (such as hemoglobin, cytochrome, quinone, thiol or redox metal to oxygen molecule to form superoxide and oxidized donor, X^+. The dismutation of superoxide requires two protons to form hydrogen peroxide:

$$2O_2^{\bullet-} + 2H^+ \rightarrow H_2O_2 + O_2$$

Superoxide radical can reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) in the presence of redox metal, $Fe^{3+}$and copper ion (Cu^{2+}). In addition, ferrous ion is oxidized by H_2O_2 to generate hydroxyl radical ($^{\bullet}OH$) and hydroxyl ion (OH^-). This reaction is called Fenton reaction (Young and Woodside, 2001).

$$O_2^{\bullet-} + Fe^{3+} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^-$$

Trace of Fe^{3+} might be able to react further with H_2O_2, although this is slower than the reaction of H_2O_2 with Fe^{2+} at physiological pH and depends on the

ligand to the ion

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^{\bullet-} + 2H^+$$

In the Fenton reaction, mixture $Fe^{3+}$of and H_2O_2, which almost certainly forms in biological systems under certain circumstances, can provoke a whole series of radical reaction.

Hydrogen peroxide (H_2O_2) is a non radical and can be generated from several enzymes including xanthine, urate and D-amino acid oxidase. In addition, any biological system that generates $O_2^{\bullet-}$ will also produces H_2O_2 by $O_2^{\bullet-}$ dismutation. Hydrogen peroxide is weak oxidizing and reducing agent and is generally poorly reactive. H_2O_2 can cross cell membrane rapidly and can probably react with iron, and possibly copper ions to form much more damaging species such as $OH^{\bullet}$. In addition, it can degrade heme proteins including myoglobin, hemoglobin and cytochrome c to release irons (Gutteridge, 1986).

Hydroxyl radical ($OH^{\bullet}$) may be formed by the generation of $O_2^{\bullet-}$ in the presence of redox active iron and hydrogen peroxide (Halliwell and Gutteridge, 1992).

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$$

This reaction is called Haber-Weiss reaction. Hydroxyl radical is can attack all classes of biological macromolecules. It can depolymerize polysaccharide (McCord, 1974), causes DNA strand breaks (Halliwell and Gutteridge, 1992), inactivates enzymes (Zhang *et al*., 1990), and initiates lipid peroxidation (Gutteridge *et al*., 1982).

Oxides of nitrogen ($NO^{\bullet}$) and nitrogen dioxide ($NO_2^{\bullet}$) contain odd number of electrons and are therefore free radicals, whereas nitrous oxide (N_2O) does not, $NO_2^{\bullet}$ is dense brown poisonous gas and a powerful oxidizing agent, $NO_2^{\bullet}$, on the other hand, is a colorless gas and a weak reducing agent. Biological interest in NO

has centered around the observation that the vascular endothelium and other cells in the body produce small amounts of the gas from the amino acid L-arginine (Yu, 1994).

2.2.3 Oxidative stress

The oxidative stress is the set of intracellular or extracellular condition that leads to the chemical or metabolic generation of reactive oxygen species (ROS), such as superoxide radical (O_2^-), hydroxyl radical ($OH^\bullet$), hydrogen peroxide (H_2O_2) or related species. It can result from diminished antioxidants and/or increased production of ROS.

The oxidative stress results in adaptation and cell injury (Halliwell and Gutteridge, 1998C). Mild oxidative stress results in up regulation of the synthesis of antioxidant defense systems in an attempt to restore the oxidant/ antioxidant balance. If the oxidative stress increases in the cell, it can damage all type of biomolecules including double stranded DNA, proteins and lipids. The primary cellular target of the oxidative stress can vary depending on the cell, the type of stress imposed and severity of stress. (Figure 4.) shows the summary of oxidative stress that can produce cell injury by multiple pathways.

Direct damage to DNA, protein and/or lipid is possible. Secondary damage occurs when oxidative stress produces rise in free intracellular metal ions, such as Ca^{2+}, Cu^{2+}, and Fe^{2+}. Ca^{2+} can stimulate proteases and nucleases, damaging both DNA and the cytoskeleton as well as increasing nitric oxide radical ($NO^\bullet$) synthesis; excess $NO^\bullet$ can inhibit mitochondrial energy generation and may lead to production of cytotoxic peroxynitrite ($ONOO^-$). Activation of cal pains may lead to increased reactive oxygen species production, e.g. by conversion of xanthine dehydrogenase to xanthine oxidase.

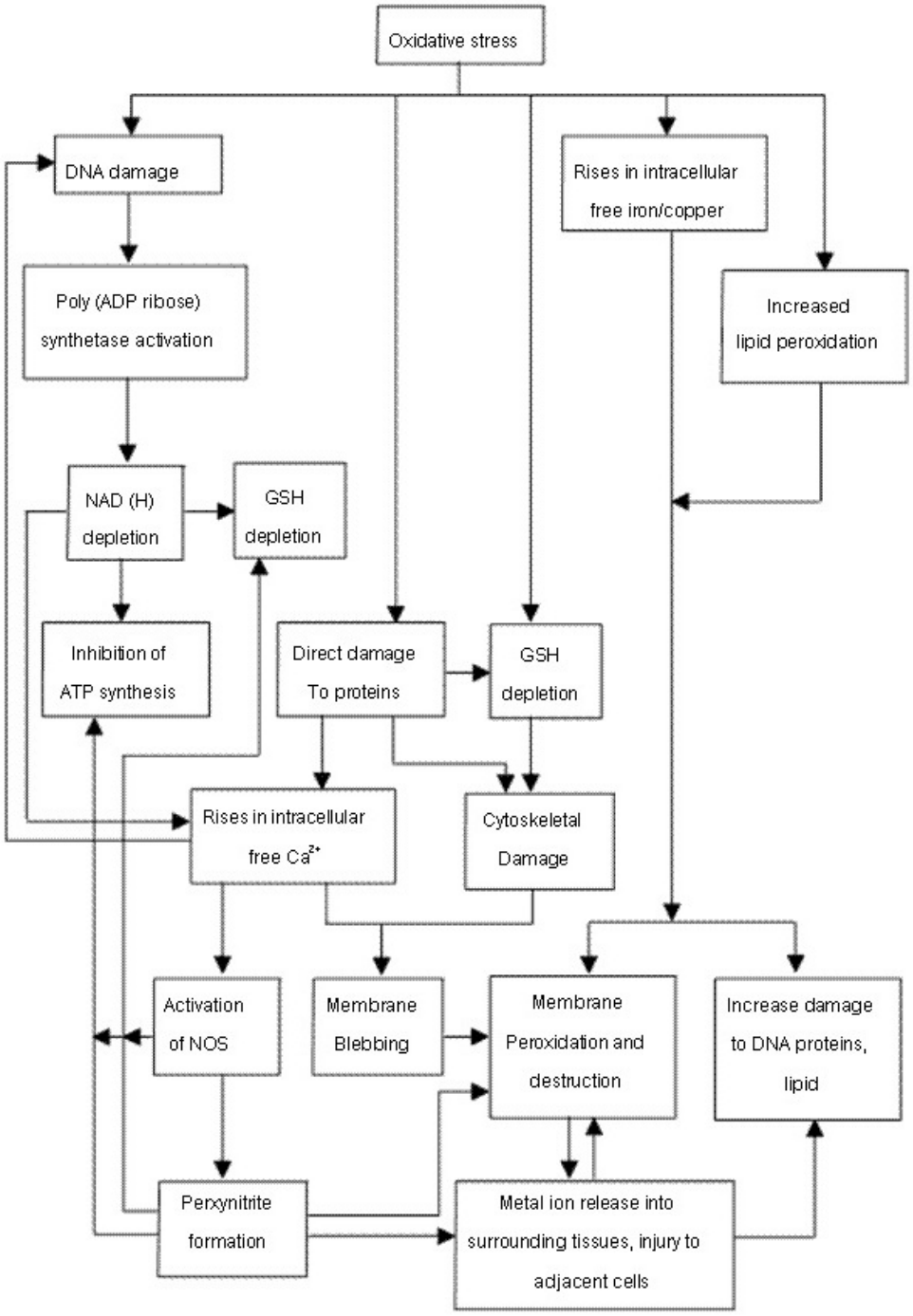


Figure 4. Summary of oxidative stress that can produce cell injury by multiple pathways (Halliwell and Gutteridge, 1998C).

2.3 Antioxidants

2.3.1 Role of antioxidants

An **antioxidant** is a chemical that reduces the rate of particular oxidation reactions in a specific context, where **oxidation reactions** are chemical reactions that involve the transfer of electrons from a substance to an oxidising agent. All living organisms maintain a reducing environment inside their cells, all cells contain complex systems of antioxidants to prevent chemical damage to the cells' components by oxidation.

2.3.2 Biological antioxidants

When the protective and controlling mechanisms can not work or are in imbalance. The excess of radicals will cause pathophysiological processes. Thus, agents which act as antioxidants are useful for treatment of diseases relating to lipid peroxidation. Antioxidants can act at different levels in the oxidative sequence. Where lipid peroxidation is concerned, they could act by decreasing local oxygen concentrations, preventing first chain initiation by scavenging initiation radicals such as hydroxyl radical, decomposing peroxides by converting them to non radical products such as alcohol, and chain breaking i.e. scavenging intermediate radical such as peroxyl and alkoxyl radicals to prevent continued hydrogen abstraction.

The antioxidants are classified according to solubility as lipophilic, such as vitamin E, coenzyme Q_{10} (ubiquinone), and carotenoids, or hydrophilic, such as ascorbate and uric acid. In addition, antioxidants contain glutathione system consists of reduced and oxidized glutathione molecules and the enzymes glutathione peroxidase and glutathione reductase. The enzymatic primary antioxidant defense system are superoxide dismutase and catalase (Grune *et al*., 2000).

Superoxide dismutase (SOD)

Superoxide dismutase catalyzes the reduction of superoxide anions to produced hydrogen peroxide and oxygen.

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

The enzyme superoxide dismutases (SOD) are classified into three distinct classes depending on the metal ion component, Cu/ZnSOD, MnSOD and FeSOD. Although some of the SOD activity appears to be extracellular, the bulk of the activity is localized intracellularly, divided between the mitochondria and cytosolic compartments. In rat hepatocytes, 70% of SOD is found in the cytosolic compartment. The activity of SOD varies among the tissues. The highest levels are seen in the liver, adrenal gland, kidney and spleen. The activity of SOD is regulated through biosynthesis, which is sensitive to tissue oxygenation (Halliwell and Gutteridge, 1998b).

Catalase

This enzyme is a major primary antioxidant defense component that primarily works to catalyze the decomposition of H_2O_2 to H_2O (Halliwell and Gutteridge, 1998e). In animals, it is present in all major body organs especially in liver and is location in organelles such as peroxisomes, mitochondria (in liver), and endoplasmic reticulum. Animal catalase consists of four protein subunits, each of which contains a ferric (Fe^{3+}) heme group bound to its active site (Lockitch, 1989).

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Vitamin E (α-tocopherol)

Vitamin E is the generic term used to describe a group of at least eight compounds that exhibit the biological activity of α-tocopherol. α-tocopherol, major constituent of the fat soluble vitamin E, is the most important free radical scavenger within membranes and lipoproteins. α -tocopherol, the most active form of vitamin E, has been demonstrated to be an essential factor in the cellular antioxidant defense system (Andreas, 1999). By donating a hydrogen atom, it function as an efficient chain breaking antioxidant that block lipid peroxidation. The α-tocopherol inhibits lipid peroxidation by scarvenging lipid peroxyl ($LOO^{\bullet}$) or alkoxyl ($LO^{\bullet}$) radicals, which are intermediates in the chain reaction (Noguchi, and Niki, 1999).

$$\alpha TH + LOO^{\bullet} \rightarrow \alpha T^{\bullet} + LOOH$$

The α-tocopherol radical ($\alpha T^{\bullet}$), although not completely unreactive, is less efficient at abstracting hydrogen than are peroxyl radicals, so the chain reaction of peroxidation is slowed. Several biological mechanisms may exist for recycling $\alpha T^{\bullet}$ back to α-tocopherol, although none of them had yet been proven vigorously to operate in vivo in humans. Likely mechanisms include the reaction of $\alpha T^{\bullet}$ with ascorbic acid at the surface of membranes and lipoproteins (Halliwell and Gutteridge, 1998b)

$$\alpha T^{\bullet} + \text{ascorbate} \rightarrow \alpha TH + \text{ascorbate}^{\bullet}$$

The primary dietary source of vitamin E is vegetable oil, secondary source includes liver, egg, cereals, and legumes (Bonorden and Pariza, 1994). The long recognized relationship between vitamin E and selenium appears to reflect the presence of other antioxidant systems, one of which contains a selenoenzyme, glutathione peroxidase (Mahan and Stump, 2004). Vitamin E and other antioxidant prevent or minimize oxidative damage in biological systems.

Vitamin C

Ascorbic acid (vitamin C), a common constituent in fruits and vegetable, is regarded as the most efficient water soluble free radical chain breaking antioxidant in human tissue and plasma (Bonorden and Pariza, 1994). It exerts antioxidant properties by directly reduces superoxide anion radical, hydroxyl radical and reacts with singlet oxygen (Halliwell and Gutteridge, 1998b). It has also been show to have a synergistic effect on the antioxidant function of vitamin E and selenium (Herman, 1979). Vitamin C can function synergistically with vitamin E in preventing membrane lipid peroxidation (Figure 5.). The synergistic mechanism is via recycling of vitamin E from its free radical (Packer *et al*., 1979).

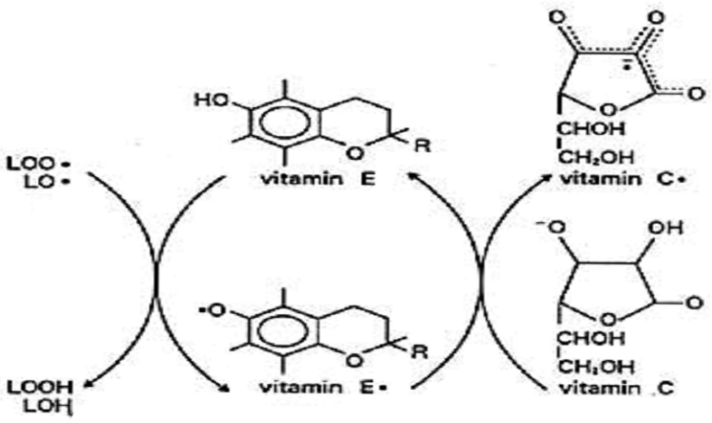


Figure 5. Chain reaction of vitamin E with lipid radicals and vitamin C (Haliwell and Gutteridge, 1998a)

Ascorbic acid can act either as an antioxidant or as a prooxidant (Herman, 1979; Combs and Gerald, 1998). In the presence of transition metal ions (e.g., Fe^{3+}, Cu^{2+}), high concentrations of vitamin C can also function as a prooxidant, acting as a reducing agent and generating $O2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$. Normally, because such metal ions are available in very limited amounts in vivo, the antioxidant properties of ascorbate predominate.

Vitamin A

Carotenoids have been long considered antioxidants because of their capacity to scavenge free radicals (Krinsky, 1979). Carotenoids protect lipid against peroxidation by quenching free radicals and other reactive oxygen species, notably singlet oxygen. β-carotene displays an efficient biological radical trapping antioxidant activity through its inhibition of lipid peroxidation induced by the xanthine oxidase system. β-carotene, like vitamin C, appears to function as both an antioxidant and a prooxidant (Halliwell and Gutteridge, 1998b).

Glutathione

Glutathione is a tripeptide composed of glutamate, glycine and cysteine, and its active group is represented by the thiol (-SH) of cysteine residue.

Glutathione is a ubiquitous molecule that is produced in all organs, especially in the liver (Lauterburg *et al*., 1984) and occurring at intracellulary concentration of 5 to 10 millimolar. Plasma and urine contain lower level of Glutathione (Halliwell and Gutteridge, 1998b).

In cells, total glutathione can be free or bound to proteins. Free glutathione is present mainly in its reduced form, which can be converted to the oxidized form during oxidative stress, and can be reverted to the reduced form by the action of the enzyme glutathione reductase. The redox status depends on the relative amounts of the reduced and oxidized forms of glutathione (GSH/GSSG) and appears to be a critical determinant in cell.

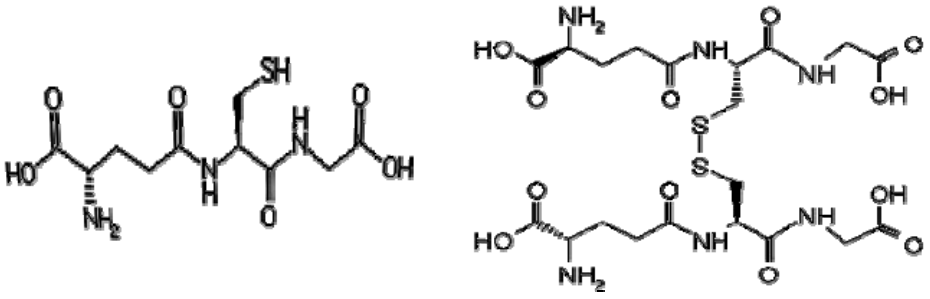


Figure 6. Structure of glutathione; (A) reduce from and (B) oxidized from

The synthesis of glutathione from its three amino acid precursors, glutamate, cysteine and glycine takes place in the cytosol. Cell can make the necessary cysteine from the essential amino acid methionine. Glutathione is synthesized within the cell in two steps. The first step, cysteine and glutamate are combined by the enzyme γ-glutamylcysteine synthetase (Whitcomb and BlocK, 1994). The γ-glutamylcysteine synthetase is feedback inhibited by GSH and does not appear saturated at normal cellular levels of cysteine, so that increased cysteine can promote GSH synthesis under certain circumstances. It has been suggested that variations in cysteine can account for the effects of starvation and refeeding on GSH level (Guoyao *et al*., 2004). The second step, adding glycine to the γ-glutamylcysteine dipeptide, is catalyzed by glutathione synthetase.

Although glutathione is synthesized inside the cell, its biodegradation occurs out side of cells. Glutathione can breakdown by γ-glutamyltranspeptidase, which located on extracellular membrane to glutamate residue on to formation of γ-glutamyl amino acids and cysteinylglycine. The latter is cleaved by dipeptidase to form cysteine and glycine. The breakdown products (glutamate, glycine and cysteine) can be reabsorbed into the cell for GSH synthesis (figure 7.).

Glutathione (GSH) performs a variety of important physiological and metabolic functions in all mammalian cells, including the detoxification of free radicals, metals, and other electrophillic compounds. One important detoxification mechanism involves the binding of GSH to electrophilic chemicals and the export of the resulting GSH S-conjugates from the cell. These conjugation reactions have been extensively characterized for a multitude of foreign chemicals, but they are also critical for the metabolism of endogenous reactive intermediates and for the formation of specific biological mediators.

The glutathione S-transferadses catalyses the conjugation of GSH with several compounds produced *in vivo* during oxidative stress. Glutathione forms conjugates with a great variety of eletrophilic compounds nonenzymatically, when the eletrophile is vary reactive, or more often through the action of glutathione S-transferadses (GST).

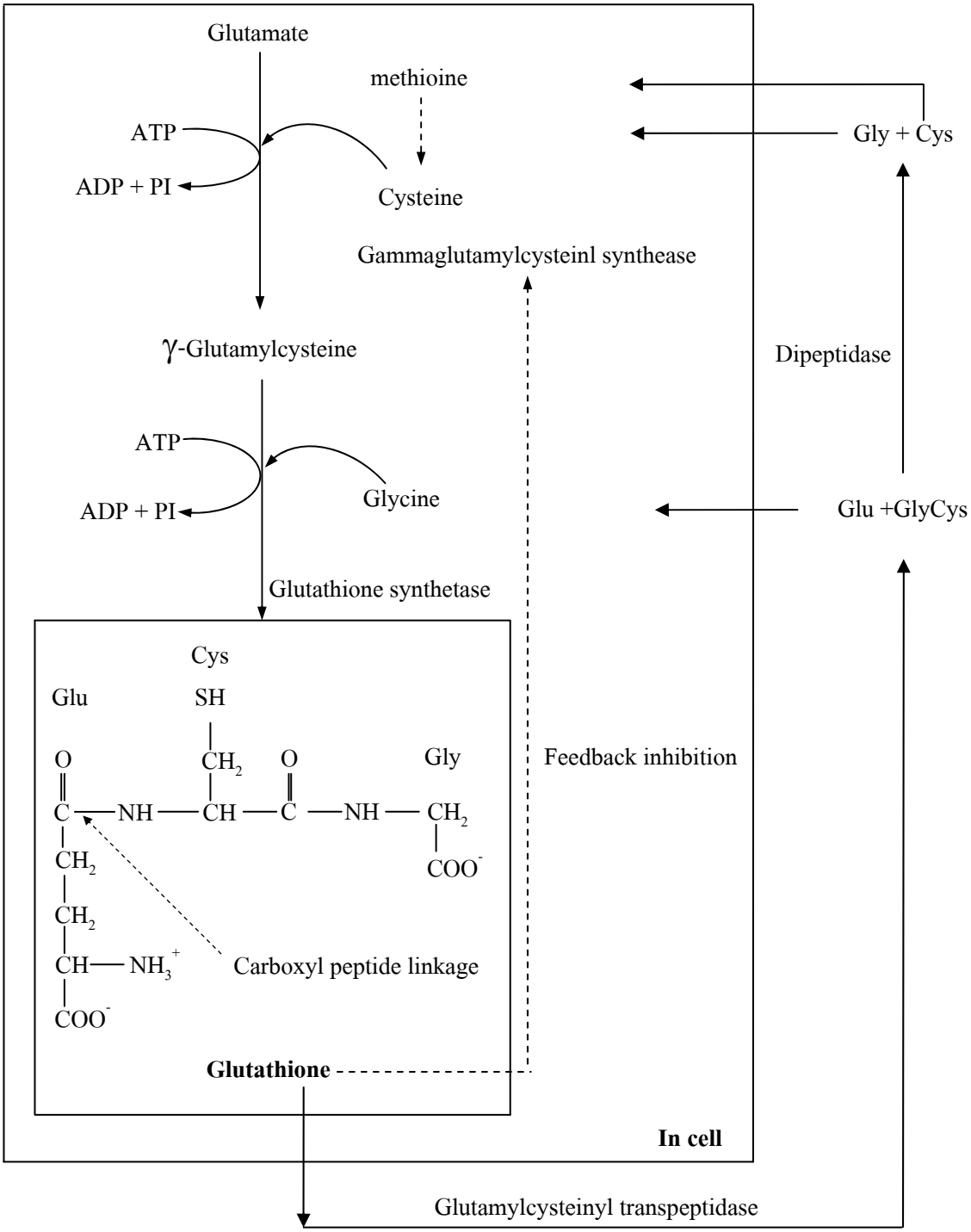


Figure 7. Glutathione synthesis and metabolism. (Glu = glutamate, Cys = cysteine, Gly = glycine)

The glutathione S-conjugates are metabolized by the same degradative enzymes that metabolize glutathione. The breakdown products of the glutathione S-conjugates are glutamate, glycine, and cysteine, which can also be reabsorbed into the cell. The glutamate and gylcine may then be used for glutathione synthesis, whereas the cysteine S-conjugates can be acetylated on the amino group of the cysteinyl resiue by intracellular *N*-acetyltransferases to from the corresponding mercapturic acid. Mercapturic acids are released into the circulation or bile, some are eventually excreted in urine, and some may undergo further metabolism (Hinchman *et al*., 1991).

The antioxidant function of GSH depends primarily on its role as a component of the enzymatic pathway that cells developed against ROS, consisting of glutathione peroxidase (GPx) and glutathione reductase (GR). Endogenously produced hydrogen peroxide or organic hydroperoxide are reduced by glutathione in presence of glutathione peroxidase. As a consequence, GSH is oxidized to GSSG, which in turn is rapidly reduced back to GSH by glutathione reductase at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH). The reduction of organic hydroperoxides by GSH may be catalyzed by either this glutathione peroxidase or by glutathione S-transferases (GST).

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + 2H_2O$$

$$2GSH + LOOH \xrightarrow{GPx} GSSG + H_2O + LOH$$

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

During the course of the reaction catalyzed by glutathione peroxidase, glutathione is recycled. In contrast, glutathione is consumed during the generation of glutathione S-conjugates by glutathione S-transferase. Both process lower the level of total in tracellular glutathione.

Therefore, in order to maintain a constant intracellular glutathione concentation, the glutathione consumed has to be replaced by resynthesis from its constituent amino acids (Cohen and Hochstein, 1963; Brigelius-flohe, 1999; Hinchman and Ballatori, 1994). Conjugation with GSH is an essential aspect of both xenobiotic and normal physiological metabolism (Strange *et al*., 2000; Eaton and Bammler, 1999). Formation of conjugates can result in depletion of GSH and has been used as a tool to study the role of GSH in antioxidant defense.

2.4 Benjakul

Benjakul (BJK) is a formular of traditional Thai medicine and composes of five dried medicinal plants, *Piper chaba* (Piperaceae) fruit, *Piper sarmentosum* (Piperaceae) root, *Piper sp*. (Piperaceae) stem that locally called Sa-karn, *Plumbago indica* (Plumbaginaceae) root and *Zingiber officinale* (Zingiberaceae) rhizome. BJK exhibits a number of beneficial effects against various types of diseases in humans. It was an ancient philosophical concept used to normalize the five primary elements (earth, water, air, fire and space) that regulate physiological function within the human body in relationship to the natural environment. It has been used at various different ratios based on traditional examination of patients to restore balance. The traditional Thai medicine holds that specific disease conditions are symptoms of an underlying imbalance and thus living in balance is the key to a long life free from disease.

Zingiber officinale rhizome *Plumbago indica* root

Piper chaba fruit *Piper sarmentosum* root *Piper sp*. ('Sa-karn') stem

Figure 8. Five dried medicinal plants of Benjakul (BJK)

Piper chaba fruit, *Piper sarmentosum* root and *Piper sp*. stem that locally called Sa-karn are all in family of Piperaceae which has over 700 species. ***Piper*** species, widely distributed in the tropical and subtropical regions of the world are used medicinally in various manners. They are erect or scandent herbs, shrubs

trees. They have high commercial, economical and medicinal importance. *Piper chaba* Hunter (syn. *P. retrofractum* Vahl.) fruit which is called 'Dee Plee', has been used as an anti-flatulent, stomachic, expectorant, antitussive, antifungal, uterus-contractile drug, sedative-hypnotic, appetizer, and counterirritant in the traditional medicine. (Virinder S. P et al, 1997). *Piper sarmentosum* is an erect herb with long creeping stems. The plant is usually found as a weed in villages and places with plenty of shade (Hsuan, 1990). Leaves alternate, simple, heart shaped and young leaves have a waxy surface. Flowers are bisexual or unisexual, in terminal or leaf opposite spikes. Fruit is small, dry, with several rounded bulges. Plant has a characteristic pungent odour (Wee, 1992; Wee and Hsuan, 1990; Hsuan, 1990; 1992). *Piper sarmentosum* roots are used to treat toothache (Wee, 1992; Duke and Ayensu, 1985; Toong and Wong, 1989) and are an effective remedy for, fungoid dermatitis on the feet, coughing, asthma and pleurisy (Toong and Wong, 1989; Duke and Ayensu, 1985). *Piper sarmentosum* (PS), a natural product, has been shown to have an antioxidant property.Sixteen compounds were isolated from the fresh roots of *Piper sarmentosum*. Seven of these have been previously isolated from the fruits and leaves of this plant: the aromatic alkene (1), 1-allyl-2-methoxy-4,5-methylenedioxybenzene (4), .BETA.-sitosterol, pyrrole amide (6), sarmentine (10), sarmentosine (13) and pellitorine (14). (+)-Sesamin (2), horsfieldin (3), two pyrrolidine amides 11 and 12, guineensine (15) and brachystamide B (16) are new for *P. sarmentosum*. Sarmentamide A, B, and C (7-9) are new natural products. Compounds 1-4 and 6-16 were tested for antiplasmodial, antimycobacterial and antifungal activities. (Author abst.) Leaves of *P. sarmentosum* was found to contain high antioxidant compounds such as Vitamin E, C, carotenoids, xantophylls, tannins and phenolics, it also has high antioxidant activity when tested using β-carotene bleaching method (Chanwitheesuk et al., 2005).

Plumbago is a genus of 10-20 species of flowering plants in the family Plumbaginaceae, native to warm temperate to tropical regions of the world. Plumbago indica, also known as Plumbago rosea or Scarlet leadwort, and Plumbago zeylanica, also know as White leadwort. Plumbago indica has red or pink flowers and Plumbago zeylanica has white ones, which appear in the late summer. Plumbago is perennial shrub that goes upto a height of 2 to 4 feet. Leaves of this plant can be measure as 3

inch long and 1 and ½ inch wide. The flower stalk is 4-12 cm long that produces red colour flowers in clusters. The fruit is a long and slender containing long seeds. The roots are long like fingers and are aromatic in taste. The root of plumbago acts as acrid, vesicant and abortefacient. The oil is used to treat rheumatism, joint pains and paralysis. It is also used to treat syphilis, leucoderma, skin disorders, indigestion, piles, worms, liver disorders and fever. Root contains plumbagin, sitosteraol glocoside. Plumbagin is used as gastric stimulant and appetizer; in large doses it is acro-narcotic poison. Locally it is vesicant ?. It has a specific action on the uterus. Root is said to increase the digestive power and promote appetite. Plumbagin stimulates the central nervous system in small doses, while with larger doses paralysis sets in leading ultimately to death. The blood pressure shows a slight fall. Plumbagin is a powerful irritant and has well marked antiseptic properties. In small doses, the drug is a sudorific ?; large doses cause death from respiratory failure. It is suggested that the action is probably due to the direct effect of the drug on the muscles. A liniment made from bruised root mixed with a little bland oil is used as a rubefacient in rheumatism, paralytic affections, in enlarged glands, buboes etc. Caution: Do not use when pregnant; use only in small doses. It can cause abortion. Taken in large doses, this herb can cause paralysis leading ultimately to death.

Zingiber officinale Rosc. (Zingiberaceae) has been used around the world in foods as a spice for over 2000 years (Bartley & Jacobs, 2000). Its dried rhizome has been used as a spice and in traditional Thai herbal medicines for the treatment of various illnesses that involve inflammation and which are caused by oxidative stress. (Awang, 1992, Wang and Wang, 2005 and Tapsell et al., 2006). The medicinal uses of ginger are diverse and used to promote digestion and as an antiflatulent or carminative to reduce gas and bloating (Lewis and Elvin-Lewis, 2003,; Chevallier, 2000 and Ody, 2000), for temporary relief and protection against gastrointestinal ulcers (McCann, 2003 and Wu et al., 1990), to improve blood circulation (Chevallier, 2000 and Ody, 2000), to lower blood glucose in the treatment of diabetes (Mascolo et al., 1989), to act as an anti-inflammatory against rheumatic pain and arthritis (Altman and Marcussen, 2001 and Bliddal et al., 2000), to treat cancer, due to its possible antitumorigenic effects (McCann, 2003). *In vitro* trials support the use of ginger preparations in the prevention of vomiting in pregnancy

(Apariman et al., 2006). The constituents of ginger are numerous and vary depending on the place of origin and whether the rhizomes are fresh or dry. The major bioactive constituents of ginger are polyphenol compounds such as [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol (Shukla and Singh, 2007) which have a high antioxidant activity (Chen et al., 1986 and Herrmann, 1994) and various pharmacological properties including anti-inflammatory, anticancer and anti-ulcer properties. The pungency of fresh ginger is due primarily to the gingerols, which are a homologous series of phenols. The most abundant is [6]-gingerol, although smaller quantities of other gingerols with different chain lengths are also present. The pungency of dry ginger mainly results from shogaols for example, [6]-shogaol, which are dehydrated forms of gingerols. Shogaols are formed from the corresponding gingerol during thermal processing (Wohlmuth et al., 2005). Although gingerols and shogaols are the major bioactive compounds present in *Zingiber officinale*, their molecular mechanisms of actions and the relationship between their structural features and the activity have not been well studied.

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals and plant material

Aspartate, alanine, α-ketoglutrate, pyruvic acid 2,4, dinitrophenyl hydrazine (2,4 DNPH) and carbon tetrachloride were purchased from Sigma Chemical Co. Reduced glutathione, sulfosalicylic acid, 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA) and 1,1,3,3 tetramethoxypropane (TMP) were purchased from Fluka. Trichloroacetic acid (TCA) was purchased from Merck. The *Piper chaba* fruit, *Piper sarmentosum* root, *Piper sp*. stem that locally called Sa-karn, *Plumbago indica* root and *Zingiber officinale* rhizome were purchased in dried forms from the traditional Thai-medicine pharmacy shop, at ministry of public health, Bangkok during 2006.

3.2. Peparation of plant extract

Five of plants, the *Piper chaba* fruit, *Piper sarmentosum* root, *Piper sp*. stem that locally called Sa-karn, *Plumbago indica* root and *Zingiber officinale* rhizome, in equal weight of 500 g each were washed thoroughly in tap water and shade dried. They were boiled with water (1:3) for 60 minutes and repeated for three times. The water was removed under reduced pressure to obtain the dried extract (8.187% yield, w/w).

3.3. Animals

Adult male Wistar rats (250-300 g) were obtained from Experimental Animal Care Center, Prince of Songkla University, Had-Yai, Thailand. They were housed under conventional laboratory conditions in a room temperature maintained at 25±1 °C and a relative humidity range of 40–75% with a regular 12 h light:12 h dark cycle. They were fed with pellet diet and water *ad libitum*. Rats were acclimated to laboratory conditions for 1 week before experiments. Experiments were approved by

a local ethical committee of Prince of Songkla University, Had-Yai, Thailand.

3.4. Experimental design

The experiment was divided into 2 groups of BJK treatment, 2 days pretreatment and 7 days pretreatment. Each pretreatment, rats were randomly divided into seven experimental groups of six animals each. The BJK pretreatment was given to group III-VII by gavage twice daily in the morning (08:00 a.m.) and in the evening (04:00 p.m.) for 2 or 7 days. CCl_4 (1.5 ml/kg 50 % in corn oil) was administered to group II-VII at 30 minute after BJK pretreatment in the morning on the second or the seventh day. The rats of the control group (group I) was received water and corn oil at the same time instead of the water extract of BJK and CCl_4, respectively. The seven groups were treated as follows:

Group I served as control group, was received water (5 ml/kg, orally) twice daily for 2 or 7 days and corn oil (1.5 ml/kg) 30 minute after water in the morning on the second or the seventh day

Group II served as CCL_4-treated group, was received water (5 ml/kg, orally) twice daily for 2 or 7 days and a single dose of carbon tetrachloride (1.5 ml/kg 50 % in corn oil) 30 minute after water in the morning on the second or the seventh day

Group III-VII served as BJK-pretreated groups, were received the water extract of Benjakul (BJK) at dose 62.5, 125, 250, 500 and 1,000 mg/kg body weight respectively (orally) twice daily for 2 or 7 days and a single dose of carbon tetrachloride (1.5 ml/kg in olive oil) 30 minute after BJK in the morning on the second or the seventh day

Animals were fasted overnight before CCl_4 administration and before the experimentation, but were allowed water ad libitum.

3.5. Collection of serum and tissue samples

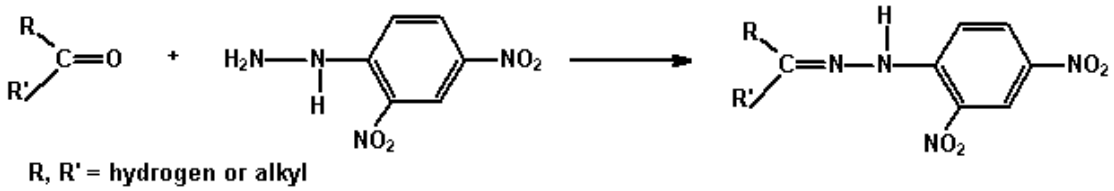
After 24 h of CCl_4 administration, blood was collected from the orbital venous plexus, under light ether anaesthesia, into non-heparinized tubes and then rats were sacrificed by cervical decapitation to collect liver. The blood samples were

allowed to clot for 45 min at room temperature. Serum was separated by centrifugation at 3,000 rpm for 15 min. The liver were rapidly excised, washed in ice-cold saline, and weight. The serum and liver samples were kept in -70 °C for biochemical determination.

3.6. Method

3.6.1 Serum transaminase (AST and ALT) assay

Principle: AST transfer an amino group from aspartate to alpha-KG and produce oxaloacetate and glutamate. ALT transfer an amino group from alanine to alpha-KG and produce pyruvate and glutamate. The products, oxaloacetate and pyruvate, form complex with 2,4, dinitrophenyl hydrazine (2,4 DNPH) and a red colour is produced on the addition of sodium hydroxide.



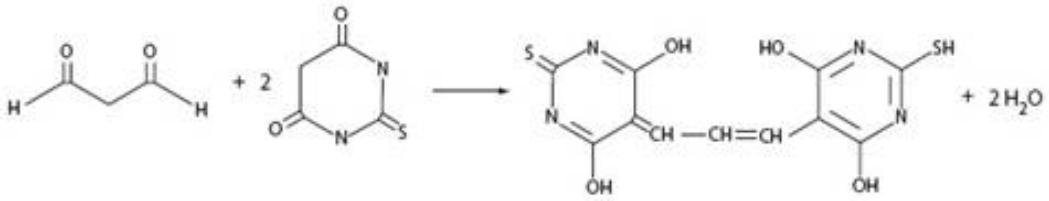
2,4, dinitrophenyl hydrazine **2,4, dinitrophenyl hydrazone (yellow to orange color)**

Figure 9. The complex of 2,4, dinitrophenyl hydrazine (2,4 DNPH)

Procedure: Serum aspartate transaminase(AST) and alanine transaminase (ALT) were determined by the method of Reitman and Frankel (1957). Each substrate (0.5 mL) [either α-l-alanine (200 mM) or l-aspartate (200 mM) with 2 mM α-ketoglutarate] was incubated for 5 min at 37 °C. A 0.1 mL of serum was added. The reaction mixture was incubated for 60 min and 30 min for AST and ALT, respectively. A 0.5 mL of 2,4-dinitrophenyl hydrazine (1 mM) was added to the reaction mixture and left for 30 min at room temperature. Finally, the colour as developed by addition of 5 ml NaOH (0.4 N) and the product formed was read at 505 nm using spectrophotometer. The AST and ALT activities were calculated in comparison with a standard AST and ALT curve and were expressed as IU/L.

3.6.2 Hepatic malondialdehyde (MDA) assay

Principle: Malondialdehyde (MDA), a product of lipid peroxidation, forms a 1:2 adduct with thiobarbituric acid in acidic condition and at 90°C. The TBA-MDA complex (pink-coloured) is measured by spectrophotometer at 532 nm. (Draper and Hadley, 1990).



MDA **TBAR** **MDA-TBAR adduct**

Figure 10. The reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA)

Procedure: Lipid peroxidation of rat liver using thiobarbituric acid was measured by a modified method of Ohkawa *et al*. (1979). One gram of rat liver tissue was homogenized in 3 ml of 50 mM potassium phosphate buffer (pH 7.0) by using a Heidolph homogenizers with a Teflon pestle. To 0.3 ml of liver homogenated in test tube, 1.5 ml of 10% trichloroacetic acid solution and 1.5 ml of 0.67% thiobarbituric acid solution were added. The mixture was boiled in waterbath at 95°C for 60 min and then cooling with tap water at room temperature. After centrifugation at 3,000 rpm for 15 min, the absorbance of sample was measured at 532 nm. 1,1,3,3 tetramethoxypropane (TMP) was used as a standard of malondialdehyde. The MDA content was calculated in comparison with a standard MDA curve and was expressed as nanomole/g of liver

3.8. Hepatic glutathione (GSH) assay

Principles: GSH can react with 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB or Ellman's reagent) to form a chromophore TNB (5-thio-2-nitrobenzoic acid) and GS-TNB. The TNB (yellow color) formed can be quantitated at 412 nm.with maximal absorbance at 412 nm.

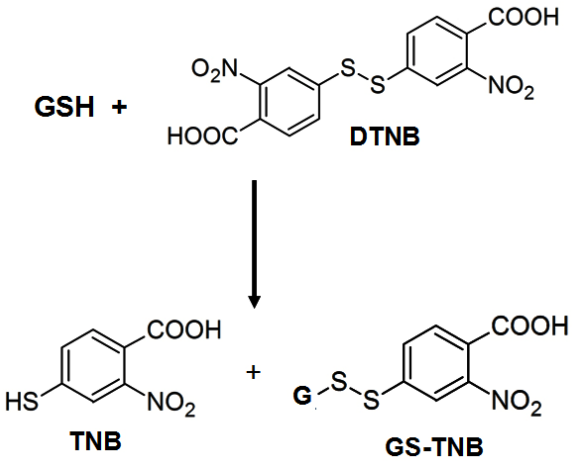


Figure 11. The reaction of 5, 5′-dithio, bis (2-nitrobenzoic acid)(DTNB) with Reduced glutathione (GSH)

Procedure: Reduced glutathione was measured according to the method of Ellman, G.L. (1959). The livers were homogenated with 5% salicylic acid (SSA) and after centrifugation at 3300 rpm for 15 min, supernatants were used for the estimation of GSH level. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5′-dithio, bis (2-nitrobenzoic acid or DTNB) and 0.4 ml double distilled water was added. Mixture was vortexed and the absorbance read at 412 nm within 15 min. The GSH content was calculated in comparison with a standard GSH curve and was expressed as micromole/g of liver

3.9. Statistical analysis

The data were expressed as mean ± S.E.M. One-way analysis of variance (ANOVA) followed by Least Significant Difference (LSD) comparisons test for multiple comparisons were used. Differences were considered statistically significant when $P < 0.05$.

CHAPTER 4

RESULTS

4.1 Effect of BJK treatment on body weight and liver weight.

Table 1 shows the body weights of rats in each group during BJK treatment for 7 days. The body weights of all rats were measured on the day 1, 3, 5 and 7 of the treatment. Compared with controls, there was no significant difference of the increment of body weight between the BJK-treated rats and the control rats.

For the relative liver weights (% of body weight) of rats after 24 hours of CCl_4 administration, shown in Table 2. It was observed that CCl_4-treated rats showed a significant increase in the relative liver weight when compared with the control group. The pretreatment of BJK at all dose for both 2 days and 7 days also showed the significant increase in the relative liver weights when compared with the control group, however there were not significant difference from the CCl_4-treated rats. The relative liver weights of BJK 1000 mg/kg treated rats for 7 days was significantly lower than that of BJK 125 mg/kg treated rats

4.2 Effect of BJK treatment on serum AST and ALT

The effects of BJK-treatment on the CCl_4-induced elevation of serum AST and ALT levels are shown in Table 3 and 4. Twenty four hours after administration of a toxic dose of CCl_4 (1.5 ml/kg, oral), caused hepatotoxicity in the rats, as indicated by the significant increment in AST and ALT levels . All dose of BJK treatment, both 2 days and 7 days treatment caused significant elevation of serum AST and ALT as compared to the control groups. Comparing to CCl_4-treated group, the treatment for 2 days showed that BJK high dose (1000 mg/kg) caused significant decrease in serum levels of AST and ALT (50 and 51 % of CCl_4-treated group) and BJK low dose (62.5 mg/kg) also caused decrease, but only serum ALT was significantly decreased (64 and 42 % of CCl_4-treated group) (Table 3).

For 7 days treatment, the serum AST and ALT of rats treated with BJK high dose (1000 mg/kg) were decreased to 55 and 57 % of CCl_4-treated group, even not significant difference, whereas with BJK low dose (62.5 mg/kg) significantly increased to 168 and 183 % of CCl_4 -treated rats (Table 4).

4.3 Effect of BJK treatment on lipid peroxidation

In order to evaluate the effect of BJK treatment on CCl_4-induced liver lipid peroxidation, the levels of MDA, one of the principal products of lipid peroxidation, were determined. A toxic dose of CCl_4 (1.5 ml/kg, oral) slightly increased MDA production in the liver homogenate, compared to control group. The treatment of BJK at all dose for both 2 days and 7 days showed no significant difference in the MDA content when compared with the CCl_4-treated rats. Only the MDA content of BJK 62.5 mg/kg-pretreated rats for 7 days showed slightly higher values than CCl_4-treated rats, however was not significantly different.

4.4 Effect of BJK treatment on hepatic glutathione

Hepatic GSH is an important intracellular antioxidant that can scavenge free radicals and could be important in the defense against radical-mediated hepatotoxicity. Table 6 shows the effect of BJK on the content of GSH in CCl_4-induced hepatotoxicity in rats. Twenty four hours after CCl_4 administration (1.5 ml/kg, oral), GSH levels were depleted approximately 15-27% of the control groups. All treatments of BJK, except 62.5 mg/kg for 7 days, showed restoration of hepatic GSH of which their amount showed significantly higher level than the CCl_4-treated rats. In addition, rats treated with BJK high dose (1000 mg/kg) for 7 days showed significant higher amount of GSH than the control rats.

Table 1 Effect of Benjakul treatment for 7 days on body weight

Treatment	Body weight (g)			
	Day 1	Day 2	Day 5	Day 7
Control (C)	254.83 ± 4.53	261.67 ± 4.22	260.17 ± 4.09	260.83 ± 3.49
CCl_4	254.67 ± 6.92	261.67 ± 6.15	259.50 ± 6.44	260.17 ± 6.58
BJK 62.5 mg/kg + CCl_4	267.50 ± 4.49	279.17 ± 5.23	283.00 ± 5.30	283.83 ± 5.95
BJK 125 mg/kg + CCl_4	262.50 ± 5.38	275.00 ± 4.65	276.17 ± 4.40	277.33 ± 4.42
BJK 250 mg/kg + CCl_4	269.17 ± 9.87	271.83 ± 10.06	274.00 ± 10.57	274.00 ± 10.57
BJK 500 mg/kg + CCl_4	281.67 ± 5.87	283.17 ± 6.99	282.50 ± 7.48	282.50 ± 7.48
BJK 1000 mg/kg + CCl_4	274.17 ± 4.36	277.50 ± 4.46	279.83 ± 4.99	278.17 ± 4.63

The rats were treated with BJK (62.5, 125, 250, 500 and 1000 mg/kg, oral) twice daily for 7 consecutive days. The control rats were given water. Body weight of rats was measured for every two days. Rats were killed on the day 8.

Each value represents the mean ± S.E.M of six rats.

a Significantly different from the control at $P < 0.05$

b Significantly different from CCl_4 at $P < 0.05$

c Significantly different from BJK + CCl_4 at $P < 0.05$

Table 2 Effect of Benjakul treatment on liver weight

Treatment	Liver weight			
	2 days BJK treatment		7 days BJK treatment	
	% of BW	% Change from C	% of BW	% Change from C
Control (C)	2.60 ± 0.08	100	2.35 ± 0.03	100
CCl_4	3.62 ± 0.14 [a]	139	3.49 ± 0.15 [a]	148
BJK 62.5 mg/kg + CCl_4	3.60 ± 0.11 [a]	138	3.53 ± 0.09 [a]	150
BJK 125 mg/kg + CCl_4	3.46 ± 0.12 [a]	133	3.66 ± 0.11 [ab]	156
BJK 250 mg/kg + CCl_4	3.43 ± 0.08 [a]	132	3.54 ± 0.13 [a]	150
BJK 500 mg/kg + CCl_4	3.73 ± 0.13 [a]	143	3.43 ± 0.08 [a]	145
BJK 1000 mg/kg + CCl_4	3.44 ± 0.08 [a]	132	3.36 ± 0.07 [ac]	143

Rats were pretreated with BJK (62.5, 125, 250, 500 and 1000 mg/kg BW) twice daily for either 2 or 7 days. A single dose of carbon tetrachloride (1.5 ml/kg, CCl_4 1:1 in corn oil, orally) was administered on the second or seventh day, respectively. Blood samples and liver tissues were collected after 24 h of CCl_4 administration. Each value represents the mean ± S.E.M of six rats.

a Significantly different from the control at $P < 0.05$

b Significantly different from CCl_4 at $P < 0.05$

c Significantly different from BJK (125 mg/kg)+ CCl_4 at $P < 0.05$

Table 3 Effect of Benjakul treatment for 2 days on CCl_4–induced hepatotoxicity

Treatment	Serum AST (U/L)	% Change from CCl_4	Serum ALT (U/L)	% Change from CCl_4	ALT:AST ratio
Control (C)	95.62 ± 2.67		30.37 ± 1.68		0.3
CCl_4	904.70 ± 198.94[a]	100	484.06 ± 114.73[a]	100	0.6
BJK 62.5 mg/kg + CCl_4	581.49 ± 90.34[a]	64	205.42 ± 15.32[ab]	42	0.3
BJK 125 mg/kg + CCl_4	545.72 ± 77.94[a]	60	304.74 ± 42.36[a]	63	0.7
BJK 250 mg/kg + CCl_4	725.01 ± 197.39[a]	80	462.94 ± 136.43[a]	96	0.6
BJK 500 mg/kg + CCl_4	734.82 ± 193.98[a]	81	440.04 ± 110.46[a]	91	0.5
BJK 1000 mg/kg + CCl_4	448.51 ± 39.88[b]	50	236.23 ± 20.65[b]	49	0.5

Rats were pretreated with BJK (62.5, 125, 250, 500 and 1000 mg/kg BW) twice daily for 2 days. A single dose of carbon tetrachloride (1.5 ml/kg, CCl_4 1:1 in corn oil, orally) was administered on the second day, respectively. Blood samples and liver tissues were collected after 24 h of CCl_4 administration. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined. Each value represents the mean ± S.E.M of six rats.

a Significantly different from the control at $P < 0.05$

b Significantly different from CCl_4 at $P < 0.05$

c Significantly different from BJK + CCl_4 at $P < 0.05$

Table 4 Effect of Benjakul treatment for 7 days on CCl_4–induced hepatotoxicity

Treatment	Serum AST (U/L)	% Change from CCl_4	Serum ALT (U/L)	% Change from CCl_4	ALT:AST ratio
Control (C)	112.80 ± 14.55		36.67 ± 3.68		0.3
CCl_4	1824.28 ± 451.73[a]	100	1439.39 ± 388.30[a]	100	0.8
BJK 62.5 mg/kg + CCl_4	3071.63 ± 508.26[ab]	168	2640.53 ± 486.97[ab]	183	0.9
BJK 125 mg/kg + CCl_4	1967.87 ± 138.58[a]	108	1678.20 ± 171.32[a]	117	0.9
BJK 250 mg/kg + CCl_4	1568.40 ± 562.34[a]	86	1290.85 ± 381.41[a]	90	0.8
BJK 500 mg/kg + CCl_4	2333.78 ± 450.40[a]	128	1771.11 ± 261.70[a]	123	0.8
BJK 1000 mg/kg + CCl_4	999.56 ± 150.08[a]	55	825.64 ± 205.62[a]	57	0.8

Rats were pretreated with BJK (62.5, 125, 250, 500 and 1000 mg/kg BW) twice daily for 7 days. A single dose of carbon tetrachloride (1.5 ml/kg, CCl_4 1:1 in corn oil, orally) was administered on the seventh day, respectively. Blood samples and liver tissues were collected after 24 h of CCl_4 administration. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined. Each value represents the mean ± S.E.M of six rats.

a Significantly different from the control at $P < 0.05$

b Significantly different from CCl_4 at $P < 0.05$

c Significantly different from BJK + CCl_4 at $P < 0.05$

Table 5 Effect of Benjakul treatment on lipid peroxidation

Treatment	Lipid peroxidation			
	2 days BJK treatment		7 days BJK treatment	
	MDA (nmole/g liver)	% Change from C	MDA (nmole/g liver)	% Change from C
Control (C)	39.02 ± 4.62	100	33.99 ± 4.08	100
CCl_4	48.17 ± 4.88	123	38.30 ± 5.15	113
BJK 62.5 mg/kg + CCl_4	42.19 ± 4.99	108	44.16 ± 1.85	130
BJK 125 mg/kg + CCl_4	43.61 ± 5.96	112	39.65 ± 6.89	117
BJK 250 mg/kg + CCl_4	50.72 ± 8.35	130	34.02 ± 3.58	100
BJK 500 mg/kg + CCl_4	45.86 ± 6.86	118	39.75 ± 4.31	117
BJK 1000 mg/kg + CCl_4	48.97 ± 7.15	125	40.26 ± 1.81	118

Rats were pretreated with BJK (62.5, 125, 250, 500 and 1000 mg/kg BW) twice daily for either 2 or 7 days. A single dose of carbon tetrachloride (1.5 ml/kg, CCl_4 1:1 in corn oil, orally) was administered on the second or seventh day, respectively. Blood samples and liver tissues were collected after 24 h of CCl_4 administration. Hepatic maldehyde (MDA) was measured.

Each value represents the mean ± S.E.M of six rats.

a Significantly different from the control at $P < 0.05$

b Significantly different from CCl_4 at $P < 0.05$

c Significantly different from BJK + CCl_4 at $P < 0.05$

Table 6 Effect of BJK treatment on hepatic glutathione

Treatment	Hepatic GSH			
	2 days BJK treatment		7 days BJK treatment	
	μmole/g liver	% Change from C	μmole/g liver	% Change from C
Control (C)	5.21 ± 0.14	100	4.63 ± 0.29	100
CCl_4	3.81 ± 0.09[a]	73	3.95 ± 0.47	85
BJK 62.5 mg/kg + CCl_4	5.92 ± 0.26[b]	114	4.33 ± 0.14	94
BJK 125 mg/kg + CCl_4	5.96 ± 0.23[b]	114	5.43 ± 0.15[b]	117
BJK 250 mg/kg + CCl_4	4.89 ± 0.39[b]	94	5.03 ± 0.58[b]	109
BJK 500 mg/kg + CCl_4	5.22 ± 0.18[b]	100	5.09 ± 0.32[b]	110
BJK 1000 mg/kg + CCl_4	5.71 ± 0.49[b]	110	6.41 ± 0.33[ab]	138

Rats were pretreated with BJK (62.5, 125, 250, 500 and 1000 mg/kg BW) twice daily for either 2 or 7 days. A single dose of carbon tetrachloride (1.5 ml/kg, CCl_4 1:1 in corn oil, orally) was administered on the second or seventh day, respectively. Blood samples and liver tissues were collected after 24 h of CCl_4 administration. Hepatic reduced glutathione (GSH) was measured.

Each value represents the mean ± S.E.M of six rats.

a Significantly different from the control at $P < 0.05$

b Significantly different from CCl_4 at $P < 0.05$

c Significantly different from BJK + CCl_4 at $P < 0.05$

CHAPTER 5

DISCUSSION

Considering the beneficial effects of BJK against various types of diseases in humans, the present study was carried out in order to evaluate whether BJK would reduce the extent of hepatic injury induced by CCl_4 in rats. The present study, BJK resulted either protection or potentiation against CCl_4-induced hepatotoxicity in rats, depending on the dose and time of treatment of BJK. Pretreatment for 2 days, BJK showed some protection against the CCl_4-induced hepatotoxicity in rats as evidenced by the significant decrease in serum AST and ALT activity in BJK high dose (1000 mg/kg) treated rats and serum ALT in BJK low dose (62.5 mg/kg) treated rats when compared to the CCl_4-treated rats. For 7 days pretreatment, BJK at high dose (1000 mg/kg) also lowered serum AST and ALT activity, even not significant, however BJK at low dose (62.5 mg/kg) showed potentiation as evidenced by the significant increase in serum AST and ALT activity when compared to the CCl_4-treated rats. The levels of hepatic MDA and hepatic GSH were determined to investigate the mechanism. However, these effects were not confirmed by histological parameters.

Liver injury induced by CCl_4 is the best-characterized system of the xenobiotic-induced hepatotoxicity and is a commonly used model for the screening the anti-hepatotoxic / hepatoprotective activity of drugs (Brattin *et al*., 1985; Recknagel *et al*., 1989; Brent and Rumack, 1993). It is now generally accepted that the hepatotoxicity of CCl_4 is the result of formation of the highly reactive trichloromethyl free radical ($^{\bullet}CCl_3$) by cytochrome P-450 system, mainly through CYP 2E1. These trichloromethyl free radicals then readily interacts with oxygen to form highly reactive trichloromethylperoxy radical (CCl_3OO) (Brattin *et al*., 1985; Recknagel *et al*., 1991; Williams and Burk, 1990; Brent and Rumack, 1993). Both radicals may covalently binds to cellular molecules such as nucleic acid, protein and lipids, especially the

polyunsaturated fatty acids to initiate a process of lipid peroxidation by attacking the methylene bridges of unsaturated fatty acid side chains. This invariably affects the permeability of mitochondrial, endoplasmic reticulum and plasma membrane, resulting in the loss of cellular calcium sequestration and homeostasis and ultimately in cell damage (Boll *et al*., 2001). Liver weight also generally increases as a consequence of CCl_4–induced hepatic damage and the consequent fibrosis and hypertrophy of the liver. The changes in liver weight after CCl_4 dosing are a valuable index of the extent of hepatic damage (Fukao, 2004; Wu *et al*., 2004). Our study showed that the liver weights of the control CCl_4-treated rats in both 2 and 7 days treatment were significantly higher than that of the control normal rats. The pretreatment of BJK at all doses for both 2 and 7 days also increased the relative liver weights to nearly the same extent as the control CCl_4-treated rats.

When liver cells are damaged, a variety of enzymes located normally in cytosol is released into the blood, thereby causing increased enzyme levels in the serum (Asha, 2001). In the other word, leakage of large quantities of enzymes into the blood stream is often associated with loss of the functional integrity of the cell membranes in liver (Rajesh and Latha, 2004) and massive necrosis of the liver (Rees and Spector, 1961). One of the most sensitive and dramatic indicators of hepatocyte injury is the release of intracellular enzymes, such as aspartate transaminases (AST) and alanine transaminases (ALT) into the circulation. The elevated activities of these enzymes are indicative of cellular leakage and loss of the functional integrity of the cell membranes in liver (Rajesh and Latha, 2004). AST is found in various tissues such as the liver, cardiac muscles, skeletal muscles, pancreas, lungs, kidney, brain, etc., whereas ALT concentration is highest in the liver and therefore, it appears to be a more sensitive test to hepatocellular damage than AST (Lin *et al*., 1997). It is well known that hepatoxic agents cause leakage of both enzymes into the blood stream. CCl_4 is known to cause marked elevation in serum AST and ALT, which is indicative of hepatocyte injury.

Our results showed that 24 h after a single toxic dose of CCl_4 (1.5 ml/kg in corn oil, oral) caused increase in serum AST and ALT levels higher than those of normal values as compared with the control group. This demonstrated that CCl_4 caused severe acute liver damage in rats. Treatment of BJK for 2 days, BJK at high dose (1000 mg/kg) significantly decreased serum AST and ALT activity (50 and 49 % of CCl_4) and BJK at low dose (62.5 mg/kg) significantly decreased serum ALT activity (42 % of CCl_4). In addition, BJK at high dose (1000 mg/kg) for 7 days pretreatment also lowered serum AST and ALT activity (55 and 57 % of CCl_4) even not significant difference when compared to the CCl_4-treated rats. These indicated that BJK showed some protection of CCl_4-induced hepatotoxicity in rats.

Because the bioactivation is needed to occur in this model of CCl_4-induced hepatotoxicity. $CC1_4$ is activated mainly by CYP 2E1 (Weber *et al*., 2003; Lee *et al*., 2001). Several studies have reported that compounds or drugs that inhibits CYP 2E1 protect against CCl_4-induced toxicity (Kim *et al*., 1997; Jeong, 1999). On the other hand, compounds that induce CYP 2E1 potentiate the hepatotoxicity of CCl_4 (Allis *et al*., 1996). However, after CCl_4 bioactivation, the resulting radicals either trichloromethyl or trichloromethylperoxy radical may bind covalently to CYP 2E1, either to the active site or to the heme group of the enzyme, thereby causing suicide inactivation (Weber *et al*., 2003; Fernández *et al*., 1982; Roberts *et al*., 1995). The present study showed the significant increase in the serum AST and ALT levels in rats treated with BJK 62.5 mg/kg for 7 days when compared to the CCl_4-treated rats. This might be due to the induction of CYP 2E1.

Lipid peroxidation, is accepted to be one of the major causes of CCl_4-induced liver injury, and is mediated by the production of free radical derivatives of CCl_4. In higher animals, lipid peroxidation was known to cause destabilization and disintegration of the cell membrane, leading to liver injury, arteriosclerosis and kidney damage (Chang *et al*., 1994). Peroxy radicals are important agents that mediate lipid peroxidation thereby damaging cell membrane. The elevation of MDA levels, which is one of the end products of lipid peroxidation in the liver tissue, and the reduction of

hepatic GSH levels are important indicators of oxidative stress in CCl_4-treated rats (Souza *et al*., 1997). Enhanced lipid peroxidation expressed in terms of MDA contents and reduction in liver GSH level in CCl_4 treated rats indicates the damage to the hepatic cells (Bhandarkar and Khan, 2004; Suja *et al*., 2004). In the present study, the CCl_4-treated rats slightly increased MDA level. The treatment with BJK at all doses for both 2 days and 7 days showed no significant difference in the MDA content when compared with the CCl_4-treated rats. Only the MDA content of BJK 62.5 mg/kg-pretreated rats for 7 days showed the slightly higher values, however it was not significantly different.

In contrast to the toxic activation of CCl_4 via the CYP 2E1 pathway, the detoxification pathway involves GSH and antioxidant enzymes. Many studies have shown that GSH plays a key role in detoxifying the reactive toxic metabolites of CCl_4 and that liver necrosis begins when the GSH stores are markedly depleted (Recknagel *et al*., 1991; Williams and Burk, 1990). Glutathione is synthesized within the cell in two steps. The first step, cysteine and glutamate are combined by the enzyme γ-glutamylcysteine synthetase. The second step, adding glycine to the γ-glutamylcysteine dipeptide to form GSH, is catalyzed by glutathione synthetase. The γ-glutamylcysteine synthetase is feedback inhibited by GSH and does not appear saturated at normal cellular levels of cysteine, so that increased cysteine can promote GSH synthesis under certain circumstances (Whitcomb and BlocK, 1994). The role of GSH in eliminating the reactive toxic metabolites of hepatotoxins involves GSH conjugation via glutathione-S-transferase (GST) and/or GSH oxidation via. glutathione peroxidase (GPx). Detoxification by GSH conjugation via GST causes decrease in GSH and GST whereas GSH oxidation causes decreasing in GSH and GPx together with increase in GSSG. GSSG can be reduced to GSH by glutathione reductase (GR), which is NADPH-dependent. Therefore, reduction in hepatic GSH indicates the damage to the hepatic cells. Carbon tetrachloride induced depletion of hepatic glutathione. In fed mice with acute liver injury induced by intraperitoneal administration of CCl_4 (1 ml/kg), hepatic GSH content was decreased to less than 70% of the control level between 2 and 6 h after the administration, and then slightly recovered by 24 h after the administration (Nishida *et al*., 1996b). Nishida K et

al (1998) also reported that hepatic GSH concentrations at 3, 6, 12, and 24 h after CCl_4-injected in mice were 68, 70, 74, and 73%, respectively, of those in the control group. Our results showed that GSH levels at 24 h after CCl_4 administration in rats were 73-85% of that in the control group. The significant increase in hepatic GSH (>100%) in rats treated with BJK 1000 mg/kg for 7 days demonstrated that BJK can protect depletion.

Benjakul, composes of five medicinal plants including *Piper chaba* fruit, *Piper sarmentosum* root, *Piper sp*. stem that locally called Sa-karn, *Plumbago indica* root and *Zingiber officinale* rhizome, contains several bioactive constituents. Among these, the major bioactive constituents of *Zingiber officinale (*ginger), polyphenol compounds such as [6]-gingerol, [8]-gingerol, [10]-gingerol and [6]-shogaol (Shukla and Singh, 2007), had been reported having a high antioxidant activity (Chen *et al*., 1986 and Herrmann, 1994). The polyphenols in the ginger extract demonstrated a higher chelatoforming capacity with regard to Fe^{3+}, leading to the prevention of the initiation of hydroxyl radicals which is known as inducer of lipid peroxidation. The ginger extract also showed an inhibiting effect with regard to the hydroxyl radicals, better than that of quercetin in the study (Chang Xin Zhou *et al*, 2007). Yemitan and Izegbu (2006) reported that pretreatment of rats with an ethanol extract of the rhizome of *Z. officinale* and oil extracted from the plant were effective in ameliorating carbon tetrachloride and acetaminophen (paracetamol)-induced acute hepatotoxicity. Ajith T.A. et al (2007) also reported that *Zingiber officinale* showed protective effect against acetaminophen-induced acute hepatotoxicity by enhancing hepatic antioxidant status. Moreover, three new diarylheptanoids from the methanol extract of ginger rhizome were recently isolated and exhibited important bioactivities such as inhibitory effects on the biosyntheses of prostaglandin and leukotriene, activities of anti-fungal and anti-oxidation and cancer chemopreventive activity.

Piperine, a major plant alkaloid present in *Piper* species, is known to possess several pharmacological actions, such as antimicrobial, antifungal, anti-inflammatory (Lee, 1984) and antioxidant effects (Piyachaturawat, 1995). It is also known to inhibit the xenobiotic metabolizing enzymes and enhance the cellular

antioxidants and cytoprotective activities in rat hepatoma cells (Allanch A, 1991). There was reported that piperine given by oral route at a dose of 100 mg/kg enhanced enzymatic and non-enzymatic antioxidants and suppressed lipid peroxidation in benzo[*a*]pyrene induced lung cancer (Selvendiran K., 2003). However, Piyachaturawat (1995) demonstrated that piperine potentiated the CCl_4-induced hepatotoxicity in rats by interacting with liver cells and increased the activity of NADPH-cytochrome c reductase.

The results showed that the pretreatment of BJK at high dose (1000 mg/kg) for both 2 and 7 days and BJK at low dose (62.5 mg/kg) for 2 days showed some protection whereas BJK at low dose (62.5 mg/kg) for 7 days showed potentiation against the CCl_4-induced hepatotoxicity in rats, evidenced by the level of serum transaminase, hepatic MDA and hepatic GSH. These results demonstrated the interactions of several effects of different active constituents of BJK including enzyme inhibiting effect, enzyme inducing effect, antioxidant activities, anti-inflammatory, which makes it difficult to elucidate.

In conclusion, BJK pretreatment showed unconsistently effects on the CCl_4-induced hepatptoxicity in rats, either protection or potentiation depending on the dose of BJK and time of pretreatment. These complex results may involved interaction of various effects of numerous constituents in different herbs in BJK and need further investigations and challenge to elucidate the mechanism involved. However, this study suggested that the co-use of modern and traditional therapies, becoming more common, need to be used carefully because the herb–toxicant or herb-drug interactions may involved both pharmacokinetics and pharmacodynamics and may give positive and negative results.

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APPENDIX

APPENDIX 1

Effect of BJK treatment twice a day for 7 days on body weight and liver weight in carbon-tetrachloride induced hepatotoxicity in rats.

Group	Treatment	Rat no.	B.W. (g) Day 1	Day 3	Day 5	Day 7	L.W. (g)	%BW
1	Control(water)	1	260	265	261	261	5.9	2.26
	Oil 1.5 ml/kg	2	256	260	258	259	6.0	2.32
		3	238	250	252	254	6.0	2.36
		4	266	275	271	265	6.5	2.45
		5	245	250	247	251	5.8	2.31
		6	264	270	272	275	6.6	2.40
	Mean		**254.83**	**261.67**	**260.17**	**260.83**	**6.13**	**2.35**
	S.D.		**11.11**	**10.33**	**10.03**	**8.54**	**0.33**	**0.07**
	S.E.		**4.53**	**4.22**	**4.09**	**3.49**	**0.14**	**0.03**
2	CCL_4 1.5 ml/kg	1	277	280	279	280	8.5	3.04
		2	225	235	233	234	8.1	3.46
		3	252	260	260	258	9.4	3.64
		4	261	270	271	274	9.3	3.39
		5	258	265	260	260	8.5	3.27
		6	255	260	254	255	10.5	4.12
	Mean		**254.67**	**261.67**	**259.50**	**260.17**	**9.05**	**3.49**
	S.D.		**16.95**	**15.06**	**15.78**	**16.13**	**0.87**	**0.37**
	S.E.		**6.92**	**6.15**	**6.44**	**6.58**	**0.36**	**0.15**
3	BJK 62.5 mg/kg	1	276	295	296	298	10.7	3.59
	+ CCl_4 1.5 ml/kg	2	279	290	296	299	10.5	3.51
		3	261	275	283	285	10.6	3.72
		4	255	265	268	264	9.9	3.75
		5	277	285	288	288	9.1	3.16
		6	257	265	267	269	9.3	3.46
	Mean		**267.50**	**279.17**	**283.00**	**283.83**	**10.02**	**3.53**
	S.D.		**10.99**	**12.81**	**12.99**	**14.58**	**0.69**	**0.21**
	S.E.		**4.49**	**5.23**	**5.30**	**5.95**	**0.28**	**0.09**

APPENDIX 1 (continued)

Effect of BJK treatment twice a day for 7 days on body weight and liver weight in carbon-tetrachloride induced hepatotoxicity in rats.

Group	Treatment	Rat no.	B.W. (g) Day 1	Day 3	Day 5	Day 7	L.W. (g)	%BW
4	BJK 125 mg/kg + CCl_4 1.5 ml/kg	1	279	285	284	284	9.0	3.17
		2	253	270	274	277	10.1	3.65
		3	244	255	256	256	9.6	3.75
		4	260	280	279	281	10.8	3.84
		5	264	275	278	281	10.0	3.56
		6	275	285	286	285	11.3	3.96
	Mean		**262.50**	**275.00**	**276.17**	**277.33**	**10.13**	**3.66**
	S.D.		**13.19**	**11.40**	**10.78**	**10.82**	**0.82**	**0.28**
	S.E.		**5.38**	**4.65**	**4.40**	**4.42**	**0.34**	**0.11**
5	BJK 250 mg/kg + CCL_4 1.5 ml/kg	1	235	236	240	240	8.0	3.33
		2	255	257	255	255	8.9	3.49
		3	290	292	292	292	11.9	4.08
		4	285	290	286	286	10.2	3.57
		5	255	259	262	262	8.3	3.17
		6	295	297	309	309	11.2	3.62
	Mean		**269.17**	**271.83**	**274.00**	**274.00**	**9.75**	**3.54**
	S.D.		**24.17**	**24.65**	**25.90**	**25.90**	**1.60**	**0.31**
	S.E.		**9.87**	**10.06**	**10.57**	**10.57**	**0.65**	**0.13**
6	BJK 500 mg/kg + CCl_4 1.5 ml/kg	1	260	259	259	259	8.5	3.28
		2	280	275	269	269	9.7	3.61
		3	280	279	282	282	9.0	3.19
		4	285	292	291	291	10.2	3.51
		5	305	310	312	312	11.5	3.69
		6	280	284	282	282	9.3	3.30
	Mean		**281.67**	**283.17**	**282.50**	**282.50**	**9.70**	**3.43**
	S.D.		**14.38**	**17.13**	**18.32**	**18.32**	**1.06**	**0.20**
	S.E.		**5.87**	**6.99**	**7.48**	**7.48**	**0.43**	**0.08**
7	BJK 1000 mg/kg + CCl_4 1.5 ml/kg	1	285	284	283	280	9.2	3.29
		2	280	277	285	279	10.0	3.58
		3	260	267	268	268	8.8	3.28
		4	265	269	268	269	8.7	3.23
		5	270	272	275	274	9.7	3.54
		6	285	296	300	299	9.6	3.21
	Mean		**274.17**	**277.50**	**279.83**	**278.17**	**9.33**	**3.36**
	S.D.		**10.68**	**10.93**	**12.22**	**11.34**	**0.52**	**0.16**
	S.E.		**4.36**	**4.46**	**4.99**	**4.63**	**0.21**	**0.07**

APPENDIX 2

Effect of BJK treatment twice a day for 7 days on AST, ALT, GSH and MDA in carbon-tetrachloride induced hepatotoxicity in rats.

Group	Treatment	Rat no.	AST IU/L	ALT IU/L	MDA nmole/g liver	GSH μmole/g liver
1	Control(water)	1	108.88	42.62	28.93	5.79
	Oil 1.5 ml/kg	2	81.36	24.47	45.38	4.22
		3	118.70	42.08	23.83	3.78
		4	85.32	31.45	29.43	5.03
		5	179.58	48.31	28.56	4.32
		6	102.95	31.09	47.81	4.66
	Mean		**112.80**	**36.67**	**33.99**	**4.63**
	S.D.		**35.64**	**9.03**	**10.00**	**0.71**
	S.E.		**14.55**	**3.68**	**4.08**	**0.29**
2	CCL_4 1.5 ml/kg	1	363.34	248.68	41.64	4.90
		2	2671.05	2196.36	42.89	4.57
		3	2671.05	2196.36	32.05	3.37
		4	2076.35	1532.95	26.19	5.35
		5	492.81	265.61	27.31	3.09
		6	2671.05	2196.36	59.71	2.43
	Mean		**1824.28**	**1439.39**	**38.30**	**3.95**
	S.D.		**1106.51**	**951.14**	**12.63**	**1.15**
	S.E.		**451.73**	**388.30**	**5.15**	**0.47**
3	BJK 62.5 mg/kg	1	3808.38	3557.50	46.63	4.26
	+ CCl_4 1.5 ml/kg	2	4303.99	3557.50	42.19	4.86
		3	1612.84	1309.23	41.83	4.18
		4	4303.99	3557.50	45.38	3.86
		5	1688.93	978.94	37.66	4.38
		6	2711.64	2882.50	49.31	4.47
	Mean		**3071.63**	**2640.53**	**44.16**	**4.33**
	S.D.		**1244.97**	**1192.84**	**4.52**	**0.33**
	S.E.		**508.26**	**486.97**	**1.85**	**0.14**
4	BJK 125 mg/kg	1	1479.23	1305.38	35.17	5.71
	+ CCl_4 1.5 ml/kg	2	2361.15	2141.82	43.89	5.84
		3	1636.75	1139.25	28.56	5.65
		4	2191.58	2071.36	33.55	5.03
		5	2104.24	1905.68	25.20	5.38
		6	2034.27	1505.68	71.55	4.97
	Mean		**1967.87**	**1678.20**	**39.65**	**5.43**
	S.D.		**339.45**	**419.64**	**16.88**	**0.37**
	S.E.		**138.58**	**171.32**	**6.89**	**0.15**

APPENDIX 2 (continued)

Effect of BJK treatment twice a day for 7 days on AST, ALT, GSH and MDA in carbon-tetrachloride induced hepatotoxicity in rats.

Group	Treatment	Rat no.	AST IU/L	ALT IU/L	MDA nmole/g liver	GSH μmole/g liver
5	BJK 250 mg/kg + CCL_4 1.5 ml/kg	1	921.83	1169.47	41.27	4.77
		2	751.84	411.33	47.38	7.34
		3	2051.99	2435.00	33.79	4.28
		4	912.84	901.08	29.06	4.75
		5	4175.00	2435.00	24.07	3.21
		6	596.89	393.23	28.56	5.86
	Mean		**1568.40**	**1290.85**	**34.02**	**5.03**
	S.D.		**1377.44**	**934.25**	**8.77**	**1.42**
	S.E.		**562.34**	**381.41**	**3.58**	**0.58**
6	BJK 500 mg/kg + CCl_4 1.5 ml/kg	1	4341.60	2181.97	50.99	3.82
		2	2108.78	2181.97	48.75	4.70
		3	1692.79	1407.84	35.54	5.12
		4	2152.03	2056.97	27.94	5.38
		5	1112.47	615.91	28.06	6.22
		6	2595.00	2181.97	47.25	5.33
	Mean		**2333.78**	**1771.11**	**39.75**	**5.09**
	S.D.		**1103.24**	**641.03**	**10.56**	**0.80**
	S.E.		**450.40**	**261.70**	**4.31**	**0.32**
7	BJK 1000 mg/kg + CCl_4 1.5 ml/kg	1	707.36	372.60	37.35	6.02
		2	1634.61	1693.33	43.14	5.42
		3	727.68	480.23	42.39	6.59
		4	706.79	576.31	46.01	7.09
		5	1148.24	1152.88	33.79	5.80
		6	1072.70	678.49	38.90	7.53
	Mean		**999.56**	**825.64**	**40.26**	**6.41**
	S.D.		**367.63**	**503.66**	**4.43**	**0.81**
	S.E.		**150.08**	**205.62**	**1.81**	**0.33**

APPENDIX 3

Effect of BJK treatment twice a day for 2 days on body weight and liver weight in carbon-tetrachloride induced hepatotoxicity in rats.

Group	Treatment	Rat no.	B.W. (g)	L.W. (g)	L.W. %BW
1	Control(water) Oil 1.5 ml/kg	1	236	5.70	2.42
		2	227	5.40	2.38
		3	231	6.00	2.60
		4	243	6.40	2.63
		5	207	6.00	2.90
		6	242	6.50	2.69
	Mean		**231.00**	**6.00**	**2.60**
	S.D.		**13.28**	**0.41**	**0.19**
	S.E.		**5.42**	**0.17**	**0.08**
2	CCL_4 1.5 ml/kg	1	258	9.6	3.72
		2	243	8.4	3.46
		3	253	9.1	3.60
		4	242	8.0	3.31
		5	255	10.8	4.24
		6	229	7.8	3.41
	Mean		**246.67**	**8.95**	**3.62**
	S.D.		**10.82**	**1.13**	**0.33**
	S.E.		**4.42**	**0.46**	**0.14**
3	BJK 62.5 mg/kg + CCl_4 1.5 ml/kg	1	243	8.1	3.33
		2	251	9.7	3.86
		3	224	8.6	3.84
		4	256	8.9	3.48
		5	218	8.3	3.81
		6	233	7.6	3.26
	Mean		**237.50**	**8.53**	**3.60**
	S.D.		**15.08**	**0.72**	**0.27**
	S.E.		**6.16**	**0.30**	**0.11**
4	BJK 125 mg/kg + CCl_4 1.5 ml/kg	1	225	7.0	3.11
		2	243	9.4	3.87
		3	251	8.1	3.23
		4	264	9.3	3.52
		5	246	8.3	3.37
		6	264	9.7	3.67
	Mean		**248.83**	**8.63**	**3.46**
	S.D.		**14.66**	**1.02**	**0.28**
	S.E.		**5.99**	**0.42**	**0.12**

APPENDIX 3 (continued)

Effect of BJK treatment twice a day for 2 days on body weight and liver weight in carbon-tetrachloride induced hepatotoxicity in rats.

Group	Treatment		Rat no.	B.W. (g)	L.W. (g)	L.W. %BW
5	BJK 250 mg/kg + CCL_4 1.5 ml/kg		1	231	7.5	3.25
			2	235	7.5	3.19
			3	245	8.5	3.47
			4	255	9.2	3.61
			5	242	8.9	3.68
			6	249	8.5	3.41
		Mean		**242.83**	**8.35**	**3.43**
		S.D.		**8.86**	**0.71**	**0.19**
		S.E.		**3.62**	**0.29**	**0.08**
6	BJK 500 mg/kg + CCl_4 1.5 ml/kg		1	269	9.1	3.38
			2	239	10.2	4.27
			3	236	8.9	3.77
			4	248	9.4	3.79
			5	225	8.3	3.69
			6	257	8.9	3.46
		Mean		**245.67**	**9.13**	**3.73**
		S.D.		**15.77**	**0.63**	**0.31**
		S.E.		**6.44**	**0.26**	**0.13**
7	BJK 1000 mg/kg + CCl_4 1.5 ml/kg		1	278	9.0	3.24
			2	237	7.7	3.25
			3	244	8.0	3.28
			4	235	8.4	3.57
			5	225	8.3	3.69
			6	244	8.8	3.61
		Mean		**243.83**	**8.37**	**3.44**
		S.D.		**18.15**	**0.48**	**0.21**
		S.E.		**7.41**	**0.20**	**0.08**

APPENDIX 4

Effect of BJK treatment twice a day for 2 days on AST, ALT, GSH and MDA in carbon-tetrachloride induced hepatotoxicity in rats.

Group	Treatment	Rat no.	AST IU/L	ALT IU/L	MDA nmole/g liver	GSH μmole/g liver
1	Control(water)	1	105.93	24.51	45.01	5.29
	Oil 1.5 ml/kg	2	94.56	29.38	38.47	4.90
		3	87.75	35.05	45.88	5.86
		4	91.96	34.78	52.61	5.12
		5	100.51	30.93	30.31	5.10
		6	92.99	27.58	21.83	5.02
	Mean		**95.62**	**30.37**	**39.02**	**5.21**
	S.D.		**6.54**	**4.12**	**11.31**	**0.34**
	S.E.		**2.67**	**1.68**	**4.62**	**0.14**
2	CCL_4 1.5 ml/kg	1	1357.81	735.27	65.32	4.16
		2	622.74	392.87	53.23	3.91
		3	543.82	262.14	49.68	3.74
		4	353.13	244.45	44.82	3.60
		5	1586.17	927.50	47.25	3.92
		6	964.53	342.14	28.69	3.54
	Mean		**904.70**	**484.06**	**48.17**	**3.81**
	S.D.		**487.29**	**281.02**	**11.95**	**0.23**
	S.E.		**198.94**	**114.73**	**4.88**	**0.09**
3	BJK 62.5 mg/kg	1	960.89	242.43	50.99	6.18
	+ CCl_4 1.5 ml/kg	2	481.94	195.67	44.06	6.66
		3	586.26	169.84	48.75	6.12
		4	359.38	162.43	55.10	6.27
		5	691.08	255.05	28.31	5.12
		6	409.38	207.11	25.94	5.19
	Mean		**581.49**	**205.42**	**42.19**	**5.92**
	S.D.		**221.29**	**37.53**	**12.22**	**0.63**
	S.E.		**90.34**	**15.32**	**4.99**	**0.26**
4	BJK 125 mg/kg	1	496.24	269.11	40.77	5.64
	+ CCl_4 1.5 ml/kg	2	673.06	486.36	44.14	6.79
		3	296.26	197.41	52.86	5.82
		4	452.77	244.72	66.50	5.13
		5	508.18	361.35	29.56	6.10
		6	847.84	269.46	27.81	6.27
	Mean		**545.72**	**304.74**	**43.61**	**5.96**
	S.D.		**190.90**	**103.75**	**14.59**	**0.57**
	S.E.		**77.94**	**42.36**	**5.96**	**0.23**

APPENDIX 4 (continued)

Effect of BJK treatment twice a day for 2 days on AST, ALT, GSH and MDA in carbon-tetrachloride induced hepatotoxicity in rats.

Group	Treatment	Rat no.	AST IU/L	ALT IU/L	MDA nmole/g liver	GSH μmole/g liver
5	BJK 250 mg/kg	1	1525.34	953.84	48.00	2.98
	+ CCL_4 1.5 ml/kg	2	344.79	278.47	50.72	4.91
		3	275.40	163.49	69.31	5.39
		4	531.20	303.12	78.65	5.59
		5	1080.78	819.07	28.56	5.10
		6	592.56	259.68	29.06	5.37
	Mean		**725.01**	**462.94**	**50.72**	**4.89**
	S.D.		**483.51**	**334.17**	**20.46**	**0.96**
	S.E.		**197.39**	**136.43**	**8.35**	**0.39**
6	BJK 500 mg/kg	1	336.57	213.17	41.15	4.88
	+ CCl_4 1.5 ml/kg	2	764.42	773.18	68.56	4.62
		3	1329.52	582.07	58.84	5.06
		4	269.21	191.49	45.86	5.58
		5	1284.85	686.74	19.71	5.67
		6	424.34	193.55	41.02	5.54
	Mean		**734.82**	**440.04**	**45.86**	**5.22**
	S.D.		**475.16**	**270.56**	**16.81**	**0.43**
	S.E.		**193.98**	**110.46**	**6.86**	**0.18**
7	BJK 1000 mg/kg	1	426.81	275.35	39.65	5.53
	+ CCl_4 1.5 ml/kg	2	373.96	189.92	71.18	4.67
		3	452.21	219.72	38.47	5.78
		4	599.09	253.84	68.93	5.31
		5	512.51	304.52	27.94	4.98
		6	326.49	174.02	47.63	8.01
	Mean		**448.51**	**236.23**	**48.97**	**5.71**
	S.D.		**97.68**	**50.58**	**17.51**	**1.19**
	S.E.		**39.88**	**20.65**	**7.15**	**0.49**

APPENDIX 5

Standard solution of reduced glutathione was prepared by dissolving 3.1 mg reduced glutathione in 0.5% sulfosalicylic acid. The stock solution was used for preparing the working standard reduced glutathione (1.56-100 µM).

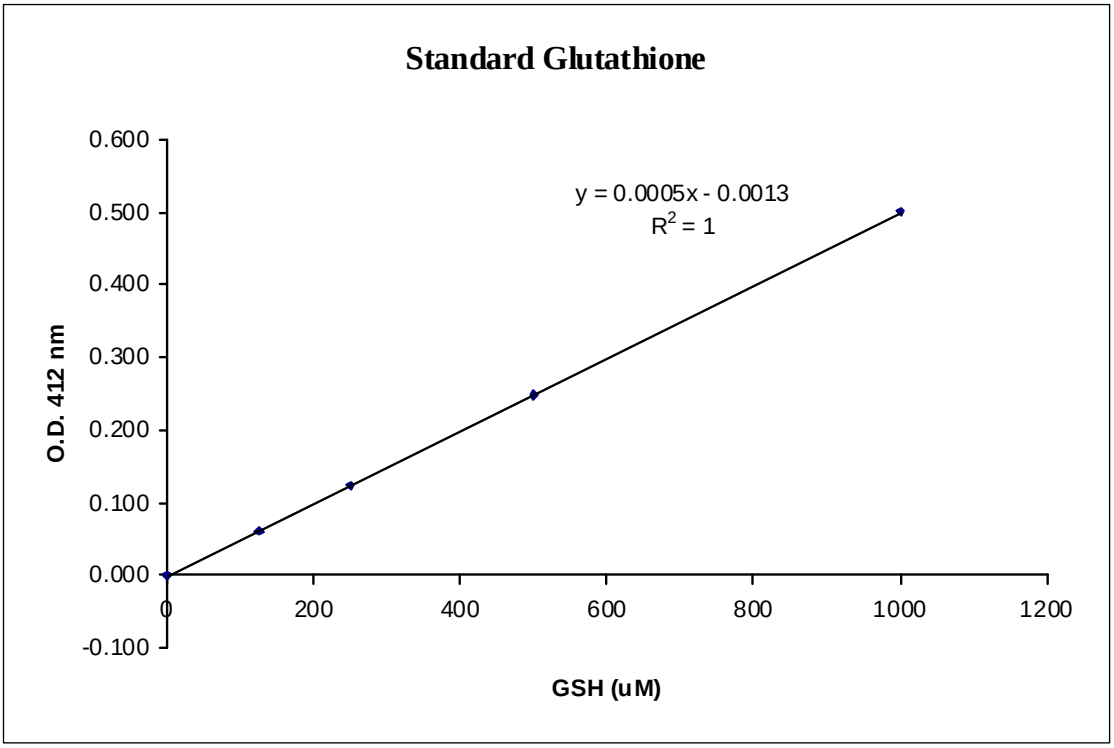

Figure 12. Standard curve of reduced glutathione

APPENDIX 6

Standard solution of 1,1,3,3 tetramethoxypropane (TMP) was prepared by pipetting 10 µl of TMP add in 990 µl distilled water. The stock solution was used for preparing the working standard TMP in concentration 1.56, 3.13, 6.25, 12.5, 25 and 50 nmole/ml.

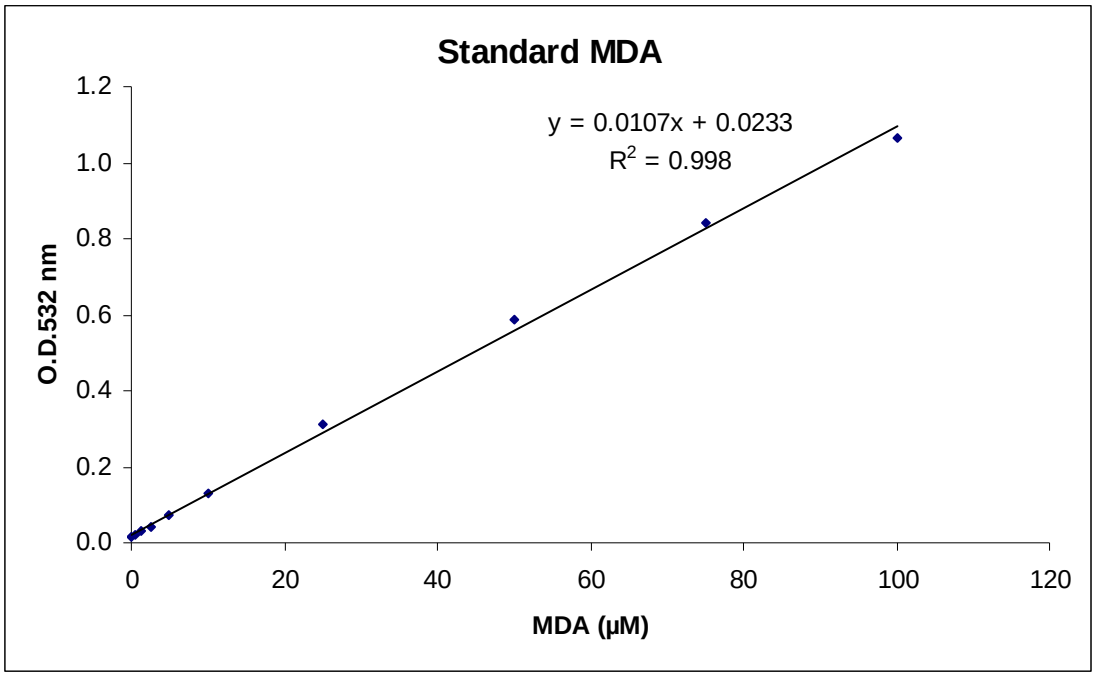

Figure 13. 1,1,3,3 tetramethoxypropane standard curve of malondialdehyde

APPENDIX 7

Standard solution of pyruvate acid was prepared by dissolving sodium pyruvate in phosphate buffer. Dilute 10 ml of this solution to 100 ml with phosphate buffer to obtain the working standard containing 2 mmole pyruvate per ml. The stock solution was used for preparing the working standard pyruvate acid and were expressed as (AST) 0, 20, 55, 95, 148, 216 IU/L and (ALT) 0, 20, 50, 83, 125 IU/L.

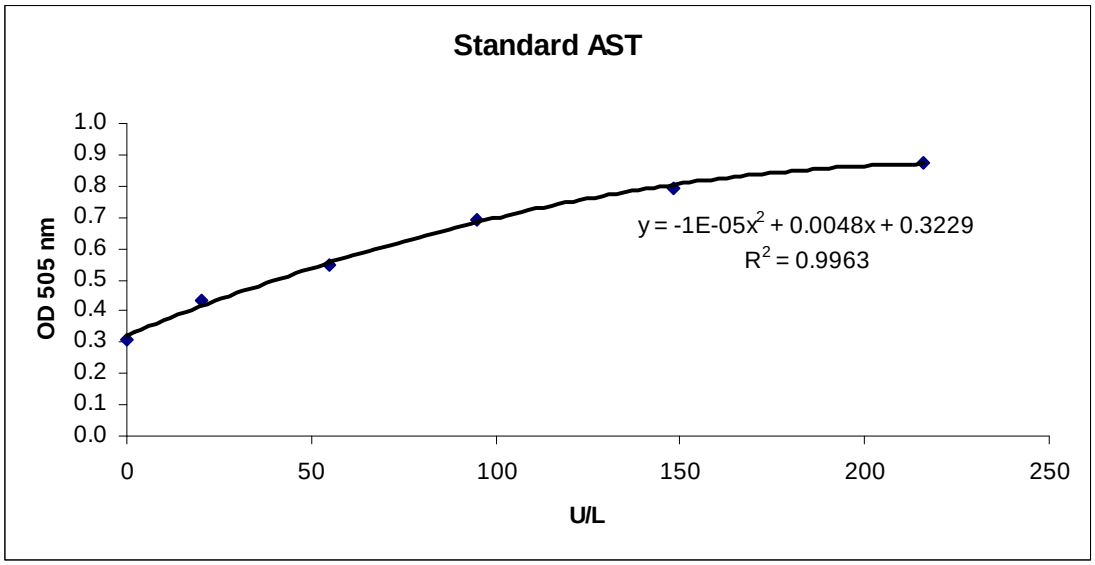

Figure 14. Standard curve of serum aspartate transaminase (AST)

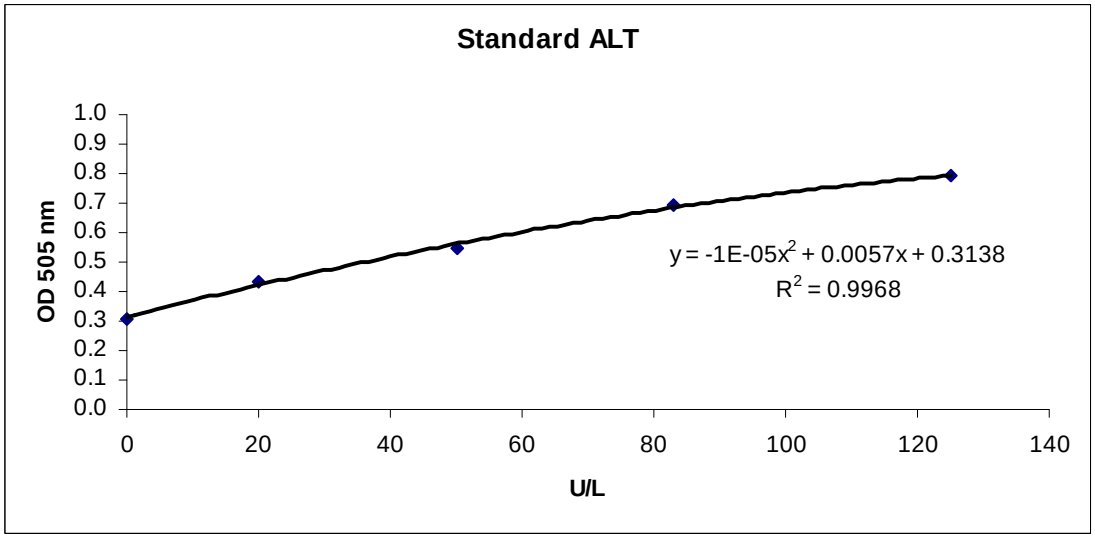

Figure 15. Standard curve of serum alanine transaminase (ALT)

VITAE

Name Mr. Akkawit Jieokok

Student ID 4822123

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Public Health	Prince of Songkla University	2002