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EFFECT OF PIPERINE ON CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY

SAROCHA KINGKAEOHOI

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
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(TOXICOLOGY)

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Thesis

entitled

EFFECT OF PIPERINE ON CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY

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Sarocha Kingkaeohoi

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ชื่อวิทยานิพนธ์ ผลของไปเปอรีนต่อพิษของคาร์บอนเตตราคลอไรด์ที่ดับ

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บทคัดย่อ

ได้ทำการศึกษาผลของไปเปอรีนต่อการเกิดพิษของคาร์บอนเตตราคลอไรด์ที่ดับในหนูพุกขาว โดยทำการป้อนไปเปอรีนขนาด 100 mg/kg BW แก่หนูเป็นเวลา 4 ชั่วโมงก่อนที่จะฉีดคาร์บอนเตตราคลอไรด์ขนาด 0.1 ml/kg BW เข้าทางช่องท้อง หลังจากนั้น 24 ชั่วโมงทำการสำรวจความเป็นพิษที่เกิดขึ้นที่ตับ พบว่าไปเปอรีนขนาดดังกล่าวออกฤทธิ์เสริมความเป็นพิษของคาร์บอนเตตราคลอไรด์ที่ตับได้อย่างเด่นชัด ทำให้ระดับของเอ็นซัยม์ glutamic pyruvic transaminase และ glutamic oxaloacetic transaminase ในกระแสโลหิต มีค่าเพิ่มขึ้นประมาณ 70-80 เปอร์เซนต์ ในขณะเดียวกันระดับไตรกลีเซอไรด์ในตับสูงขึ้นและระดับไตรกลีเซอไรด์ในเลือดมีค่าลดลงในปริมาณที่สอดคล้องกัน นอกจากนี้ยังพบว่าการออกฤทธิ์ของไปเปอรีนในการเสริมความเป็นพิษของคาร์บอนเตตราคลอไรด์จะขึ้นกับความเข้มข้นของสารที่ให้ ความเข้มข้นที่เหมาะสมคือไปเปอรีนขนาด 100 mg/kg BW ให้ไป 4 ชั่วโมงก่อนให้คาร์บอนเตตราคลอไรด์

จากการศึกษากลไกของการออกฤทธิ์ของไปเปอรีนในการเสริมความเป็นพิษของคาร์บอนเตตราคลอไรด์ที่ตับ พบว่าเมื่อป้อนไปเปอรีนขนาด 100 mg/kg BW เป็นเวลา 4 ชั่วโมงก่อนฉีดคาร์บอนเตตราคลอไรด์ขนาด 0.1 ml/kg BW เข้าหน้าท้องหนู ไปเปอรีนมีผลเสริมฤทธิ์การกระตุ้นการเกิดไลปิดเปอร์ออกซิเดชั่นของคาร์บอนเตตราคลอไรด์ที่ตับ และยังพบว่าไปเปอรีนมีฤทธิ์เสริม

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การกระตุ้นการทำงานของเอนไซม์ซึ่งเป็นตัวสำคัญในการเปลี่ยนแปลงคาร์บอนเตตราคลอไรด์ให้เป็นเมทาบอไลท์ที่เป็นพิษต่อตับคือเอนไซม์ NADPH-cytochrome c reductase

เมื่อเปลี่ยนวิธีการให้ไปเปอรีนและคาร์บอนเตตราคลอไรด์แก่สัตว์ทดลองโดยการป้อนไปเปอรีนให้หนู และที่ 4 ชั่วโมงต่อมานำตับของหนูดังกล่าวมาศึกษาโดยใส่คาร์บอนเตตราคลอไรด์ลงในหลอดทดลองโดยตรง พบว่าไปเปอรีนมีผลกระตุ้นการเกิดไลปิดเปอร์ออกซิเดชั่นและการทำงานของเอนไซม์NADPH-cytochrome c reductase ได้บ้าง ผลต่อค่าทั้งสองนี้คล้อยตามกัน นอกจากนี้เมื่อให้ไปเปอรีนพร้อมกับคาร์บอนเตตราคลอไรด์แก่ตับในหลอดทดลอง 30 นาทีต่อมาพบว่าก็ให้ผลเสริมฤทธิ์การกระตุ้นในลักษณะเดียวกัน แต่อย่างไรก็ตาม การให้ไปเปอรีนและคาร์บอนเตตราคลอไรด์ในสัตว์ทดลองให้ฤทธิ์เสริมสูงกว่าอย่างมาก ซึ่งจากผลการทดลองนี้น่าจะแสดงว่าตัวไปเปอรีนเองมิใช่ไปเปอรีนต้องไปผ่านขบวนการเปลี่ยนแปลงในตับก่อนที่มีผลกระตุ้นการเกิดพิษของคาร์บอนเตตราคลอไรด์ในตับให้เพิ่มขึ้น โดยไปเปอรีนอาจจะไปจับที่ผนังเซลล์ของตับ (liver membrane) แล้วมีผลทำให้เกิดการทำลายของเซลล์ตับมากขึ้นเมื่อได้รับคาร์บอนเตตราคลอไรด์ซึ่งเป็นสารที่เป็นพิษต่อเซลล์ตับ

จากการทดลองนี้อาจสรุปได้ว่าไปเปอรีนออกฤทธิ์เสริมพิษของคาร์บอนเตตราคลอไรด์ได้ โดยออกฤทธิ์เกี่ยวกับการกระตุ้นการทำงานของเอนไซม์ที่มีบทบาทในการเปลี่ยนคาร์บอนเตตราคลอไรด์ให้เป็นสารที่เป็นพิษต่อเซลล์ตับ จึงทำให้เซลล์ตับถูกทำลายมากขึ้นและเกิดพิษต่อตับมากขึ้น

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Thesis Title Effect of Piperine on Carbon Tetrachloride Induced Hepatotoxicity

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ABSTRACT

The effect of piperine on CCl_4-induced hepatotoxicity was investigated in male Wistar rats. The hepatotoxicity was assessed by measuring plasma glutamic pyruvic transaminase (PGPT) and glutamic oxaloacetic transminase (PGOT) levels at 24 h following the CCl_4 administration (0.1 ml/kg BW, i.p.). Piperine had ability to potentiate the hepatotoxicity of CCl_4 whereas piperine itself had no hepatotoxic effect. The maximum potentiation occurred when piperine (100 mg/kg BW) was orally administered 4 h prior to CCl_4 at which the activities of PGPT and PGOT were elevated by 70-80%. Concurrent to the rise in PGPT and PGOT levels, an accumulation of hepatic triglycerides was increased and plasma triglycerides level was decreased. The potentiating effect of piperine on these action of CCl_4

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showed a dose-dependent pattern and the maximum dose in producing effect was 100 mg/kg BW, p.o.

The potentiating mechanism of piperine was investigated. Piperine also potentiated the stimulating action of CCl_4 on lipid peroxidation and on NADPH-cytochrome c reductase activity. The extent of potentiation on these two parameters were similar and correlated well with the elevation of plasma transaminase activities.

The effect and mechanism of piperine on CCl_4-induced hepatotoxicity were further explored by modifying schedule of treatment. Piperine pretreatment for 4 h slightly enhanced the *in vitro* effect of CCl_4 in stimulating lipid peroxidation and activity of NADPH-cytochrome c reductase but there was a statistical difference ($P<0.05$). A similar enhancing effect of piperine on both CCl_4-stimulated lipid peroxidation and NADPH-cytochrome c reductase also significantly occurred when the liver microsomes were exposed to both piperine and CCl_4. The potentiating effect was increased with the concentration of piperine and the presence of piperine in the incubation mixture before adding CCl_4 was essential for producing potentiation. However, the potentiating action of piperine on CCl_4-induced hepatotoxicity *in vitro* was very much lower than that of *in vivo* system. From these *in vitro* studies, it suggests that piperine itself, not its metabolite, exerts the potentiating action. And as the degree of potentiation on various concentrations of

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CCl_4 was similar, piperine probably affected the liver function by binding to the liver membrane non-competitively.

Therefore, it is concluded that piperine potentiates CCl_4-induced hepatotoxicity by directly interacting with liver cell. Its mechanism associates with the increase in activity of NADPH-cytochrome c reductase which accelerates biotransformation of CCl_4 to the highly reactive metabolite, thereby increasing lipid peroxidation and enhancing its hepatotoxicity.

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TABLE OF CONTENTS

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LIST OF TABLES

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TABLE PAGE

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LIST OF FIGURES

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FIGURE PAGE

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LIST OF ABBREVIATIONS

BW	=	body weight
cm	=	centrimetre
g	=	gram
h	=	hour
Kg	=	kilogram
LD_{50}	=	Lethal dose for 50% for population
LTG	=	Liver triglyceride
M	=	Molarity
mg	=	milligram
min	=	minute
ml	=	millilitre
nmole	=	nanomole
N	=	Normality
OD	=	Optical density
%	=	percent
pH	=	log concentration of H^+
PO	=	Peroral
SEM	=	Standard error of mean
Std	=	Standard
ug	=	microgram
ul	=	microlitre
w/v	=	weight by volume

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CHAPTER I

INTRODUCTION

Carbon Tetrachloride

Carbon tetrachloride has been used extensively as solvents in many chemical industries and also in the manufacturing of the household materials including fire extinguisher fluids, insecticides spray, degreasing cleaner, polishing waxes etc. It is a clear colourless with high density and has a similar odour to that of chloroform. It is slightly soluble in water and readily soluble in chloroform, alcohol, ether and benzene (1). Absorption rate CCl_4 into the body is varied with species and the route of administration. After being absorbed, it will be distributed throughout the body and accumulated in fat components of various tissues such as bone marrow, liver, pancreas etc.

Carbon tetrachloride is known as a potent hepatotoxin. Its ability of to induce hepatotoxicity is related to its own metabolism and also to the general ability of the liver to metabolize drugs. The degree of hepatotoxicity of CCl_4 is varied and depends on several factors including species, age, sex and nutritional status.

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The differences in susceptibility of various animals species to CCl_4 have been reported to be due to

the system that converted CCl_4 into active metabolite . Several lines of evidence support this assumption. In 1970, Fowler (2) found that carbon tetrachloride was not metabolized in chicks. Likewise, Friedman and his colleagues (3) demonstrated that at very high doses of carbon tetrachloride only slight fatty infiltration was found in chick liver. In contrast, the administration of the hydrocarbon at a much lower dose to rats produced a marked increase in liver triglyceride content and hepatic necrosis. The lipid peroxidation of both *in vivo* and *in vitro* treated with carbon tetrachloride in chicks could not be detected. But in rats, the degree of lipid peroxidation significantly increased.

Age and sex of animals also play an important role in the toxicity of carbon tetrachloride. Dawkins (4) demonstrated that the administration of carbon tetrachloride to newborn rats (12-15 hours after birth) produced no histological evidence of necrosis and fat accumulation. Both newborn and adult rats showed the same maximum concentration of the compound in the body but in the newborn it occurred much earlier and fell more rapidly. These phenomena were suggested to account for the resistance of newborn liver to carbon tetrachloride poisoning. Reuber and Glover (5) found that the older animals treated with carbon tetrachloride developed more severe cirrhosis of the liver than the younger ones. The age differences might be related to the increased necrosis and decreased regeneration in the liver of the older

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animals One of the possible explanation is that in the newborn there is a low level of total fat and phospholipid where carbon tetrachloride preferentially accumulate (6, 7). Another possibility is due to a very low activities of hepatic microsomal enzymes in newborn which may be responsible for the conversion of carbon tetrachloride into its toxic metabolites.

Sex differences on hepatotoxicity of carbon tetrachloride have been described by Bengmark and Olsson (8). They found that female rats were more susceptible to toxic agents than the male ones. Pretreatment female animals with testosterone propionate reduced the severity of hepatic injury and stimulated regeneration of liver cells. This might be due to the protein sparing effect of testosterone, since it has been shown by Bernelli-Zazzera and coworkers in 1958 (9) that the sex hormone increased the incorporation of glycine into liver slices to a rate comparable to that found in the regenerating liver.

The nutritional status and dietary factors also play a role in the toxicity of carbon tetrachloride. Tamura and coworkers (10,11) discovered that a subcutaneous injection of carbon tetrachloride (0.1 ml/animal) to male albino rats caused a marked decrease in the level of vitamin E and unsaturated fatty acid in liver. The activity of oxidative phosphorylation also decreased while the content of saturated fatty acid was increased. The pretreatment with vitamin E (10 mg/rat, daily for 3 days) prevented against these changes and significantly

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inhibited the rise in the hepatic fatty acid after the administration of carbon tetrachloride. In agreement with these findings, Gallagher (12, 13) has demonstrated that in female Wistar rats, a pretreatment with a single intraperitoneal dose of vitamin E (95 mg/rat, approximately 600 mg/kg BW) could completely protect death induced by the oral administration of 4.0 ml/kg BW carbon tetrachloride, provided that the vitamin was given at least 40 hours before the hydrocarbon administration.

From various factors mentioned above, it can be seen that the degree of hepatotoxicity produced by carbon tetrachloride depends upon its conversion into toxic metabolites in the liver.

Metabolism of CCl_4

Metabolism of CCl_4 consists of series of reactions. The first step is the homolytic cleavage of molecule resulting in the free radical ($.CCl_3$) or trichloromethyl free radical which is derived from the clevage bond between carbon and chloride atom (14). The NADPH linked microsomal drug metabolizing system which refered to as NADPH-cytochrome c reductase, the initial phase of hepatic microsomal electron transport chain contribute a role to conversion of CCl_4 to $.CCl_3$ (15). After this reaction, the free radical will attack at the hydrogen atoms on the methylene bridges separating the double bonds in polyunsaturated fatty acid side chains of microsomal lipids which are sensitive to free radical

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attack, as shown in Figure A. Following the attack of free radical, the formation of organic free radicals (R) and chloroform occurs. After the initial attack, there is a resonance shift of organic free radical and resulting in the diene conjugation (16). The free radicals then react with the molecular oxygen to form the corresponding organic peroxide free radicals (ROO.) rapidly (17). After this process, they will attack the other unsaturated side chain of fatty acids (RH) to yield a hydroperoxides (ROOH) which will undergo many complex reactions. One of these reactions is the intramolecular cyclization of hydroperoxide radical to form an unstable cyclic compound which will cleavage later and resulting in malondialdehyde (Figure A).

Pathophysiology of liver induced by carbon tetrachloride

Carbon tetrachloride produces many changes in the hepatocyte which almost immediately occur after administration of this toxin. After carbon tetrachloride was metabolized to a free radical,it could act in two ways either in a direct way, by covalent binding to membrane protein and lipid; or in an indirect way, through interactions with membrane unsaturated fatty acids to promotion of lipid peroxidation. Currently, it is believed that lipid peroxidation in cellular membranes plays a significant role in the pathogenic mechanisms of liver injury produced by carbon tetrachloride (18). As described by M. Comporti (18), within 15 minutes the

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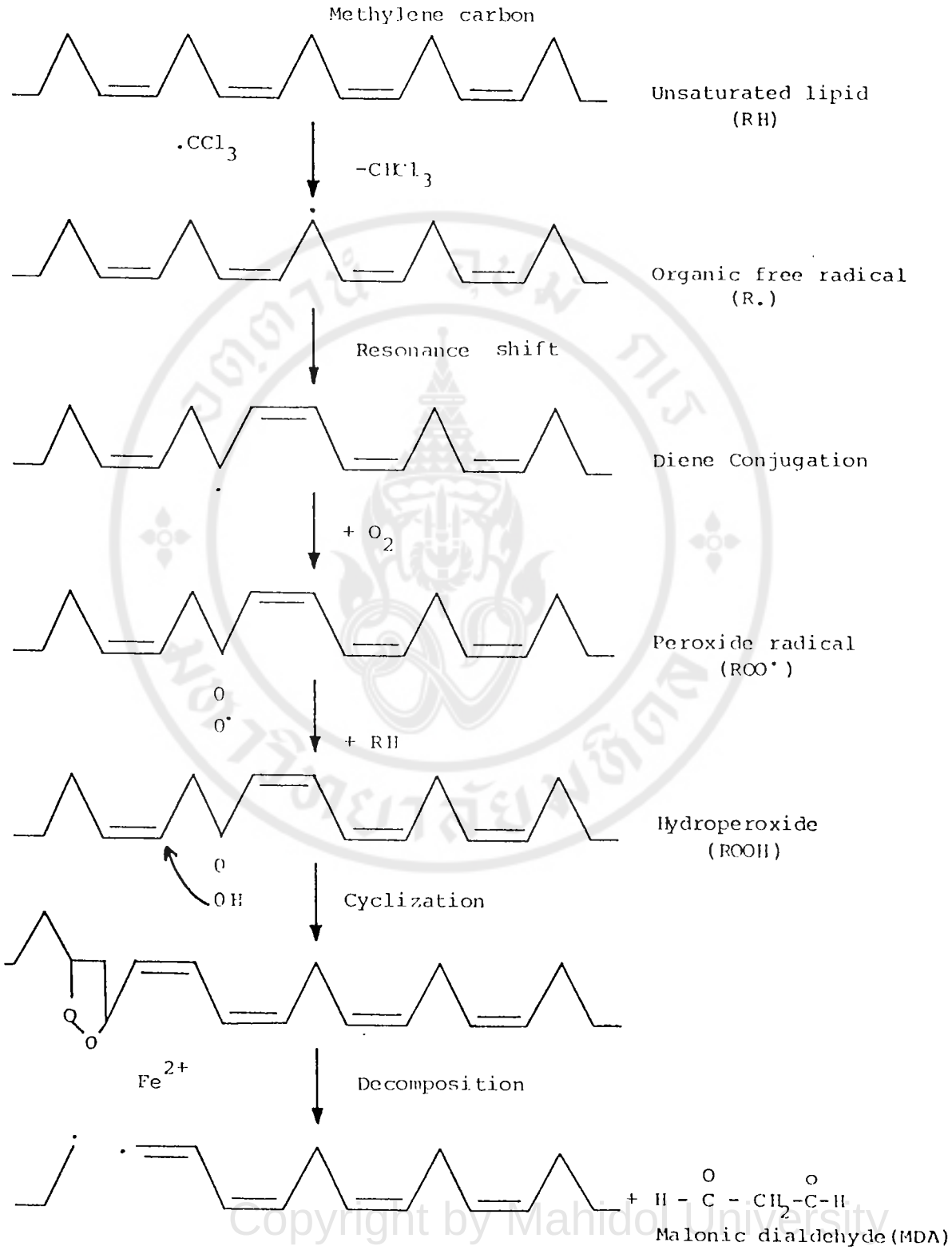

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Figure A. Schematic diagram showing mechanism of CCl_4-lipid peroxidation process.

endoplasmic reticulum (ER) showed evidence of injury. In the cisternae, the vacuole formation occurred, the ribosomes dissociated from the RER and scattered through the cytoplasm. The smooth endoplasmic reticulum (SER) developed a chaotic fragmented (17, 18, 19, 20). Then both SER and degranulated RER formed tubular aggregrates. By 5 to 6 hours later, the destruction of lysosome appeared and the mitochondria changes could be observed in 10 to 20 hours after CCl_4 administration (17). The mitochondria enlarge, the cristae was disappeared and degranulation (17). Golgi apparatus lost the dense particle reflecting a depression of lipoprotein assembly (17, 21).

Pathogenesis of fatty liver induced by CCl_4

A fatty liver generally occurs as a result of an imbalance between the rate of synthesis and the rate of utilization of hepatic triglyceride. Many lines of evidence obtained from a number of investigations indicated that the basic pathogenetic mechanism underlying the fatty liver induced in the rat by administration of CCl_4 was due to the blockade in the secretion of hepatic triglycerides (22, 23, 24). Since the secretion of lipoproteins to the plasma appeared to be the major pathway of utilization of hepatic triglyclerides, the action of CCl_4 was the blockade of utilization pathway. Normally the hepatic triglylcerides were released into the plasma as a moiety of serum lipoproteins. The primary

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defect was in either the synthesis of plasma lipoproteins or the secretion of plasma lipoprotein or both. In case of fatty liver induced by CCl_4, many available evidences indicate that the interference was at 2 level.

1) CCl_4 might interfere the synthesis of protein moiety. This evidence based on the results obtained by Smucker and coworker (25,26) who have shown that synthesis of hepatic and plasma lipoproteins, including the protein moiety of plasma lipoprotein called apoprotein were inhibited by CCl_4 and that the inhibition was established at a time prior to, or coincident with the onset of fatty liver.

2) CCl_4 might cause the defect at the level of conjugation of various moiety or coupling process to form lipoprotein called nascent VLDL and then being excreted into the plasma.(25,26)

Effect of stimulators and inhibitors of drug metabolism on CCl_4-toxicity

Carbon tetrachloride is metabolized in the liver by the drug metabolizing system or the mixed function oxidase system which ultilizes nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P-450 electron transport chain at the level of smooth endoplasmic recticulum (15). As the hepatotoxicity of CCl_4 is dependent on the ability of the liver to metabolize the drugs, there is a possibility that stimulators and inhibitors of the drug metabolizing enzymes may modify the hepatic

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damage produced by CCl_4. The phenobarbital (PB), an inducer of microsomal enzymes, has been reported to enhance the lethality and hepatotoxicity of CCl_4. Both microsomal NADPH cytochrom c reductase activity and the amount of co-binding pigment were elevated after giving PB (27). Therefore, the potentiation of PB on CCl_4 hepatotoxicty was arise from the increased production of free radical metabolites by the microsomal cytochrome P-450 (28). However, though the potentiation of PB on CCl_4 toxicity is well documented, not all inducers of mixed function oxidase activity can potentiate CCl_4-toxicity. 3-Methylcholantrene (MC) and some others polycyclic hydrocarbons which are inducers of drug metabolizing enzymes exerted a protective effect against the hepatic damage produced by CCl_4. The spectum of enzyme induced, character of co-binding pigment and the mechanisms of induction by MC were also reported to be different from those by PB (27). MC increases co-binding pigment without a concomittant increase in NADPH-cytochrome c reductase (27).

Cignoli and Castro (1971) tested ability of several drug metabolizing enzyme inhibitors in preventing carbon tetrachloride induced liver necrosis and fatty infiltration in the rat. Beta-diethylaminoethyl diphenyl propylacatate HCl (SKF 525A), a well known inhibitor of drug metabolizing enzyme was found to prevent CCl_4-induced necrosis and also prevented the loss of ethylmorphine-N-de methylase activity as well as the decrease in the amount

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of cytochrome P-450. The effects of other inhibitors of drug metabolism namely Sch 5706, Sch 5712, CFT 1201, DPEA, Lilly 18947 and imipramine were also examined (29). Among these compounds tested, Sch 5706 and CFT 1201 offered the greatest protection against necrosis. NADPH and imipramine did not protect at all. It was suggested that the protective effect of those compounds were not only attributable to their ability to inhibit drug metabolism, but also the anti-oxidant ability or ability to inhibit lipid peroxidation and the competition for TPNH and oxygen with lipid peroxidation. The competing effect of substrates of drug metabolizing enzymes on microsomal lipid peroxidation was previously observed *in vitro* by Orrenius *et al* (30).

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PIPERINE

Piperine (1-piperoyl piperidine) is a major alkaloid present in various piper species of Piperaceae family. Its structure is shown in Figure B.

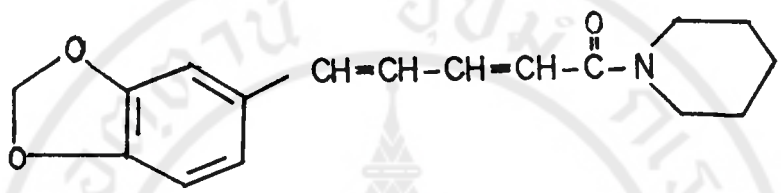

Figure B. Chemical structure of piperine

Among the piper species, black pepper (Piper nigrum Linn.) and long pepper (Piper longum Linn.) are commonly used in many regions of the world as food additive. They are also employed in folklore medicine for treatment of asthma, bronchitis, dysentery, pyrexia and insomnia (31, 32). However, the biological activities of plant in piper species and piperine have been investigated by a few groups of scientists.

In 1971 and 1973, Singh and coworkers (33) reported that piperine as well as crude extract of Piper longum possessed a stimulating effect on respiration and induced convulsion in mice and dogs. In contrast, Neogi and coworkers (34) and Lee et al. (35) showed the contradictory results that piperine decreased respiratory rate and possessed central depressant activity. Piperine produced diverse pharmacological activities. Antipyretic,

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analgesic, anti-inflammatory activity (35), and anti-fertility (36) properties of piperine were also reported.

Toxicity of Piperine

Piperine has been reported to be acutely toxic to various experimental animals (37). The LD_{50} value for a single intraperitoneal injection to adult female mice is 60 mg/kg BW whereas value for intraperitoneal and intragastric administration to adult female rat is 33.5 and 514.0 mg/kg BW, respectively. The cause of death was reported to be similar to the earlier finding of Singh et al., (33). Piperine induced excitation and convulsion and the animals died with the tonic spasm of musculature.

Subacute toxicity of piperine was also reported. Piperine administered at the dose of 100 mg/kg BW/day intragastrically administration for one week was not toxic to animals (37). The toxic effect was found at a dose of 250 mg/kg BW/day or more. Several histopathological changes including severe hemorrhage, necrosis and edema occurred along the gastrointestinal tract, urinary bladder and adrenal gland. These changes were suggested to be responsible for the death of animals in that course of treatment.

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From previous studied found that piperine could potentiated effect of other compound. In 1990, Mujumdar et al., discovered that pretreatment of piperine at the doses of 10, 25, 50 mg/kg BW to rats potentiated PB

sleeping time in dose-dependent manner. They also found that piperine treated in rats, treated chronically with PB, significantly potentiated PB sleeping time (38).

Metabolism of Piperine

Bhat and Chandrasekhara (39) found that when piperine was administered to male albino rat by gavage or intraperitoneally, most of the administered piperine was absorbed (about 97%). Only three percent of the administered dose was excreted as piperine in the feces and it was not detectable in the urine. However, it is not transformed during absorption by the gut but probably is later metabolized rapidly by other tissues.

In 1987, the same group of investigators demonstrated that after oral administration of piperine to rats, it is biotransformed to piperic acid, piperonylic acid, piperonyl alcohol, piperonal and vanillic acid and their conjugates (40). It is speculated that piperine absorbed into the portal vein is first hydrolysed to piperic acid and piperidine mainly in the liver and then the former is transformed to piperonylic acid after successive oxidation of the side chain. The oxidative cleavage of the side chain of piperine may also lead to the formation of piperonal. Subsequently the methylene dioxyphenyl (MDP) aldehyde, piperonal can undergo oxidation to piperonylic acid and/or reduction to piperonyl alcohol. The other principal route of metabolism of piperine is through clevage of the

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methylenedioxy group. The major metabolites would be catechols which are then O-methylated to methyloxyphenols, of which only vanillic acid has been identified. The proposed pathway of piperine metabolism is shown in Figure C (40).

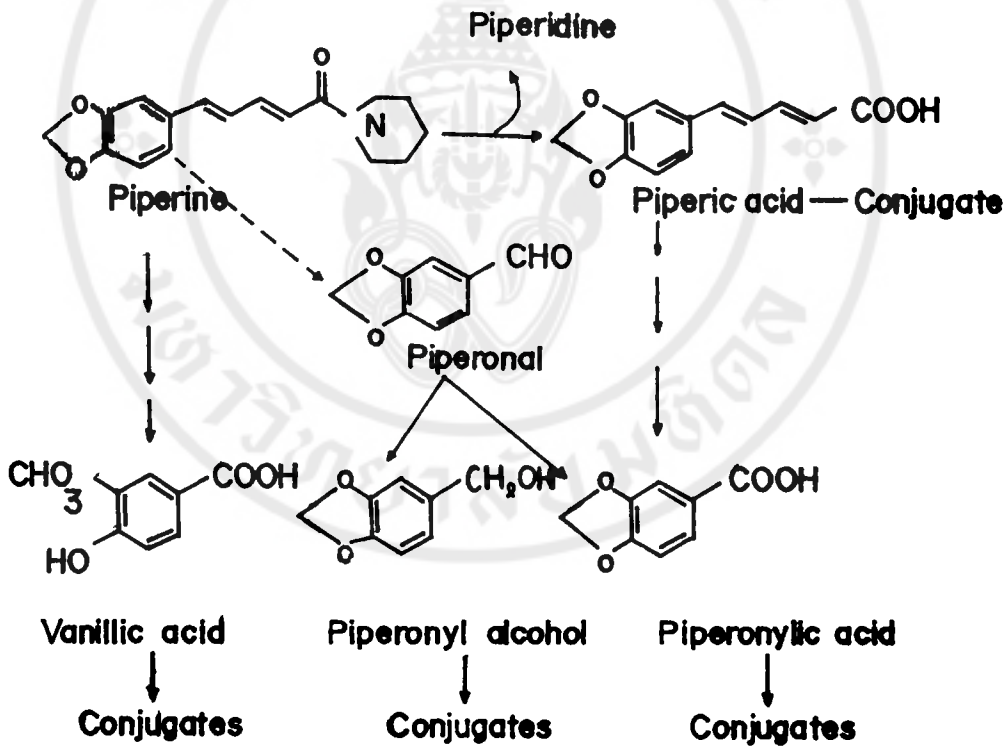

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Figure C. Proposed pathway for the biotransformation of piperine in rats.

Effect of piperine on drug metabolising enzymes

Since plants in piper species are extensively used as an essential ingredient of several ayurvedic formulations which have been used for treatment of a wide range of diseases. Therefore, the biological activity of these plants have been a scientific curiosity for a long time. Until recently Atal et al. (41). Zutshi et al. (42) and Bano et al. (43) found that Piper longum as well as piperine increase the bioavailability of some drugs such as vasicine, sparteine, phenytoin by inhibiting their metabolisms. According to these action, in 1985, Atal et al., provided the evidence that piperine is a potent unspecific inhibitor of drug metabolism. They found a correlation between in vitro and in vivo inhibition of drug metabolism. For in vivo inhibition, a single oral dose of piperine (125 mg/kg BW) caused a maximal inhibition of hepatic arylhydrocarbon hydroxylase (AHH) in rat at 1 h and by 6 h after administration, the enzymatic activity returned to the control value. For the in vitro, they found that piperine caused a concentration related-inhibition on ethylmorphine-N-demethylase, 7-ethoxy coumarin O-deethylase and benzo(a)pyrene hydroxylase activity. The concentration of piperine which caused about 50% inhibition was less than 50 uM for almost all enzymes studied. Piperine also caused a similar quantitative inhibition of various forms of monooxygenases when the enzymes were induced by either PB or MC or existed in the native form. It was concluded that

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piperine is a potent unspecific inhibitor of drug metabolism (44). These results are in agreement with the study of Ganesh Bhat and Chandrasekhara(39) who found that pretreatment of rats with piperine (a MDP or Methylene dioxyphenyl compound) decreased N, N dimethylaminobenzal dehyde demethylase activity whereas pretreatment with safrole, another MDP compound, increase daniline hydroxylase activity and induced cytochrome P-450. Though, MDP compounds are known to induce cytochrome P-450 whereas piperine is not at all an inducer (39). Glucuronosyl transferase activity was not significantly altered by pretreatment with either piperine or safrole. These findings also demonstrated that hepatic cytochrome P-450 composition from animals-treated with piperine was different from those-treated with safrole. Piperine and safrole probably reacted with different forms of cytochrome P-450.

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CHAPTER II

EXPERIMENTAL OBJECTIVES

Recently, it has been demonstrated that piperine exhibits a non-specific inhibition on drug metabolism(44). It can enhance the bioavailability of serveral tested drugs (41). Now, the potential of using the piperine for this purpose is increasing. Therefore, it is interesting to delineate more detail on the interaction of piperine with the liver cell function particularly on the drug metabolizing system.

Carbon tetrachloride is a chemical known to be metbolized by a series of drug metabolizing enzymes in the liver. Its toxicity on the liver is well documented in both human and animals. CCl_4 hepatotoxicity in animals is often used as an experimental model for studying liver cell injury. This model is also going to be employed in the present study.

The drug metabolizing system of the liver is composed of a series of enzymes and each is differently modified by inhibitors. Several inhibitors of drug metabolizing system were demonstrated to be able to prevent the liver against the CCl_4-induced necrosis (29). Accordingly, piperine which is considered as an inhibitor may modify the toxicity of CCl_4. As the influence of piperine on CCl_4-induced hepatotoxicity has not been

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investigated and CCl_4 poisoning still occurs in human, therefore it is the aim of the present investigation.

The aims of the present study are as follows :

1) To determine the time-course effect of piperine in modifying the CCl_4-induced hepatotoxicity.

2) To determine the dose-response effect of piperine in modifying the CCl_4-induced hepatotoxicity

3) To investigate the mechanism of action of piperine in modifying the CCl_4-induced hepatotoxicity.

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CHAPTER III

MATERIALS AND METHODS

A. Animals

Adults male wistar rats, weighing between 180-200 g were supplied by the National Animal Center, Salaya Campus, Mahidol University, Nakornpathom. They were maintained in stainless steel hanging cages. All animals were given free access to food (Standard rat chow, Gold Coins Co. Ltd., Singapore, supplied by F.E. Zuellig Co., Bangkok) and water ad libitum.

B. Chemicals

Carbon tetrachloride (CCl_4) was purchased from Malinkrodt Chemical Works, (New York, U.S.A). Piperine and reagents for the determination of PGPT, PGOT, liver triglyceride, NADPH-cytochrome c reductase were purchased from Sigma Chemical, Company (St. Louis, Missouri, U.S.A.). Serum triglyceride was determined by using the kits from Bio-medical Laboratory (Bangkok, Thailand). All others chemicals and solvents were also commercially obtained and were of analytical grade.

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C. Preparation of CCl_4 solution and piperine solution

For in vivo experiment, CCl_4 was diluted with corn oil to allow administration doses of 0.05, 0.1, 0.15,

0.25 and 0.4 ml/kg BW, respectively. The maximum volume for administration was not more than 0.1 ml/animal. For in vitro experiment, CCl_4 was diluted with absolute ethanol to the final concentrations of 3.1, 4.7, 6.3, 7.8, 9.4 and 12.5×10^{-3} M, respectively. To prepare piperine solution for oral administration to animals, it was suspended in corn oil. And for in vitro experiment, piperine was dissolved in absolute ethanol to the final concentrations of 5, 10, 20, 50 and 100×10^{-3} M, respectively.

D. Enzymatic Assays

1. Determination of enzyme glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT)

Measurements of the two serum transaminases were carried out according to the method described by Reitman and Frankel in 1975 (45). Blood samples were collected from abdominal aorta and were immediately centrifuged at the 3,000 rpm for 10 minutes and the plasma was removed. For PGOT assay, 0.1 ml of plasma was pipetted into incubation mixture containing 200 mM of DL-aspartate and 2 mM of 2-ketoglutarate in 0.5 ml of 0.1 M sodium-potassium phosphate buffer, pH 7.4. The final volume was 0.6 ml. For PGPT assay, the components were the same as that for PGOT assayexceptDL-alanine (200 mM) was used in stead of DL-aspartate. The mixture was incubated in a metabolic incubator with aconstant shaking rate at 37°C for 30 min

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and 60 min for the determination of PGPT and PGOT, respectively. The reaction was stopped by the addition of 0.5 ml of 1.0 mM 2,4 dinitro-phenylhydrazine solution. The reaction tubes were allowed to stand for 20 min at room temperature, then 5.0 ml of 0.4 N. NaOH was added and mixed well. Five minutes after the addition of NaOH solution, the final product was measured colorimetrically at 505 nm. The activities of PGOT and PGPT were determined from the calibration curves using sodium pyruvate (2 mM) as a standard. The activities of both plasma transaminase were expressed as Sigma-Frankel unit. One Sigma-Frankel unit is corresponded to the formation of 4.82×10^{-4} umoles of glutamate per min at pH 7.5 and 25° C.

2. Determination of NADPH-cytochrome c reductase

NADPH-cytochrome c reductase is one of the enzyme involved in microsomal electron transport of the drug metabolizing enzyme system. The activity of NADPH-chto-chrome c reductase was determined by the method of Mazel (46). After decapitation, the liver was rapidly removed, weighed and chilled in ice-cold 0.9% NaCl. The liver was perfused with ice-cold 0.9% NaCl via portal vein before homogenization. The tissue was homogenized in 3 volume of 1.15% KCl by using a Teflon-glass homogenizer (A.H. Thomas Co., U.S.A.). This homogenate was centrifuged at 9,000 x g for 25 min. The supernatant was further centrifuged at 105,000 x g for 60 min. The microsomal pellet was obtained and suspended in 3 vol

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(w/v) of ice-cold 0.1 M potassium phosphate buffer (pH 7.4). This fraction was employed for the determination of NADPH-cytochrome c reductase activity (46). 2.0 ml of the solution I which was prepared by dissolving NADPH 5.7 mg, KCN 9.7 mg and nicotinamide 366 mg in 100 ml of 0.05 M phosphate buffer, pH 7.6 was added in sample test tube and 2.0 ml of solution III which was similarly prepared to that of solution I but lack of NADPH and it was used as a blank. 0.5 ml of solution II which was prepared by dissolving cytochrome c 3.68 mg in 1 ml of distilled water was added into each test tube and stand allowed to for 2 min. Thereafter, 0.5 ml of microsomal suspension was added into the mixture and mixed rapidly. These content was poured into a-1 cm light path cuvet. The rate of cytochrome c reduction was measured by recording changes in optical density of the sample against the blank at 550 nm during the first 4 min in which the reaction rate is linear. NADPH cytochrome c reductase activities were calculated. One unit of the enzyme activity is defined as the enzyme which produces a change in O.D. of 1.0 at 550 nm in 1 min using spectrophotometer and expressed as nmoles of cytochrome c reduced/mg protein/min, which can be calculated from the equation below (46).

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$$\text{Activity} = \frac{\text{Change in O.D. for 1 min} \times \text{Total volume} \times 1000}{\text{the molar extrinction coefficient} \times \text{Amount of protein}}$$

E. Determination of Protein

The amount of protein in the liver homogenate and the microsomal fraction were determined by Lowry method (47). 5 ml of alkaline solution which was freshly prepared by mixing 50 ml of solution A (2% w/v Na_2CO_3, 0.2 w/v sodium tartate, 0.6 w/v NaOH make to 1000 ml with distilled water) with 1 ml of 0.5% $CuSO_4.5H_2O$ was added to 50 ul protein sample. The solution was mixed and left at room temperature for 10 min. Thereafter, 0.5 ml of diluted Folin-Ciocalteu reagent (1:2 dilution in water) was added to the copper-treated protein. The content of each tube was vigorously mixed after addition and the tube was incubated for 45 min at room temperature. The blue color obtained was read absorbance at 650 nm against a reagent blank. The amount of protein in liver homogenate and microsomal fraction was determined from the calibration curve using bovine serum albumin as standard.

F. Determination of triglyceride

Plasma triglyceride level was determined according to the method of Biggs et al.(48). The diagnostic kit for assay plasma triglyceride was purchased from Bio-medical Laboratory (Bangkok, Thailand). The triglycerides in 0.2 ml of plasma was extracted by 5 ml of extracting solvent which contained heptane and isopropanol and then 1.0 ml of acidifying agent was added. After the mixture was shaken vigorously, 0.5 ml of the heptane extract layer (upper layer) was transfered to mix with 0.5 ml of

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transesterifying agent and incubated at 37°C for 5 min. Thereafter, 0.5 ml of oxidizing agent, 3.0 ml of ammonium acetate buffer and 0.1 ml of acetylacetone were added and were incubated at 37°C for 20 min. At the end of the incubation period, it was cooled with tap water. The O.D. of the final product was measured at 410 nm.

Liver triglyceride content was determined according to the method of Mendez in 1975 (49). The reaction mixture contained 0.5 ml of liver homogenate, 2.0 ml of heptane, 1.0 ml of 0.04 M H_2SO_4 and 3 ml of isopropanol. The mixture was shaken vigorously for 30 second. After allowing for the phase separation, 0.2 ml of heptane layer (upper layer) was transfered to another tube. Then 2.0 ml of isopropanol and 0.6 ml of saponification reagent were added, mixed and allowed to stand at room temperature for 5 min. Thereafter, 1.5 ml of metaperiodate reagent and 1.5 ml of acetylacetone reagent were added, mixed and incubated at 65-70°C for 15 min. After allowing them to cool at room temperature, the O.D. of final product was measured at 415 nm. Triglyceride content was determined from the calibration curve using triolein solution as standard. The plasma and liver triglyceride levels were expressed in terms of mg% and mg/g liver, respectively.

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G. Measurement of lipid peroxidation

Lipid peroxidation was measured by the method of Ohkawa et al. (50). After decapitation, the liver was

rapidly removed, weighed and chilled in ice-cold 0.9% NaCl. The liver was perfused with ice-cold 0.9% NaCl via portal vein to minimize hemoglobin contamination. Then, it was homogenized in 9 volume of 1.15% KCl by using a Teflon-glass homogenizer (A.H. Thomas Co., U.S.A.). This whole homogenate was employed for determination of lipid peroxidation. The reaction mixture contained 0.1 ml of sample, 0.2 ml of 8.1% sodium dodecyl sulfate (SDS) and 1.5 ml of 20% acetic acid solution (pH 3.5). The mixture was finally made up to 4.0 ml with distilled water, and heated at about 95° C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) were added. The mixture was shaken vigorously. After centrifugation at 4,000 rpm for 10 min, the absorbance of the product in the organic layer (upper layer) was measured at 532 nm. The amount of lipid peroxide was determined from the calibration curve using TMP (1,1,3,3-tetramethoxypropane) ranging 10-50 mM as standard. The extent of lipid peroxidation was expressed as nmole MDA/100 mg protein.

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Statistical Analysis

All data were analysed and expressed as mean±standard error of mean ($\bar{X}$±SEM). Statistical differences between two groups were examined by using unpaired Student's t-test. All statistical analyse were made by using Epistat programme (Tracyl Gustafson). If the statistical probability (P-value) was less than 0.05, the difference was estimated to be statistical significance.

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EXPERIMENTAL PROTOCOLS

Study I. Effect of piperine on CCl_4-induced hepatotoxcity

Experiment I.1 Dose response effect of CCl_4-induced hepatotoxicity in rat.

This experiment was designed to investigate the dose response effect of CCl_4 in inducing hepatotoxicity. Various doses of CCl_4 ranging from 0.05, 0.1, 0.15, 0.25 to 0.4 ml/kg body weight were intraperitoneally administered to the overnight fasted-adult male Wistar rats. 24 h after CCl_4 administration, these animals were sacrificed. Blood samples were collected from abdominal aorta. Plasma levels of liver enzymes glutamic pyruvic transaminase (PGPT) and glutamic oxaloacetic transaminase (PGOT) were determined and used as indices of liver cell damage.

Experiment I.2. Time-course of the effects of piperine pretreatment on the CCl_4-induced hepatotoxicity.

This experment was designed to investigate the action of piperine in modifying CCl_4-induced hepatotoxicity. A submaximal dose of CCl_4 (0.1 ml/kg BW) was chosen from experiment I.1 of this study. The overnight fasted animals were intragastrically administered with piperine at a dose of 100 mg/kg body weight at varying

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intervals of 1,2,3,4,5,6 and 20 h prior to CCl_4 injection and then they were sacrificed at 24 h after CCl_4 administration. Blood samples were collected from abdominal aorta. Plasma levels of glutamic pyruvic transaminase (PGPT), glutamic oxaloacetic transaminase (PGOT) and plasma triglyceride level were determined. The liver was quickly removed and kept frozen for further analysis of liver triglycerides.

Experiment I.3 Dose-response effect of piperine pre-treatment on CCl_4-induced hepatotoxicity.

This experiment was designed to investigate the relationship between the given amount of piperine and the extent of the effect. From experiment I.2, the period for producing enhancing effect of piperine was chosen for this experiment (4 h). In this section, the dose-response effect of piperine in enhancing CCl_4-induced hepatotoxic effect was investigated by pretreatment with piperine only for 4 h before CCl_4. Various doses of piperine ranging from 5, 10, 50, 100 and 150 mg/kg BW were intragastrically administered to an overnight fasted animals. Four hours later CCl_4 was intraperitoneally injected and the animals were sacrificed at 24 h after CCl_4 administration. Blood samples were collected from abdominal aorta for analyses of plasma glutamic pyruvic transaminase (PGPT) and glutamic oxaloacetic transaminase (PGOT) and plasma triglyceride. The liver

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was quickly removed and kept frozen in a refrigerator for further analysis of liver triglyceride.

Study II Effect of piperine on the CCl_4-stimulated lipid peroxidation in rat liver

Experiment II.1 Effect of piperine pretreatment on CCl_4-stimulated lipid peroxidation in rat

This experiment was designed to investigate the effect of piperine treatment at a dose of 100 mg/kg BW at 4 h before giving CCl_4 (0.1 ml/kg BW) on lipid peroxidation, since the lipid peroxidation in cellular membrane is a major pathogenic mechanism of CCl_4 for liver injury.

The overnight fasted rats were intragastrically administered with piperine (100 mg/kg BW) for at 4 h before CCl_4 (0.1 ml/kg BW) injection. One hour after CCl_4 administration, they were decapitated, the livers were then rapidly removed and perfused with ice-cold 0.9% NaCl solution. After washing with 0.9% NaCl, the tissue homogenate was prepared in a ratio of 1 g of wet tissue weight to 9 ml of 1.15% KCl by using a Teflon-glass homogenizer (A.H.Thomas Co.,U.S.A.). The whole homogenatewas kept on ice for the determination of malondialdehyde level.

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Experiment II.2 Effect of piperine pretreatment *in vivo* on CCl_4-stimulated lipid peroxidation in rat liver homogenate

To evaluate whether piperine treatment *in vivo* has any potentiating effect on CCl_4-stimulated lipid peroxidation *in vitro*, the effect of CCl_4 *in vitro* was initially explored by using different concentrations of CCl_4 and incubating for 30 min, thereafter, the formation of malondialdehyde was measured.

The overnight fasted rats were sacrificed by decapitation. The liver was rapidly removed and perfused with ice-cold 0.9% NaCl solution. The liver homogenate was prepared similarly to the previous experiment (II.1). The whole homogenate was kept on ice before adding CCl_4 to obtain the final concentrations of 3.1, 4.7, 6.3, 7.8, 9.4 and 12.5×10^{-5} M, respectively. At 30 min after adding CCl_4, the amount of malondialdehyde production was measured.

After the effect of CCl_4-stimulated lipid peroxidation *in vitro* was established, the effect of piperine treatment *in vivo* on CCl_4-induced changes of lipid peroxidation in rat liver homogenate was further evaluated. The overnight fasted rats were intragastrically administered with piperine at the dose of 100 mg/kg body weight at 4 h before decapitation. The liver was rapidly removed and perfused with ice-cold 0.9% NaCl solution. The liver homogenate was prepared similarly to the previous experiment (II.1). The whole homogenate was kept on ice

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before adding CCl_4 at the concentrations of 3.1, 6.3, 9.4 and 12.5×10^{-5}M. At 30 min after adding CCl_4, malondialdehyde level was measured.

Experiment II.3 Effect of piperine on the CCl_4 stimulated lipid peroxidation in rat liver homogenate

Experiment II.3.1 Time-course of the effect of piperine administration on CCl_4 stimulated lipid peroxidation in rat liver homogenate

This experiment was designed to evaluate whether piperine or its metabolites in the body produce the enhancing effect on CCl_4. The overnight fasted rats were decapitated, the livers were rapidly removed and perfused with ice-cold 0.9% NaCl solution. The whole homogenate was kept on ice before adding piperine (50×10^{-5}M) and CCl_4 (6.3×10^{-5}M). Piperine was added to the homogenate at varying time intervals of 30,20 and 10 min prior to CCl_4, simultaneous with CCl_4 and 10, 20 and 30 min after CCl_4. At 30 min after adding CCl_4, malondialdehyde level was measured.

Experiment II.3.2 Dose-response effect of piperine administration on CCl_4-stimulated lipid peroxidation in rat liver homogenate

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From the previous experiment (II.3.1), to produce the enhancing effect on CCl_4 it was essential to add

piperine prior to or simultaneous with CCl_4. To investigate the relationship between the dose and the effect, various concentrations of piperine, 10,20,30,40, 50, 75, or 100×10^{-5} M and CCl_4 at concentration of 6.3×10^{-5} M were simultaneously added into the rat liver homogenate. After incubation for 30 min, the amount of malondialdehyde production was measured.

Study III. Effect of piperine on NADPH-cytochrome c reductase in CCl_4-induced rat

Experiment III.1 Effect of piperine pretreatment on NADPH-cytochrome c reductase in CCl_4-induced rat in vivo

From the previous experiment (I.3), 4 h of piperine pretreatment at a dose of 100 mg/kg body weight produced maximal potentiating effect on CCl_4 hepatotoxicity. This protocol of treatment would be employed here to explore its effect on NADPH cytochrome c reductase activity induced by CCl_4. The overnight fasted animals were intragastrically administered with piperine (100 mg/kg BW) for 4 h before CCl_4 (0.1 ml/kg BW) injection and they were decapitated 24 h after CCl_4 administration. The liver was rapidly removed, weighed and chilled in ice-cold 0.9% NaCl. The liver was perfused with ice-cold 0.9% NaCl solution via portal vein before homogenization. Tissue was homogenized in 3 volume of 1.15% KCl by using a Teflon-glass homogenizer (A.H.

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Thomas, Co., U.S.A.). This homogenate was centrifuged at 9,000xg for 25 min. The supernatant was further centrifuged at 105,000xg for 60 min. The obtained microsomal pellet was resuspended in 3 volume (w/v) of ice-cold 0.1 M potassium phosphate buffer (pH 7.4) and was employed for the determination of NADPH-cytochrome c reductase activity.

Experiment III.2 Effect of piperine pretreatment *in vivo* on CCl_4-induced NADPH cytochrome c reductase activity in rat liver microsomes

To evaluate whether piperine treatment *in vivo* has any potentiating effect on CCl_4-induced NADPH cytochrome c redictase *in vitro*, the effect of CCl_4 *in vitro* was initially explored by incubating the microsomes with different concentrations of CCl_4 for 30 min, thereafter, the activity of NADPH-cytochrome c reductase was then measured.

The overnight fasted rats were decapitated. The liver was rapidly removed, weighed and chilled in ice-cold 0.9% NaCl. The liver was perfused with an ice-cold 0.9% NaCl solution via portal vein. The liver microsomal fraction was prepared similar to the previous experiment (III.1). The microsome was kept on ice before adding CCl_4 to obtainthe final concentrations of 3.1, 4.7, 6.3, 7.8, 9.4 and 12.5×10^{-3} M. At 30 min after addition of CCl_4, NADPH-cytochrome c reductase activity was measured.

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After the effect of CCl_4 on NADPH-cytochrome creductase activity in vitro was established, the effect of piperine treatment in vivo on CCl_4-induced change of NADPH-cytochrome c reductase in microsomal rat liver was further explored. The overnight fasted rats were intragastrically administered with piperine at the dose of 100 mg/kg body weight for 4 h before decapitation. Then the liver was rapidly removed and perfused with ice-cold 0.9%NaCl solution. The liver microsomal fraction was similarly prepared to the previous experiment (III.1). The microsome was kept on ice before adding CCl_4 to obtain the final concentrations of 3.1,6.3,9.4 and 12.5×10^{-5}M. 30 min after addition of CCl_4 was added, NADPH-cytochrome c reductase activity was measured.

Experiment III.3 Dose-response effect of piperine administration on CCl_4-induced NADPH-cytochrome c reductase in rat liver microsomes.

From the previous experiment (II.3.1), it was essential to add piperine prior to or simultaneously with CCl_4 to produce the enhancing effect on CCl_4-induced hepatotoxicity. To investigate the relationship between the dose and the effect,various concentrations of piperine 10, 20, 30, 40, 50, 75 or 100×10^{-5}M and CCl_4 at a concentration of 6.3×10^{5}M were simultaneously added to rat liver the microsomes. After incubation for 30 min, NADPH-cytochrome c reductase activity was measured.

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CHAPTER IV

RESULTS

Study I. Effect of piperine on CCl_4-induced hepatotoxicity

Experiment I.1 Dose-response effect of CCl_4-induced hepatotoxicity in rat

Although, the hepatotoxicity of CCl_4 in rat has long been established, the results obtained in different laboratories are varied and depended upon several factors e.g. species (2, 3, sexes (8, 9) the nutritional status and dietary factors etc. (10, 11). Therefore, in the present study, the dose-response effects of CCl_4 in inducing the hepatotoxicity under our laboratory conditions were initially explored. Subsequently, the effects of piperine in modifying CCl_4-hepatotoxicity were evaluated.

CCl_4 was intraperitonealy injected into rats at the doses of 0.05, 0.1, 0.15, 0.25 and 0.4 ml/kg BW, respectively. All animals were sacrificed at 24 h after the CCl_4 administration. Plasma levels of hepatic enzymes glutamic pyruvic transaminase (PGPT) and glutamic oxaloacetic transaminase (PGOT) were measured and used as indices of liver cell damage. As shown in Table I and Figure 1, both PGPT and PGOT activities were significantly increased with the increasing doses of CCl_4 ($P<0.001$).

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The maximal effect of the CCl_4 was observed from a dose of 0.15 ml/kg body weight onward. The dose-response effect was found in the range of 0.05-0.40 ml/kg BW. The PGPT activity was increased from 45.7±0.3 units in the corn oil-control to 172.0±2.1, 347.0±3.2, 605.0±1.2, 591.0±2.4 and 577.0±0.9 units in the animals treated with CCl_4 at the doses of 0.05, 0.10, 0.15, 0.25 and 0.40 ml/kg BW, respectively. Likewise, the PGOT activity was similarly increased. The PGOT was increased from 97.9+2.8 units in the corn oil-control to 202.0±4.8, 382.0±4.1, 378.0±4.2, 660.0±2.2 and 647.0±2.0 units in the animals treated with CCl_4 at the doses of 0.05, 0.10, 0.15, 0.25 and 0.40 ml/kg BW, respectively.

Experiment I.2 Time-course of the effects of piperine pretreatment on CCl_4-induced hepatotoxicity.

From the experiment I.1, the dose dependent effect of CCl_4 in inducing hepatotoxicity was found to be in the range of 0.05 to 0.40 ml/kg BW. To investigate the action of piperine in modifying CCl_4-induced hepatotoxicity, a submaximal dose of CCl_4 (0.1 ml/kg BW) was chosen for subsequent studies. Piperine at a dose of 100 mg/kg BW was intragastrically administered to animals at varying intervals of 1,2,3,4,5,6 and 20 h prior to CCl_4 injection and they were sacrificed at 24 h after CCl_4 administration. In Table II and Figure 2, the activities of PGPT and PGOT in the CCl_4-treated animals were

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significantly elevated from 45.6±0.3 and 97.9±2.8 in controls to 344.0±4.2 and 379.0±6.2 units, respectively. Treatment with piperine alone did not produce any alteration. When piperine was given before CCl_4 administration, piperine pretreatment for 1 and 2 hhad no significant effect on CCl_4-induced hepatotoxicity whereas piperine pretreatment for 3-6 h significantly enhanced CCl_4-induced hepatotoxicity. The enhancing effect appeared to be maximal at 4 h of piperine pretreatment and it did not exist by 20 h after the treatment. In Table II and Figure 2, PGPT activity was significant increased from 344.0±4.2 unit in animals receiving CCl_4-treatment alone to 382.5±2.9, 590.5±2.1, 392.0±1.4 and 374.8±1.3 units in the animals which piperine was given prior to CCl_4 for 3,4,5 and 6 h, respectively. Likewise, the PGOT activity was significant increased from 379.0±6.2 unit in animals receiving CCl_4-treatment alone to 456.5±1.5, 697.0±3.2, 479.5±2.3 and 463.0±1.6 units in the animals which piperine was given prior to CCl_4 for 3,4,5 and 6 h, respectively ($P<0.001$). Changes in profile pattern of both enzyme activities were essentially similar, as shown in Figure 2.

In Table II and Figure 3 show the level of plasma triglycerides and liver triglycerides content in CCl_4-treated animals which were pretreated with piperine.The significant enhancing effect of piperine also occurred between 3 to 6 h of the pretreatment periods and the maximal effect was found at 4 h of the

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preteatment. The level of plasma triglyceride was decreased from 154.0±2.3 mg% in corn-oil control to 91.7±1.5, 69.5±0.5, 87.3±0.9 and 96.3±0.7 mg% in the animals which piperine was given prior to CCl_4 for 3, 4, 5 and 6 h, respectively, ($P<0.001$). Concurrent to the decrease in plasma triglyceride level, the liver triglyceride content was significantly increased. It was increased from 6.8±0.2 mg/g liver in corn oil-control to 11.3±0.2 mg/g liver in the CCl_4-treated animals and further increased to 13.6±0.3, 17.5±0.3, 14.1±0.2 and 12.7±0.2 mg/g liver in the piperine pretreated animals for 3,4,5 and 6 h, respectively, ($P<0.001$). However, treatment with CCl_4 together with piperine did not alter the liver weight ($P<0.05$).

From the results of this section, it was apparent that piperine produced an enhancing effect on CCl_4-induced hepatotoxicity, whereas piperine had no hepatotoxic effect on its own. Piperine administration when it was given between 3 to 6 h prior to CCl_4 administration and the maximal effect was obtained at 4 h prior to CCl_4 administration. At which, the activities of PGPT, PGOT and liver triglyceride content were highest and the plasma triglyceride level was lowest.

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Experiment I.3 Dose-response effect of piperine pretreatment on the CCl_4-induced hepatotoxicity.

From the results in experiment I.2 the maximal enhancing effect of piperine on CCl_4 was found at 4 h of pretreatment period. In this section, the dose-response effect of piperine in enhancing the CCl_4-induced hepatotoxicity was investigated in 4 h piperine-pretreated animals only. Various doses of piperine ranging from 5, 10, 50, 100 and 150 mg/kg BW were intragastrically administered to animals. Four hours later CCl_4 was intraperitonealy injected and the animal was sacrificed at 24 h after the CCl_4 administration. Table III,Figures 4 and 5 showed the effect of the piperine-treatment in the absence of CCl_4. Piperine did not produce any significant change on the liver functions. The PGPT, PGOT, plasma triglyceride level, liver triglyceride content and liver weight in piperine treated animals were comparable to those of corn oil-control animals. Treatment of the rats with piperine at the doses of 5 and 10 mg/kg BW at 4 h before CCl_4 administration did not significantly alter the liver function from those receiving by CCl_4-treatment alone. The significant alteration by piperine was observed at the doses of 50 and 100 mg/kg BW but not at a dose of 150 mg/kg body weight. Pretreatment with piperine at a dose of 100 mg/kg BW significantly increased the PGPT and PGOT activities from 344.0±4.2 and 379.0±6.2 units in CCl_4-treated animals to 583.0±3.4 and 678.0±6.4 units in

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animals with piperine-pretreatment, respectively, ($P<0.001$). Likewise, the plasma triglyceride level and liver triglyceride content were significantly changed from 154.0±2.3 mg% and 6.8±0.2 mg/g liver in the CCl_4-treated animals without piperine pretreatment to 70.0±0.9 mg% and 17.2±0.2 mg/g liver in animals with piperine pretreatment, respectively ($P<0.001$). Neither CCl_4 treatment alone nor CCl_4 together with piperine treatment significantly altered the liver weight.

From the results, it seems that piperine produced a dose-dependent effect in enhancing CCl_4-induced hepatotoxicity. The maximal effect was observed at a dose of 100 mg/kg BW. No effect of piperine at a dose of 150 mg/kg BW probably related to its precipitation in the gastrointestinal tract which might result in a less absorption.

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Table I. Effects of CCl_4 on plasma activities of enzymes glutamic pyruvic transaminase (PGPT) and glutamic oxaloacetic transaminase (PGOT). Rats were intraperitoneally injected with various doses of CCl_4 and were sacrificed at 24 h after CCl_4 administration.

Parameters	Carbon tetrachloride (ml/kg B.W.)					
	0	0.05	0.10	0.15	0.25	0.40
PGPT (units)	45.7±0.3	172.0±2.1**	347.0±3.2**	605.0±1.2**	591.0±2.4**	577.0±0.9**
PGOT (units)	97.9±2.8	202.0±4.8**	382.5±4.1**	678.0±4.2**	660.0±2.2**	647.0±2.0**

Each value is mean±SEM, obtained from 10 animals.

**P<0.001, when compare to the control (corn oil).

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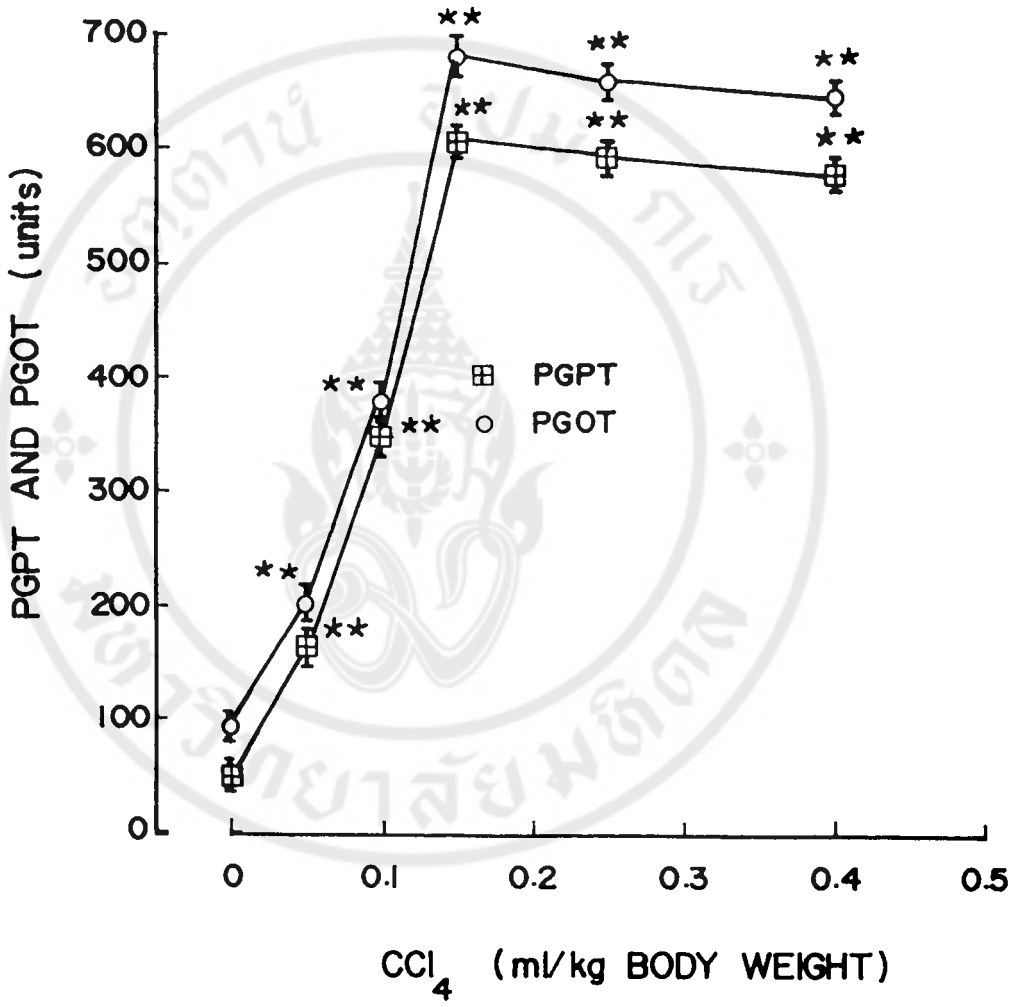

Figure 1. Effect of CCl_4 on the activities of plasma enzymes glutamic pyruvic transaminase (PGPT) and glutamic oxaloacetic transaminase (PGOT). Rats were intraperitoneally injected with various doses of CCl_4 and sacrificed at 24 h after CCl_4 administration. Each value is mean±SEM, obtained from 10 animals. **P<0.001 when compare to the control received corn oil.

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Table II. Effect of piperine pretreatment on CCl_4-induced changes of plasma levels of glutamic pyruvic transaminase (PGPT), glutamic oxaloacetic transaminase (PGOT) activities, and plasma triglyceride (TG), liver triglyceride (TG) and liver weight. Piperine (100 mg/kg BW) was intragastrically administered at varying intervals before CCl_4 administration (0.1 ml/kg BW, i.p.) and the animals were sacrificed at 24 h after CCl_4 administration.

Treatment	Piperine before CCl_4 (h)	PGPT (units)	PGOT (units)	Plasma TG (mg%)	Liver TG (mg/g liver)	Liver weight (g/100 g BW)
Corn oil	-	45.6±0.3	97.9±2.8	154.0±2.3	6.8±0.2	4.2±0.1
Piperine	-	45.0±0.4	94.0±1.4	158.0±1.4	6.8±0.2	4.0±0.1
CCl_4	-	344.0±4.2	379.0±6.2	107.0±1.2	11.3±0.2	4.1±0.1
Piperine + CCl_4	1	340.0±3.1	384.0±4.2	107.7±0.8	12.0±0.1	4.2±0.1
Piperine + CCl_4	2	341.0±2.9	387.0±5.9	110.5±1.2	12.0±0.3	4.1±0.1
Piperine + CCl_4	3	382.5±2.9**	456.5±1.5**	91.7±1.5**	13.6±0.3**	4.0±0.1
Piperine + CCl_4	4	590.5±2.1**	697.0±3.2**	69.5±0.5**	17.5±0.3**	4.2±0.1
Piperine + CCl_4	5	392.0±1.4**	479.5±2.3**	87.3±0.9**	14.1±0.2**	4.1±0.1
Piperine + CCl_4	6	374.8±1.3**	463.0±1.6**	96.3±0.7**	12.7±0.2**	4.0±0.1
Piperine + CCl_4	20	340.5±2.8	374.0±5.9	107.5±0.9	11.4±0.2	4.0±0.1

Each value is mean±SEM, obtained from 10 animals.
**P<0.001, when compare to CCl_4-treated group.

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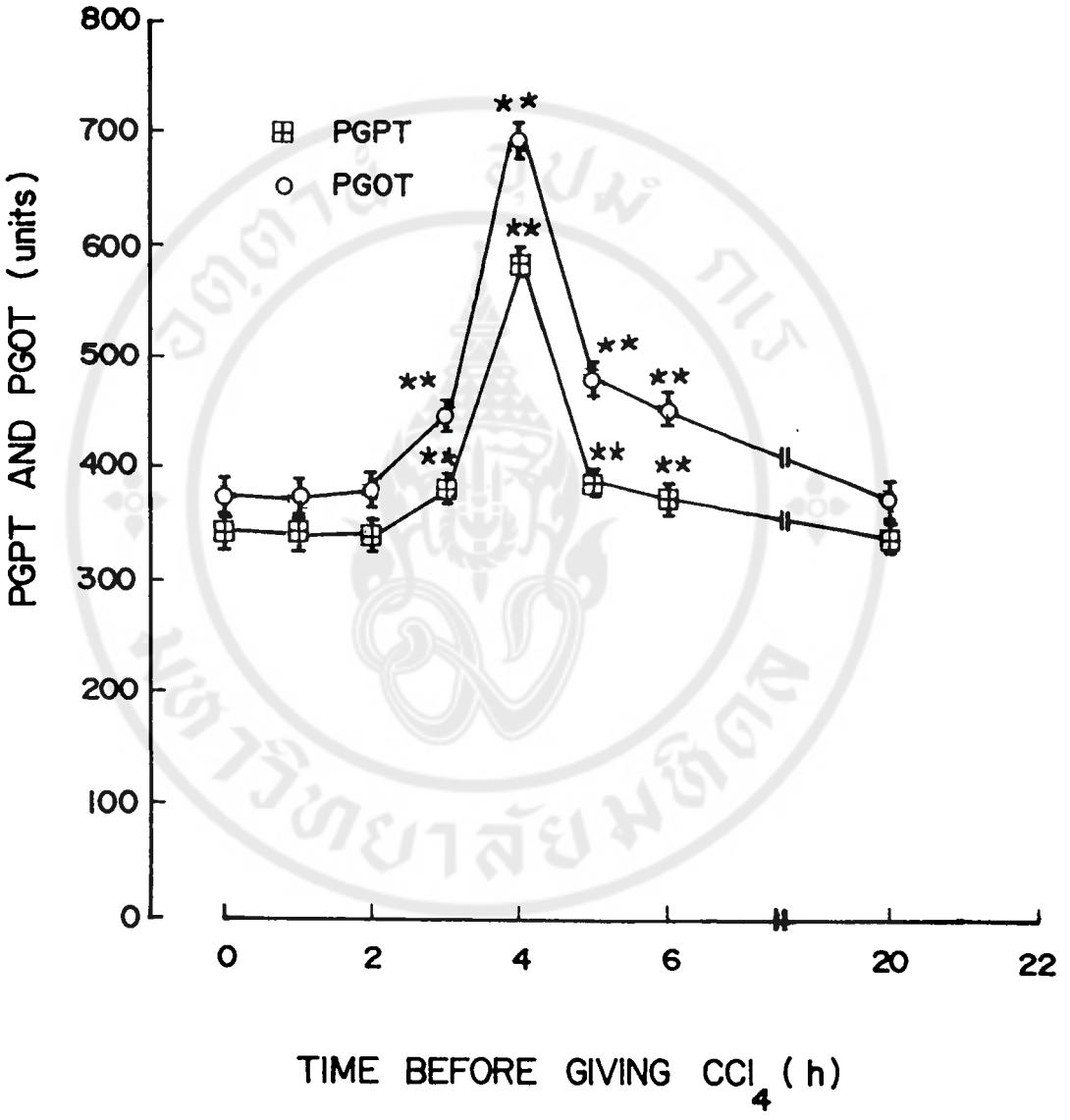

Figure 2. Effect of piperine pretreatment on CCl_4-induced changes of plasma glutamic pyruvic transaminase (PGPT) and glutamic oxaloacetic transaminase (PGOT) activities. Piperine (100 mg/kg BW) was intragastrically administered at varying time intervals before CCl_4 (0.1 ml/kg BW, ip) administration and the animals were sacrificed at 24 h after the CCl_4 administration. Each value is mean±SEM, obtained from 10 animals. **P<0.001 when compare to the control which received CCl_4.

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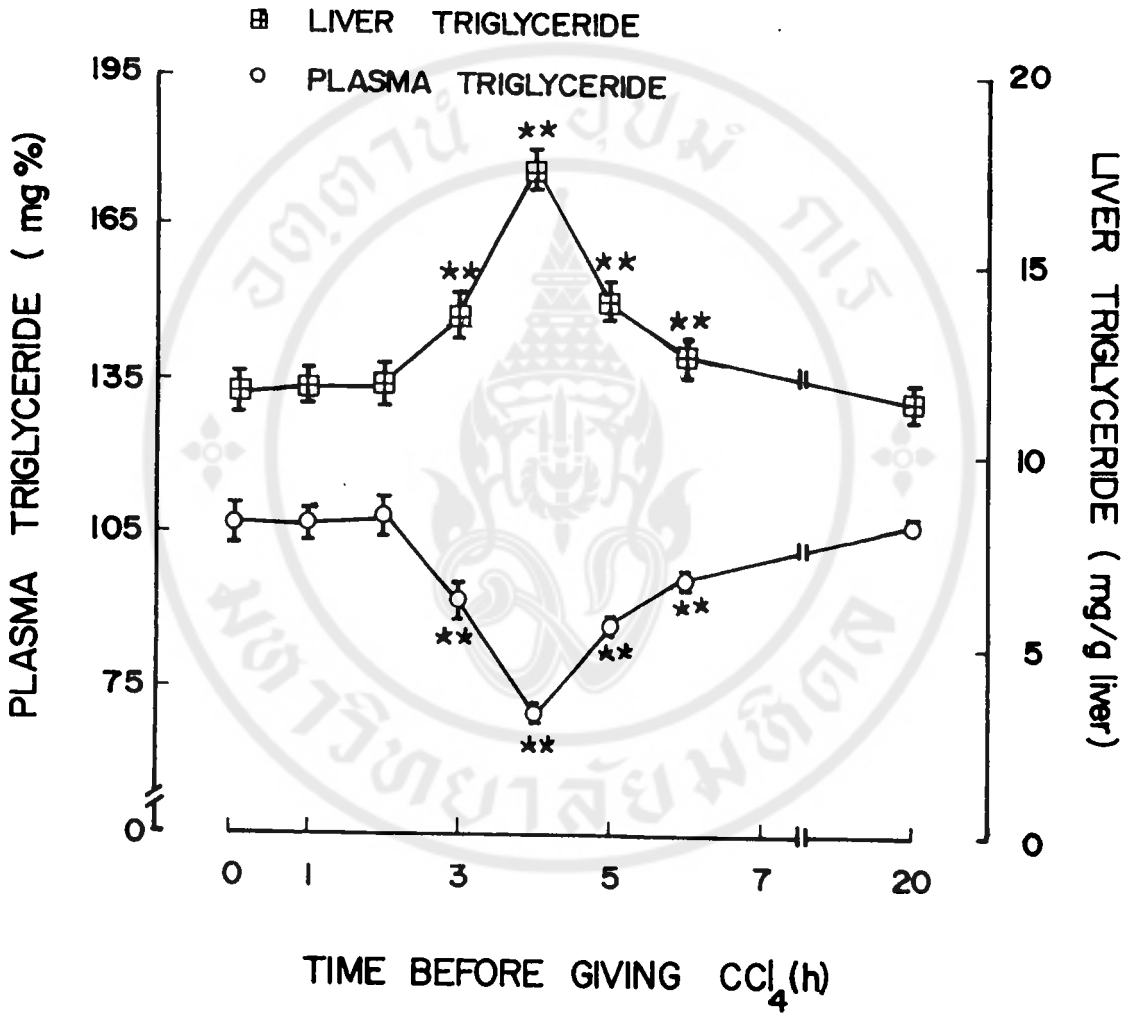

Figure 3. Effect of piperine pretreatment on CCl_4 induced changes of plasma levels of triglyceride and liver triglyceride. Piperine (100 mg/kg. BW) was intragastrically administered at varying time intervals before CCl_4 (0.1 ml/kg BW; i.p.) administration and the animals were sacrificed at 24 h after CCl_4 administration. Each value is mean±SEM, obtained from 10 animals. **P<0.001 when compare to the control which received CCl_4.

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Table III. Effect of piperine pretreatment on plasma glutamic pyruvic transaminase (PGPT), glutamic oxaloacetic transaminase (PGOT), and plasma triglyceride level (TG), liver triglyceride (TG) content and liver weight in CCl_4-treated rats. Various doses of piperine were administered 4 h prior to CCl_4 administration.

Treatment	Piperine (mg/kg BW)	PGPT (units)	PGOT (units)	Plasma TG (mg%)	Liver TG (mg/g liver)	Liver (g/100 g BW)
Corn oil	-	45.6±0.3	97.9±2.8	154.0±2.3	6.8±0.2	4.1±0.2
Piperine	100	45.0±0.4	94.0±1.4	158.0±1.4	6.8±0.2	4.0±0.1
CCl_4	-	344.0±4.2	379.0±6.2	107.0±1.2	11.3±0.2	4.2±0.1
Piperine + CCl_4	5	348.5±1.4	381.0±6.2	106.5±1.1	11.7±0.2	4.1±0.1
Piperine + CCl_4	10	350.0±2.8	380.0±5.0	106.0±1.3	11.8±0.2	4.1±0.1
Piperine + CCl_4	50	414.0±2.0**	471.0±4.6**	89.4±1.2**	12.8±0.2**	4.1±0.1
Piperine + CCl_4	100	583.0±3.4**	678.0±6.4**	70.0±0.9**	17.2±0.2**	4.0±0.1
Piperine + CCl_4	150	341.5±1.9	383.0±5.9	106.0±1.3	12.1±0.1	4.1±0.1

Each value is mean±SEM, obtained form 10 animals.
**P<0.001, when compare to CCl_4 treated group without piperine.

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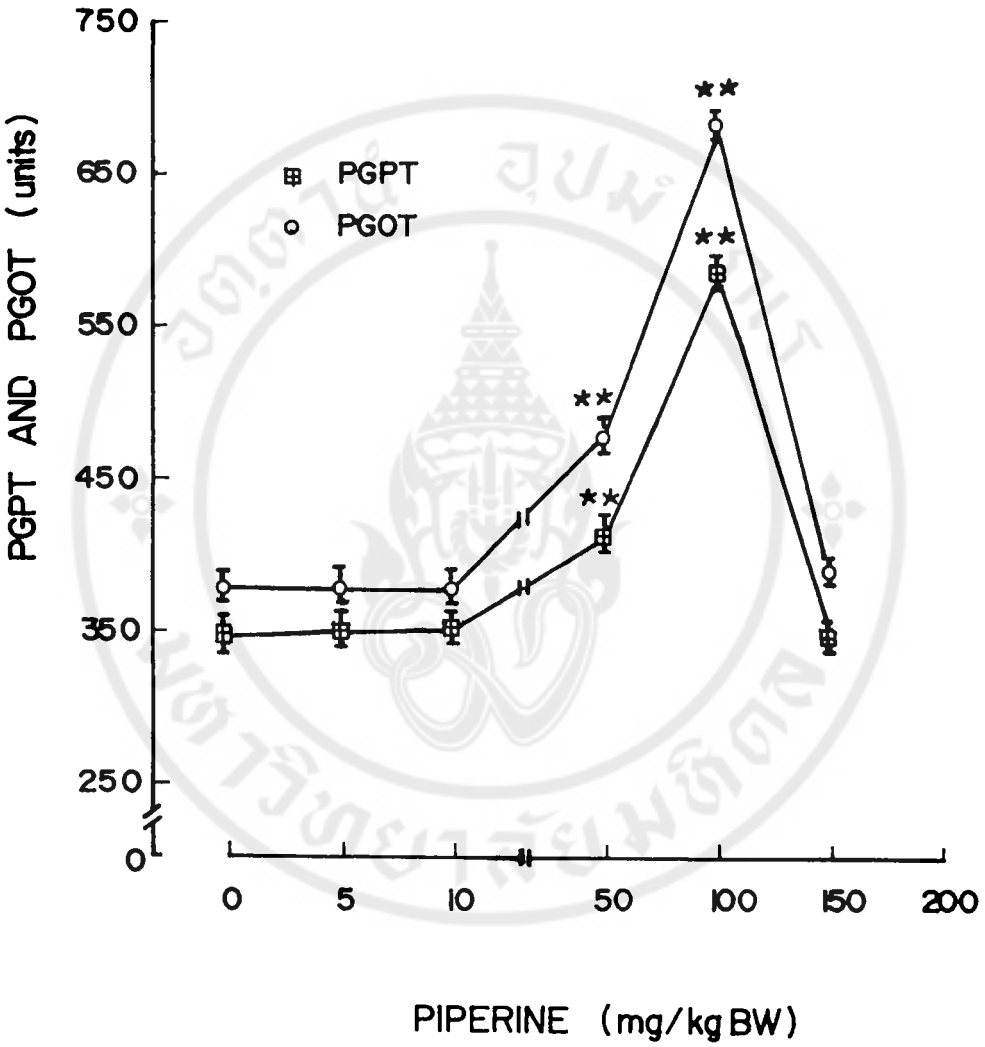

Figure 4. Effect of piperine pretreatment on activities of plasma glutamic pyruvic transaminase (PGPT) and oxaloacetic transaminase (PGOT) in CCl_4-treated rats. Various doses of piperine were administered 4 h prior to CCl_4 administration. Each value is mean±SEM, obtained from 10 animals. **P<0.001 when compare to CCl_4-treated group without piperine.

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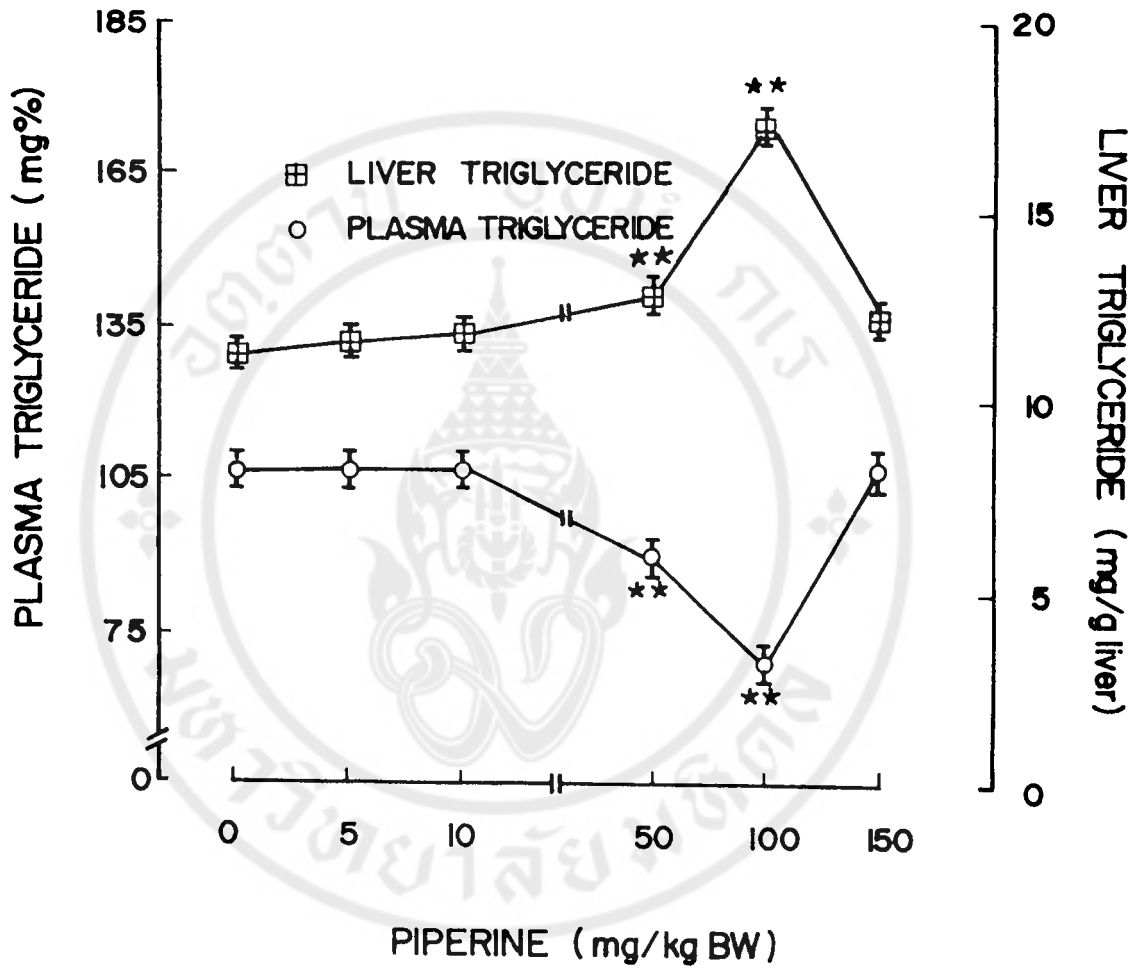

Figure 5. Effect of piperine pretreatment on plasma triglyceride level and liver triglyceride content in CCl_4-treated rats. Various doses of piperine were administered at 4 h prior to CCl_4 administration. Each value is mean±SEM, obtained from 10 animals. **P<0.001 when compare to CCl_4-treated group.

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Study II. Effect of piperine on the stimulation of lipid peroxidation by CCl_4 in rat liver

Experiment II.1 Effect of piperine pretreatment on the stimulation of lipid peroxidation by CCl_4 treatment

From the previous experiment, piperine treatment at the dose 100 mg/kg BW 4 h before giving CCl_4 produced the maximal enhancing effect. This protocol of treatment would be employed here to explore its effect on lipid peroxidation stimulated by CCl_4, since the lipid peroxidation in cellular membrane is believed to be a critical toxic event of CCl_4 for liver injury. Four hours before CCl_4 administration the animals were treated with piperine and they were sacrificed 1 h after CCl_4 administration. As the production of lipid peroxides correlates with malondialdehyde (MDA) production, the extent of lipid peroxidation would be determined from the amount of MDA (17). As shown in Table IV and Figure 6, the lipid peroxidation of the liver homogenate in normal control animals was found to be 81.0±0.3 nmole MDA/100 mg protein. Treatment with piperine had no effect on MDA production. The lipid peroxidation in the piperine-treated group (82.0±0.2 nmole MDA/100 mg protein) was also comparable to that of the control. In contrast, treatment with CCl_4 at the dose of 0.1 ml/kg BW at 1 h before sacrifice significantly increased the level of lipid peroxidation (109.0±1.2 nmole

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MDA/100 mg protein). The increased in lipid peroxidation by CCl_4 was further enhanced by giving piperine 4 h prior to CCl_4. By which the lipid peroxidation was increased to 198.0±1.8 nmole MDA/100 mg protein and it was estimated to be increased by 80% from the CCl_4-treated animals.

The results showed that piperine pretreatment at 4 h before CCl_4 could significantly enhance CCl_4-stimulated lipid peroxidation. As piperine itself has no effect and the obtained result when giving CCl_4 and piperine together was always greater than the effect of CCl_4 alone, it is anticipated that piperine potentiate the effect of CCl_4.

Experiment II.2 Effect of piperine pretreatment on CCl_4 stimulated lipid peroxidation in rat liver homogenate

To evaluate whether piperine treatment *in vivo* hasany potentiating effect on CCl_4 stimulated lipid peroxidation *in vitro*, the liver homogenate was exposed to different concentrations of CCl_4 *in vitro* for 30 min, thereafter the formation of malondialdehyde was measured. As shown in Table V and Figure 7, the amount of MDA production in the liver homogenate was progressively increased with the concentration of CCl_4. It was significantly increased from 84.0±2.4 nmole MDA/100 mg protein in control to 115.0±2.8, 134.0±3.5, 144.1±2.1, 157.0±4.0 and 137.7±4.1 nmole MDA/100 mg protein after exposure to CCl_4 at concentrations of 4.7, 6.3, 7.8, 9.4

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and 12.5×10^{-5} M, respectively. The maximal MDA production was observed at a concentration of 9.4×10^{-5} M. CCl_4 which produced 86.9%±4.9% increase from the control. Table VI and Figure 8 show the results obtained from piperine pretreatment. Profile of changes in lipid peroxidation stimulated by different concentrations of CCl_4 in the liver homogenate obtained from piperine pretreated animals were similar to those in control animals. The potentiating effect of piperine was observed when the homogenate was exposed to CCl_4 at concentrations of 6.3 and 9.4×10^{-5} M. The amount of malondialdehyde were increased from 133.5±0.7 and 155.4±1.0 nmole/100 mg protein in the homogenate exposued to CCl_4 at the concentrations of 6.3 and 9.4×10^{-5} M, respectively, to 143.1±0.9 and 162.3±1.2 nmole/100 mg protein in the homogenate obtained from piperine pretreated animals and exposed to the corresponding concentrations of CCl_4.

Experiment II.3 Effect of piperine on the CCl_4 stimulated lipid peroxidation in rat liver homogenate *in vitro*.

Experiment II.3.1 Time-course effect of piperine administration on CCl_4 stimulated lipid peroxidation in rat liver homogenate.

To evaluate whether piperine itself or its metabolites in the body produce the potentiating effect on CCl_4, in this experiment, the liver homogenate from control rat was directly exposed to both piperine and CCl_4

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in the incubation medium. Piperine was used at a concentration of $50x10^{-5}$M. The potentiating effect of piperine on CCl_4 stimulated lipid peroxidation was found only when piperine was added into the reaction mixture before or simultaneous with the addition of CCl_4. As shown in Table VII and Figure 9, the extent of lipid peroxidation was significantly increased in the liver homogenated which was concurrently exposed to piperine and CCl_4. MDA production was increased from 125.8±0.6 nmole/100 mg protein in the presence of CCl_4 ($6.3x10^{-5}$ M) to 152.8±0.6 nmole/100 mg protein in the presence of CCl_4 together with piperine. Preincubation of the liver homogenate with piperine for 10, 20 and 30 min also significantly increased lipid peroxidation. However,the result was comparable to that when it was exposed to both substances. In contrast, an addition of piperine after exposure to CCl_4 could not produce any potentiating effect. MDA production was not different from those receiving CCl_4 alone.

Experiment II.3.2 Dose-response effect of piperine administration on CCl_4 stimulated lipid peroxidation in rat liver homogenate

From the previous experiment (II.3.1), to obtainthe potentiating effect on CCl_4 it was essential to add piperine prior to or simultaneously with CCl_4. To investigate the relationship between the dose and the effect, various concentrations of piperine (10,20,30,40

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50,75, or 100x10^{-5}M) and CCl_4 at concentration of 6.3x10^{-5}M were simultaneously added into the incubation medium. After 30 min of incubation, the amount of malondialdehyde was determined. As shown in Table VIII and Figure 10, malondialdehyde production was progressively increased with the increased concentrations of piperine. However, the significant potentiating effect was observed only at piperine concentration of 40, 50 and 75x10^{-5}M. Malondialdehyde production was increased from 132.0±1.1 nmole/100 mg protein in the presence of CCl_4 alone to 141.0±1.1, 148.5±0.8 and 142.3±0.9 nmole/100 mg protein in the presence of CCl_4 together with piperine at concentrationsof 40, 50 and 75x10^{-5}M, respectively. In this study, the maximal effect was observed at the concentration of 50x10^{-5}M piperine. The higher concentrations of piperine could not produce further effect, which probably was limited by piperine solubility as the slight turbidity was observed at a dose of 75x10^{-5}M piperine.

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Table IV Effect of piperine pretreatment on CCl_4 stimulated lipid peroxidation in rats. Piperine (100 mg/kg BW) was intragastrically given at 4 h before CCl_4 administration (0.1 ml/kg BW; i.p.) and the animals were sacrificed at 1 h after CCl_4 administration.

Rat No.	Malondialdehyde (nmole/100 mg protein)			
	Corn oil	Piperine	CCl_4	Piperine + CCl_4
1	82.0	83.0	110.0	204.0 (85.5)
2	82.0	83.0	114.0	203.0 (78.1)
3	80.0	81.0	115.0	204.0 (77.3)
4	81.0	82.0	105.0	190.0 (80.9)
5	81.0	82.0	105.0	190.0 (80.9)
6	81.0	82.0	108.0	196.0 (81.4)
7	81.0	82.0	108.0	196.0 (81.4)
8	80.0	82.0	105.0	196.0 (86.7)
9	80.0	81.0	110.0	198.0 (80.0)
10	80.0	81.0	109.0	199.0 (82.5)
$\bar{X}$	81.0±0.3	82.0±0.2	109.0±1.2**	198.0±1.8**‡‡ (81.5±1.0)

Each value is mean ±SEM, obtained from 10 animals.

**P<0.001 when compare to control corn oil

‡‡P<0.001 when compare to the CCl_4-treated group.

Number in parentheses indicates percent increase from the CCl_4-treated animals.

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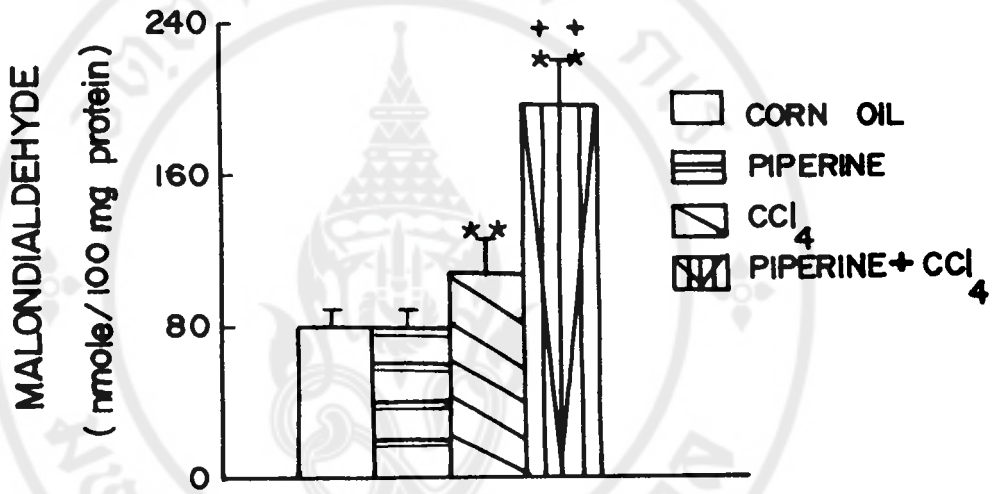

Figure 6. Effect of piperine on CCl_4-stimulated lipid peroxidation in rats. Piperine (100 mg/kg BW) was intragastrically given at 4 h before CCl_4 administration (0.1 ml/kg BW, i.p.) and the animals were sacrificed at 1 h after CCl_4 administration. Each value is mean ± SEM, obtained from 10 animals. **P<0.001 when compare to the control corn oil. ''P<0.001 when compare to CCl_4-treated group.

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Table V. Effect of CCl_4 exposure on malondialdehyde (MDA) production by rat liver homogenate. CCl_4 was added into homogenate and malondialdehyde formation was measured after 30 min of incubation.

Treatment	MDA (nmole/100 mg protein)
Control	84.0±2.4
Solvent	84.5±2.4
CCl_4 $3.1x10^{-5}$ M	85.0±2.5
CCl_4 $4.7x10^{-5}$ M	115.0±2.8**
CCl_4 $6.3x10^{-5}$ M	134.0±3.5**
CCl_4 $7.8x10^{-5}$ M	144.1±2.1**
CCl_4 $9.4x10^{-5}$ M	157.0±4.0**
CCl_4 $12.5x10^{-5}$ M	137.7±4.1**

Each value is mean±SEM, obtained from 7 animals

**P<0.001, when compare to the control.

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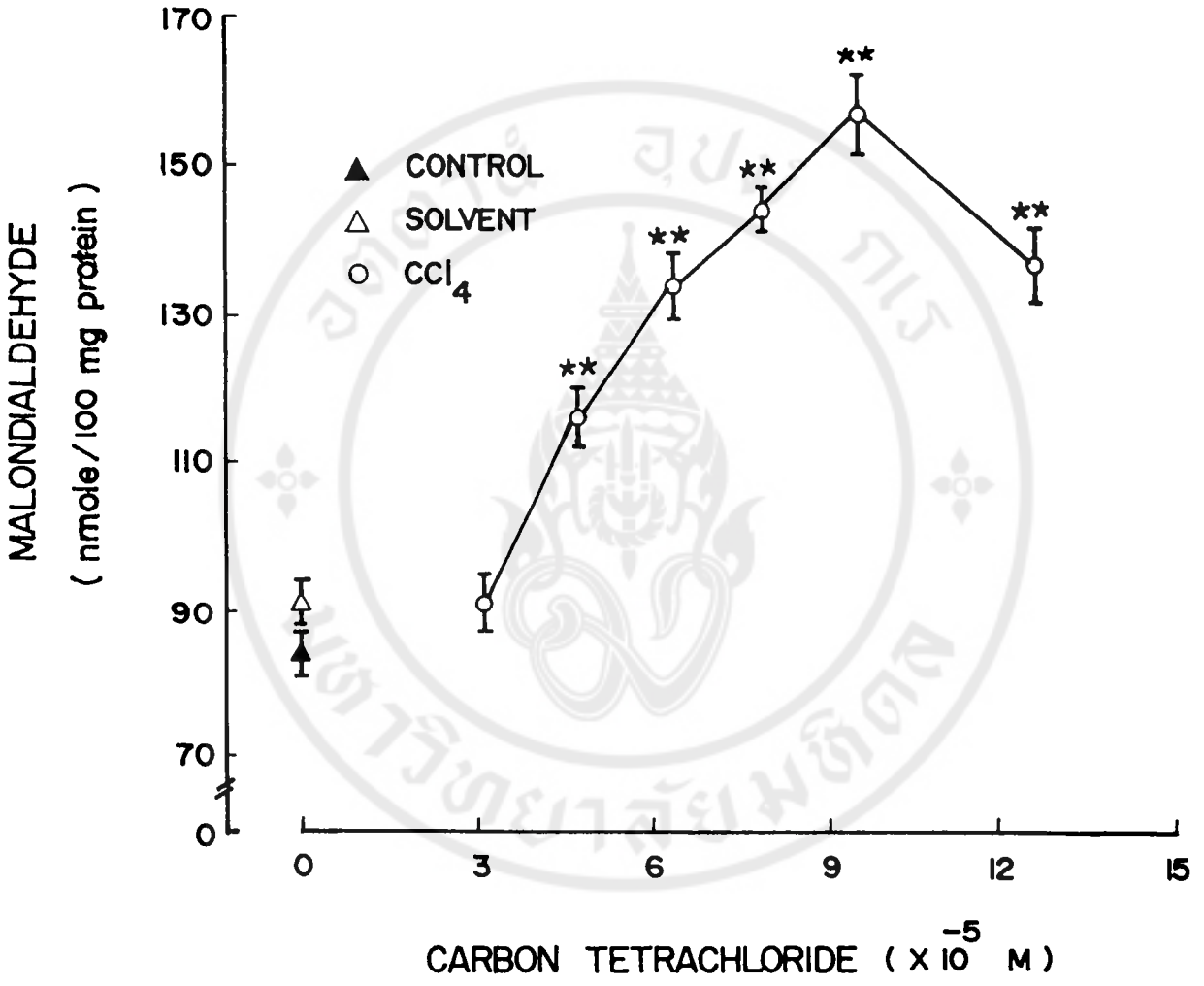

Figure 7. Effect of CCl_4 on malondialdehyde (MDA) production in rat liver homogenate. CCl_4 was added into the homogenate and MDA formation was measured after 30 min of incubation. Each value is mean±SEM, obtained from 7 animals. **P<0.001 when compare to the control

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Table VI. Effect of piperine treatment *in vivo* on the production of malondialdehyde (MDA) by rat liver homogenate. The animals were pretreated with piperine (100 mg/kg BW, PO) at 4 h before sacrifice. The rat liver homogenate was exposed to CCl_4 for 30 min.

CCl_4 ($\times 10^{-6}$ M)	MDA (nmole/100 mg protein)	
	-	Piperine
Solvent	85.0±0.5	88.0±0.8
0	83.0±0.5	85.0±0.5
3.1	91.2±0.6	92.0±0.7
6.3	133.5±0.7	143.1±0.9*
9.4	155.4±1.0	162.3±1.2*
12.5	136.4±1.3	140.0±0.5

Each value is mean±SEM, obtained from 9 animals

*$P<0.05$ when compare with the corresponding CCl_4 treatment.

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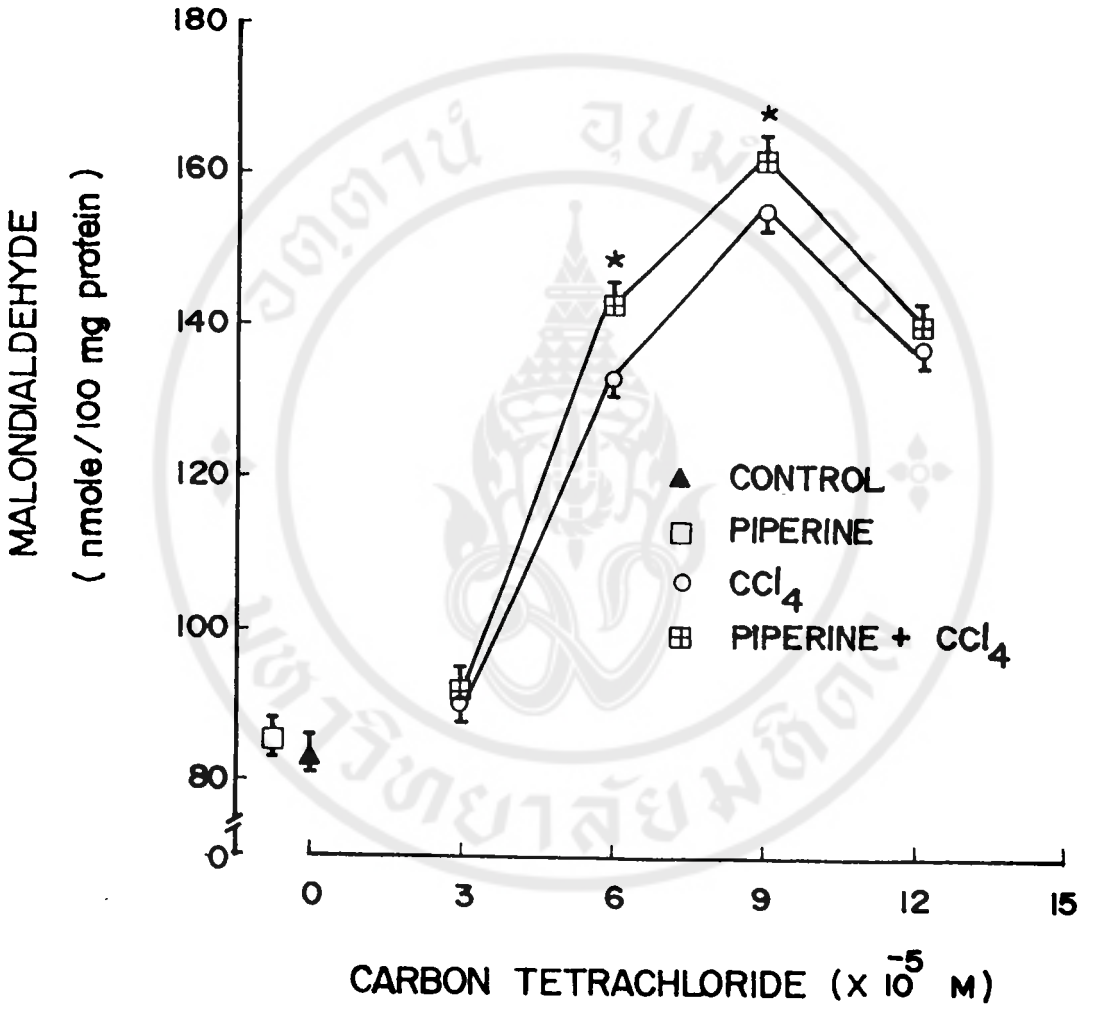

Figure 8. Effect of piperine pretreatment on the production of malondialdehyde in rat liver homogenate. The animals were intragastrically given piperine (100 mg/kg BW) at 4 h before sacrificed. The rat liver homogenate was incubated with CCl_4 for 30 min. Each value is mean±SEM, obtained from 9 animals. *P<0.05 when compare to the corresponding CCl_4 treatment

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Table VII. Effect of piperine on CCl_4- of malondialdehyde (MDA) production in rat liver homogenate. The homogenate was exposed to piperine ($50x10^{-5}M$) and CCl_4 $6.3x10^{-5}M$ at different period of time and the level of MDA production was assayed at 30 min after CCl_4 administration.

Treatment	Exposure time to piperine (min)		MDA nmole/100 mg protein
	Before CCl_4	After CCl_4	
Control			80.0±0.5
Solvent			80.5±0.4
Piperine			81.0±0.3
CCl_4			125.8±0.6
Piperine+CCl_4	10		151.0±0.5*
Piperine+CCl_4	20		151.0±0.3*
Piperine+CCl_4	30		150.0±0.6*
Piperine+CCl_4	Simultaneous		152.8±0.6*
Piperine+CCl_4		10	131.3±0.6
Piperine+CCl_4		20	125.3±0.6
Piperine+CCl_4		30	125.8±0.3

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Each value is mean±SEM, obtained from 4 animals

*P<0.05, when compare to CCl_4 treatment

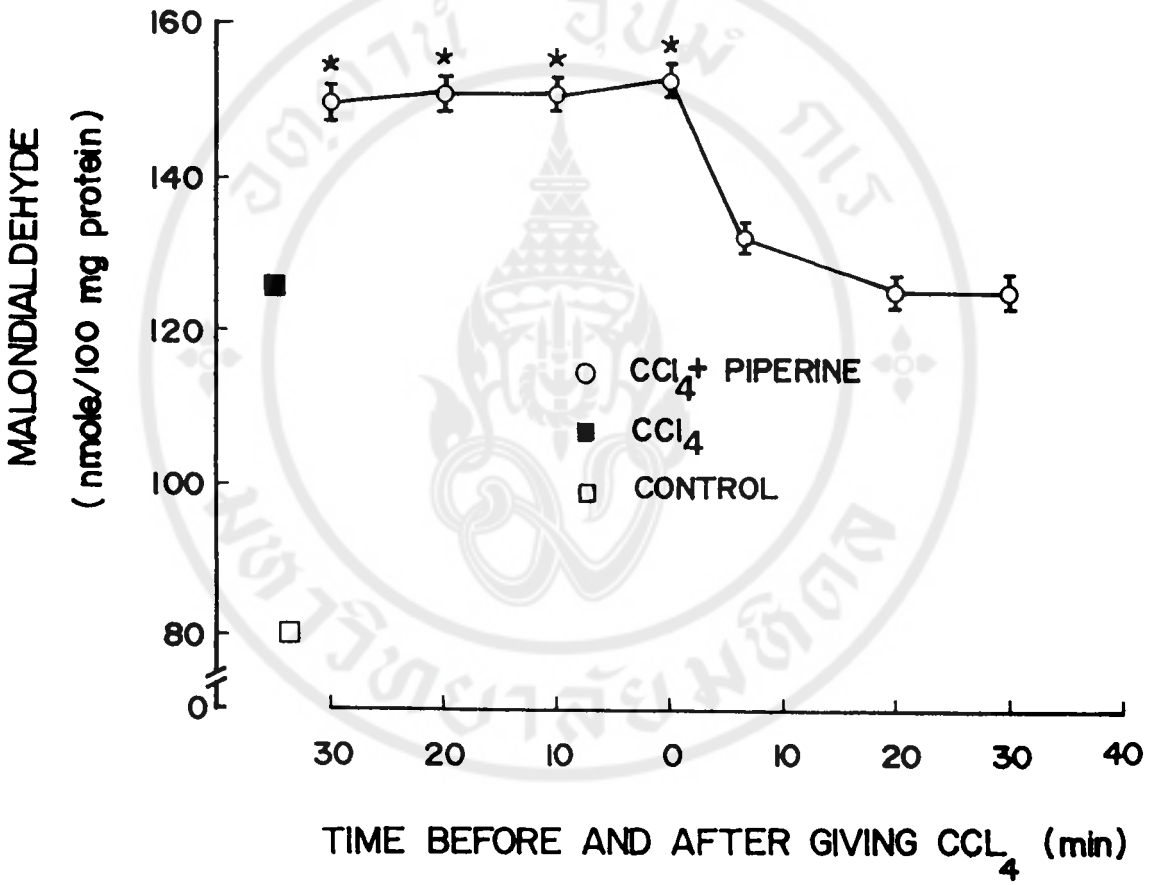

Figure 9. Effect of piperine on the CCl_4-stimulated malondialdehyde simultaneous (MDA)production when piperine $50x10^{-5}$M pre- and post-treatment of CCl_4 $6.3x10^{-3}$M to rat liver homogenate and the MDA formation was measured 30 min of incubation. Each value is mean±SEM, obtained from 4 animals. *$P<0.05$ when compare CCl_4 treatment to CCl_4-piperine treatment.

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Table VIII. Effect of piperine on CCl_4 stimulated malondialdehyde (MDA) production in rat liver homogenate. Piperine and CCl_4 were added simultaneously and level of MDA production was measured after 30 min of incubation.

Treatment	MDA (nmole/100 mg protein)
Control	80.8±0.3
Solvent	81.5±0.7
Piperine (50×10^{-5}M)	81.3±0.6
CCl_4 (6.3×10^{-5}M)	132.0±1.1
CCl_4 + piperine 10×10^{-5}M	132.5±0.8
CCl_4 + piperine 20×10^{-5}M	135.5±0.6
CCl_4 + piperine 30×10^{-5}M	137.3±0.9
CCl_4 + piperine 40×10^{-5}M	141.0±1.1*
CCl_4 + piperine 50×10^{-5}M	148.5±0.8**
CCl_4 + piperine 75×10^{-5}M	142.3±0.9*
CCl_4 + piperine 100×10^{-5}M	136.3±0.9

Each value is mean±SEM obtained from 4 animals

**P<0.001 when compare to CCl_4 treatment

*P<0.05 when compare to CCl_4 treatment

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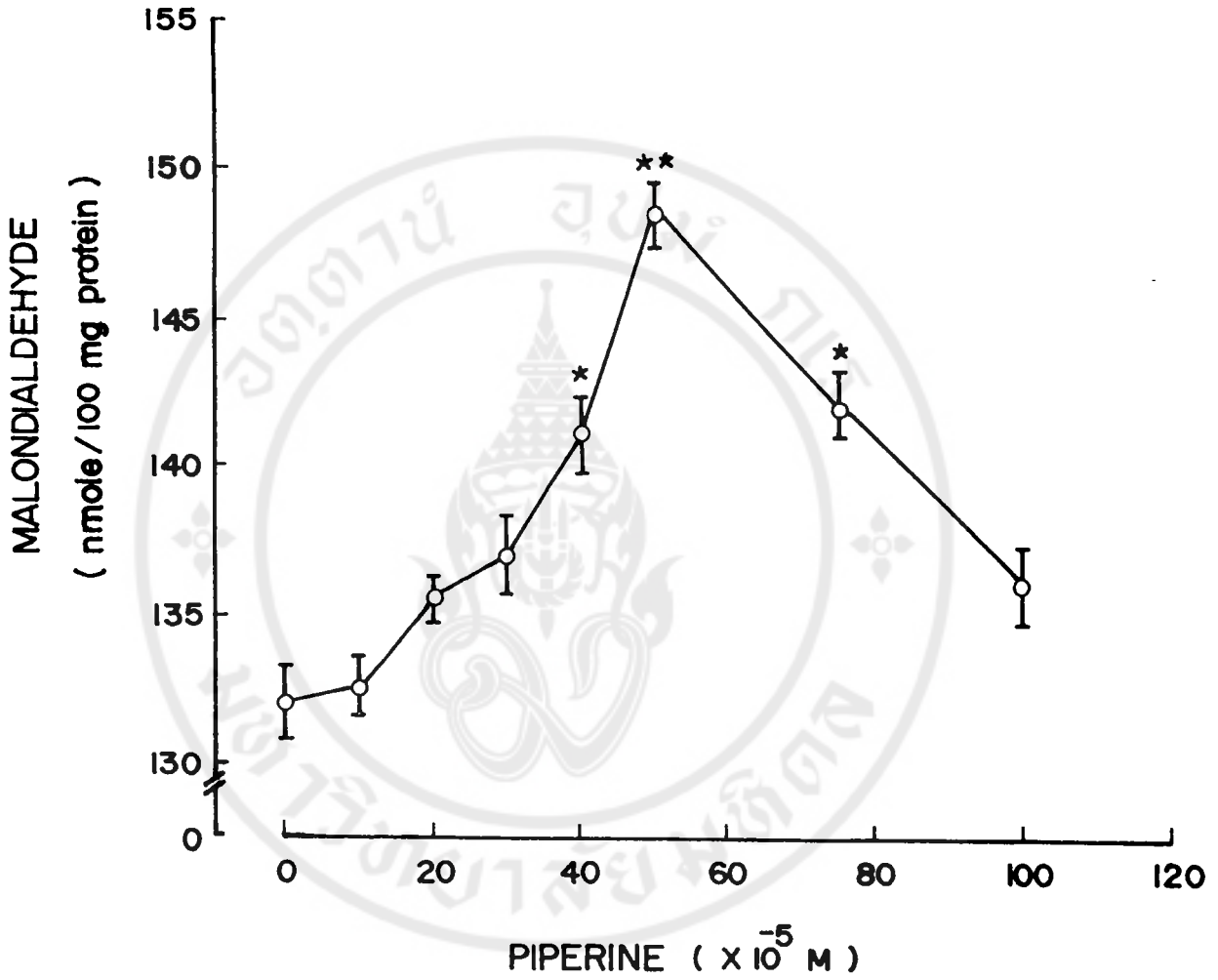

Figure 10. Effect of piperine on CCl_4-stimulated malondialdehyde(MDA) production in rat liver homogenate. Piperine and CCl_4 were added simultaneously and the amount of MDA was measured after 30 min of incubation. Each value is mean±SEM, obtained from 4 animals. **P<0.001 when compare between CCl_4 treatment with CCl_4 + piperine treatment. *P<0.05 when compare between CCl_4 treatment with CCl_4'+ piperine treatment.

Study III. Effect of piperine on NADPH cytochrome c reductase in CCl_4 treated rat

Experiment III.1 Effect of piperine pretreatment on NADPH -cytochrome c reductase in CCl_4 treated rat *in vivo*

From the previous experiment (I.3), piperine treatment at the dose of 100 mg/kg BW at 4 h before giving CCl_4 produced maximal enhancing effect. This protocol of treatment would be employed here to explore its effect on NADPH cytochrome c reductase activity induced by CCl_4, since NADPH-cytochrome c reductase played a major role in the hepatotoxic effect of CCl_4 by converting the hydrocarbon into its toxic metabolites ($\cdot CCl_3$). The animals were treated with piperine at 4 h before CCl_4 and they were sacrificed 1 h after CCl_4 administration. As shown in Table IX and Figure 11, NADPH-cytochrome c reductase activity in control animals was found to be 82.8±1.2 nmole/min/mg protein. Treatment with piperine alone had no significant effect. NADPH-cytochrome c reductase activity in the piperine treated group (83.7±1.8 nmole/min/mg protein) was comparable to that of the control. In contrast, treatment with CCl_4 at the dose of 0.1 ml/kg bw at 24 h before sacrificed significantly increased NADPH-cytochrome c reductase activity (155.0±2.6 nmole/min/mg protein). The increase in NADPH cytochrome c reductase activity by CCl_4 was further enhanced by giving piperine at 4 h prior to CCl_4. The activity was increased

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to 287.5±4.3 nmole/min/mg protein which was increased by 86% from the CCl_4-treated animals.

The results show that piperine pretreatment for 4 h could significantly enhance NADPH cytochrome c reductase activity induced by CCl_4.

Experiment III.2 Effect of piperine pretreatment *in vivo* on CCl_4-induced NADPH-cytochrome c reductase activity in rat liver microsomes

To evaluate whether piperine treatment *in vivo* has any potentiating effect on CCl_4-induced NADPH-cytochrome c reductase *in vitro*, the effect of CCl_4 *in vitro* was initially explored. Different concentrations of CCl_4 were incubated with the enzyme for 30 min, thereafter NADPH-cytochrome c reductase activities were measured. As shown in Table X and Figure 12, the level of NADPH-cytochrome c reductase in the liver microsomes was progressively increased with the concentration of CCl_4. It was significantly increased from 82.8±1.2 nmole/min/mg protein in control to 115.0±0.6, 135.5±1.4, 141.5±1.1, 149.0±1.5 and 138.0±1.1 nmole/min/mg protein after expsoure to CCl_4 at the concentrations of 4.7, 6.3, 7.8, 9.4 and 12.5×10^{-5} M, respectively. The maximal effect was observed at a concentration of 9.4×10^{-5} M CCl_4. Table XI and Figure 13 show the effect of piperine treatment on CCl_4-induced NADPH-cytochrome c reductase activity in the liver microsomes. Profile of changes in NADPH-cytochrome c

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reductase activity by different concentrations of CCl_4 in piperine pretreated animals was similar to those in control animals. The enhancing effect of piperine was observed when the microsomes was exposed to CCl_4 at the concentrations of 6.3 and 9.4×10^{-5}M. NADPH-cytochrome c reductase activities were increased from 136.0±1.1 and 149.0±1.5 nmole/min/mg protein in the microsomes exposed to CCl_4 at the concentrations of 6.3 and 9.4×10^{-5}M, respectively, to 146.0±1.0 and 166.0±1.7 nmole/min/mg protein in the microsomes obtained from piperine pretreated animals and exposed to the corresponding concentrations of CCl_4.

Experiment III.3 Dose-response effect of piperine administration on CCl_4-induced NADPH cytochrome c reductase in rat liver homogenate

To investigate the relationship between the concentration of piperine and the effect, various concentrations of piperine (10, 20, 30, 40, 50, 75 or 100×10^{-5}M) and CCl_4 at the concentration of 6.3×10^{-5}M were simultaneously added. After 30 min of incubation, the activity of NADPH cytochrome c reductase was determined. As shown in Table XII and Figure 14, NADPH-cytochrome c reductase activity was progressively increased with increasing concentrations of piperine. However, significant enhancing effect was observed only at piperine concentrations of 40, 50 and 75×10^{-5}M. NADPH-cytochrome c

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reductase activity was increased from 135.5±1.4 nmole/min/mg protein in the presence of CCl_4 alone to 148.6±0.8, 163.0±0.8 and 155.0±1.3 nmole/min/mg protein in the presence of CCl_4 together with piperine at concentrations of 40, 50 and 75×10^{-5}M, respectively. In this study, the maximal effect of piperine was observed at the concentration of 5.0×10^{-6}M. The effect of higher concentrations of piperine were limited by its solubility as the slight turbidity was observed at a dose of 75×10^{-5}M piperine.

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Table IX. Effect of piperine pretreatment on CCl_4-induced NADPH-cytochrome c reductase activity in rats. Piperine (100 mg/kg BW) was intragastrically given at 4 h before CCl_4 (0.1 m/kg BW; i.p.) administration and the animals were sacrificed at 24 h after CCl_4 administration.

Rat No.	NADPH cytochrome c reductase (nmole/min/mg protein)			
	Corn oil	Piperine	CCl_4	Piperine + CCl_4
1	80.0	82.0	153.0	298.0
2	84.0	86.0	148.0	280.0
3	84.0	86.0	153.0	286.0
4	85.0	86.0	160.0	287.0
5	78.0	76.0	156.0	275.0
6	85.0	87.0	157.0	299.0
mean	82.8±1.2	83.7±1.8	155.0±2.6**	287.5±4.3**++

Each value is mean±SEM obtained from 6 animals

**P<0.001, when compare to the control corn oil

++P<0.001, when compare to CCl_4-treated group.

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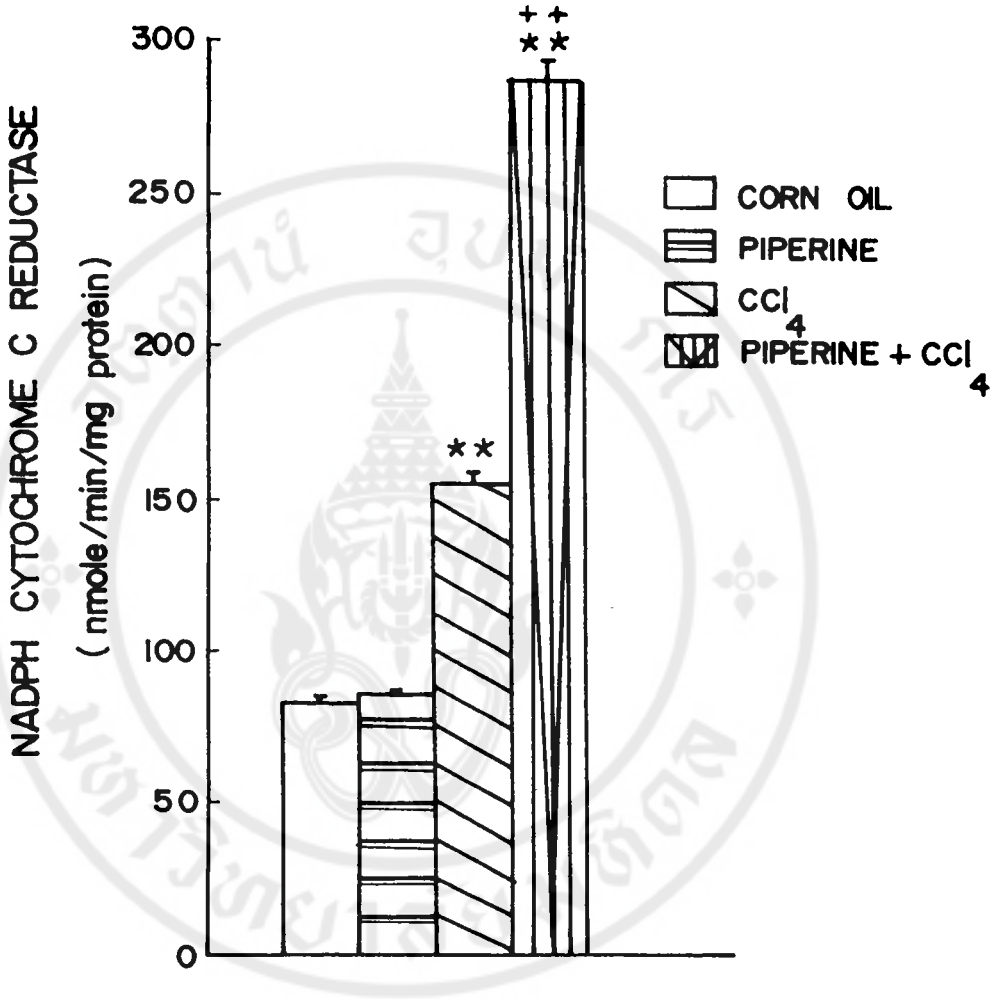

Figure 11. Effect of piperine pretreatment on CCl_4-induced NADPH-cytochrome c reductase activity in rats. Piperine (100 mg/kg BW) was intragastrically given 4 h before CCl_4 (0.1 ml/kg BW,i.p.) administration and the animals were sacrificed at 24 h after CCl_4 administration. Each value is mean±SEM, obtained from 6 animals. **P<0.001 when compare with control corn oil, ++P<0.01 when compare with CCl_4 - treatment group.

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Table X. Effect of CCl_4 exposure on NADPH-cytochrome c reductase activity in rat liver microsomes CCl_4 was added into the microsomes and the NADPH-cytochrome c reductase activity was measured after 30 min of incubation.

Treatment	NADPH cytochrome c reductase (nmole/100 mg protein)
Control	82.8±1.2
Solvent	83.0±1.2
CCl_4 $3.1x10^{-5}$ M	84.0±0.6
CCl_4 $4.7x10^{-5}$ M	115.0±0.6**
CCl_4 $6.3x10^{-5}$ M	135.5±1.4**
CCl_4 $7.8x10^{-5}$ M	141.5±1.1**
CCl_4 $9.4x10^{-5}$ M	149.0±1.5**
CCl_4 $12.5x10^{-5}$ M	138.0±1.1**

Each value is mean±SEM obtained from 6 animals

**P<0.001 when compare to the control.

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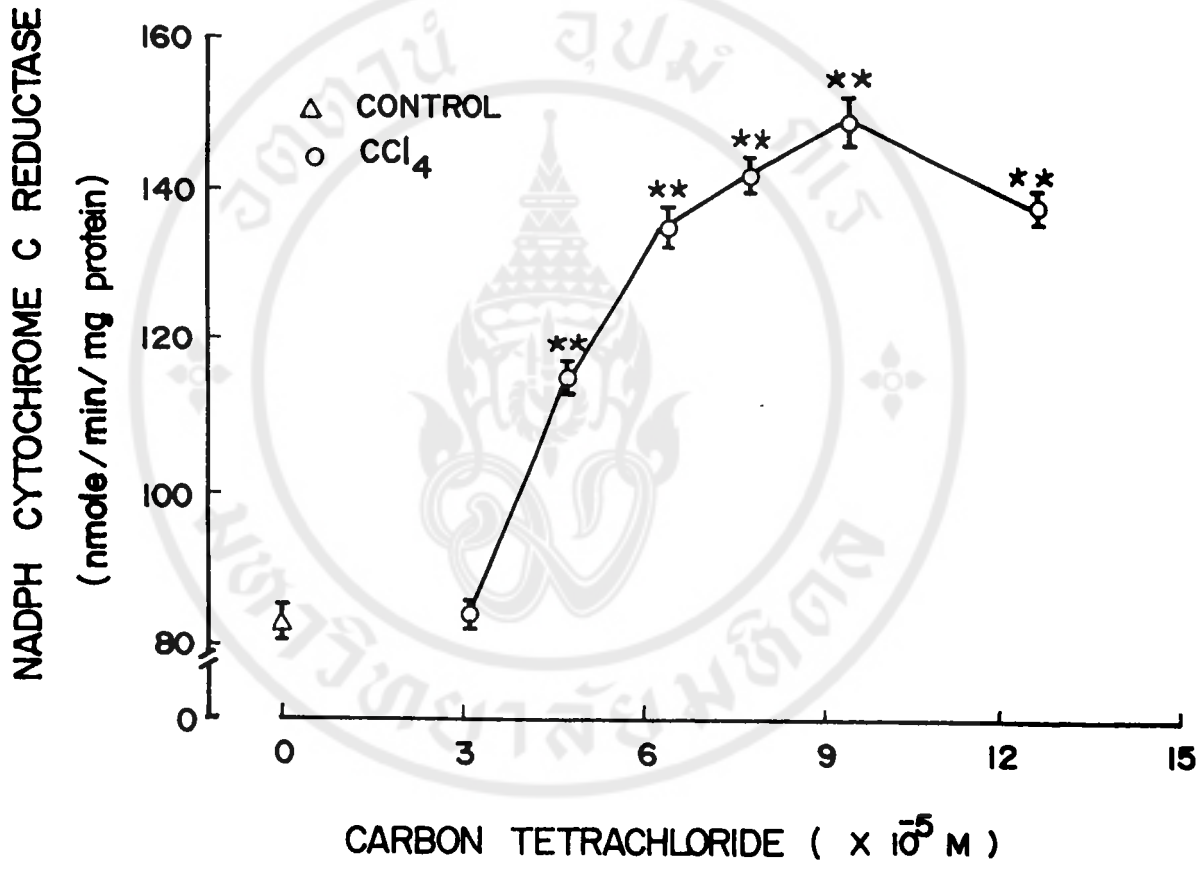

Figure 12. Effect of CCl_4 exposure on NADPH-cytochrome c reductase activity in rat liver microsomes CCl_4 was added into the microsomes and the NADPH-cytochrome c reductase activity was measured after 30 min of incubation. Each value is mean±SEM, obtained from 6 animals. **P<0.001 when compare to the control.

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Table XI. Effect of piperine treatment *in vivo* on the activity of NADPH-cytochrome c reductase in rat liver microsomes. The animals were pretreated with piperine (100 mg/kg BW,PO) at 4 h before sacrificed. The rat liver microsomes was exposed to CCl_4 for 30 min.

Dose of CCl_4 ($\times 10^{-5}$ M)	NADPH-cytochrome c reductase (nmole/min/mg protein)	
	CCl_4	CCl_4 + piperine
0	82.8±1.2	83.7±1.8
3.1	84.0±0.6	87.5±1.2
6.3	136.0±1.1	146.0±1.0*
9.4	149.0±1.5	166.0±1.7*
12.5	138.0±1.1	140.0±1.2

Each value is mean±SEM obtained from 6 animals

*P<0.05, when compare to the corresponding CCl_4 treatment.

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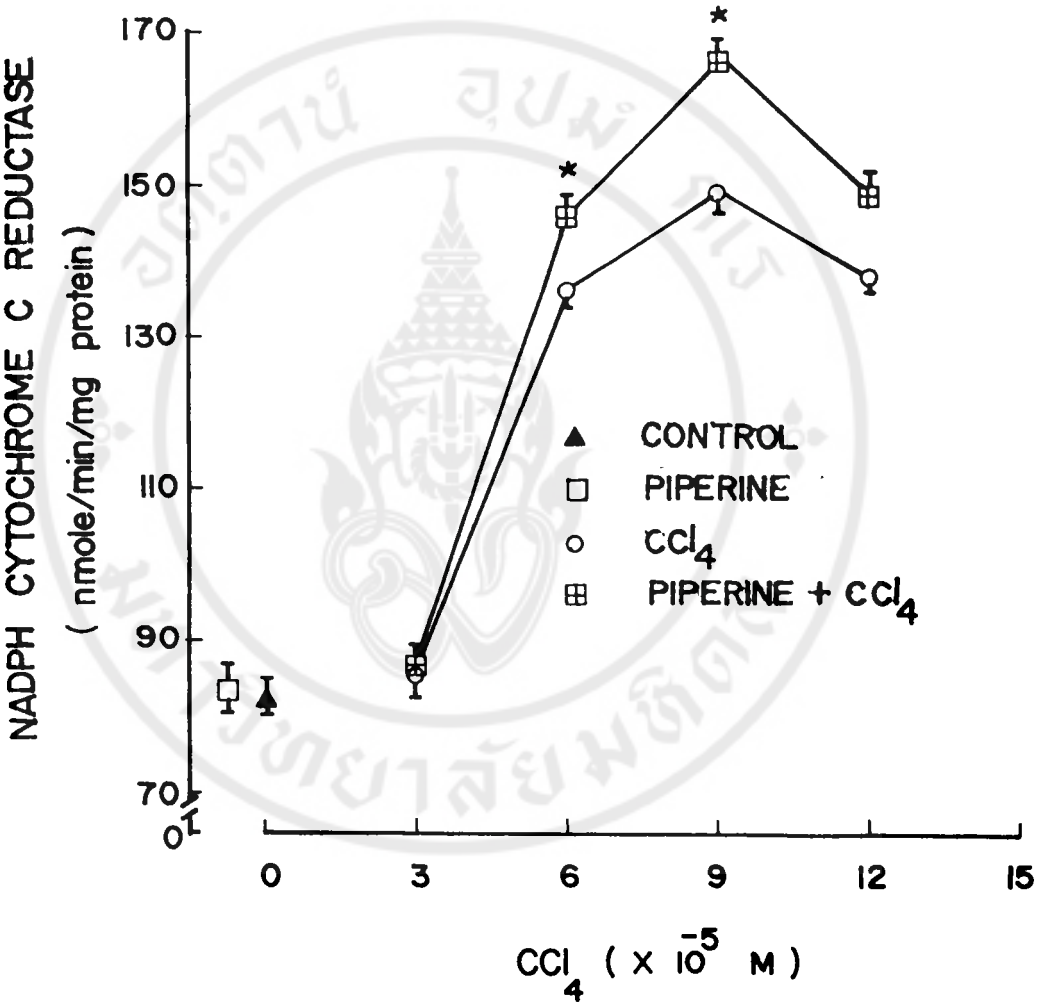

Figure 13. Effect of piperine pretreatment on the activity of NADPH-cytochrome c reductase in rat liver microsomes. The animals were intragastrically treated with piperine (100 mg/kg BW) for 4 h before sacrificed. The rat liver microsomes was incubated with CCl_4 for 30 min. Each value is mean±SEM, obtained from 6 animals. *$P<0.05$ when compare to the corresponding CCl_4 treatment.

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Table XII. Effect of piperine on CCl_4-induced change of NADPH-cytochrome c reductase activity in rat liver microsomes. Piperine and CCl_4 were added simultaneously and malondialdehyde level was measured after 30 min of incubation.

Treatment	NADPH-cytochrome c reductase (nmole/min/mg protein)
Control	82.8±1.2
Solvent	83.0±1.2
Piperine (50×10^{-5}M)	84.3±0.6
CCl_4 (6.3×10^{-5}M)	135.5±1.4
CCl_4 + piperine 10×10^{-5}M	135.3±1.3
CCl_4 + piperine 20×10^{-5}M	137.4±0.8
CCl_4 + piperine 30×10^{-5}M	139.0±0.8
CCl_4 + piperine 40×10^{-5}M	148.6±0.8*
CCl_4 + piperine 50×10^{-5}M	163.0±0.8**
CCl_4 + piperine 75×10^{-5}M	155.0±1.3*
CCl_4 + piperine 100×10^{-5}M	144.0±0.8

Each value is mean±SEM, obtained from 4 animals

**P<0.001, when compare to CCl_4 treatment.

*P<0.05, when compare to CCl_4 treatment.

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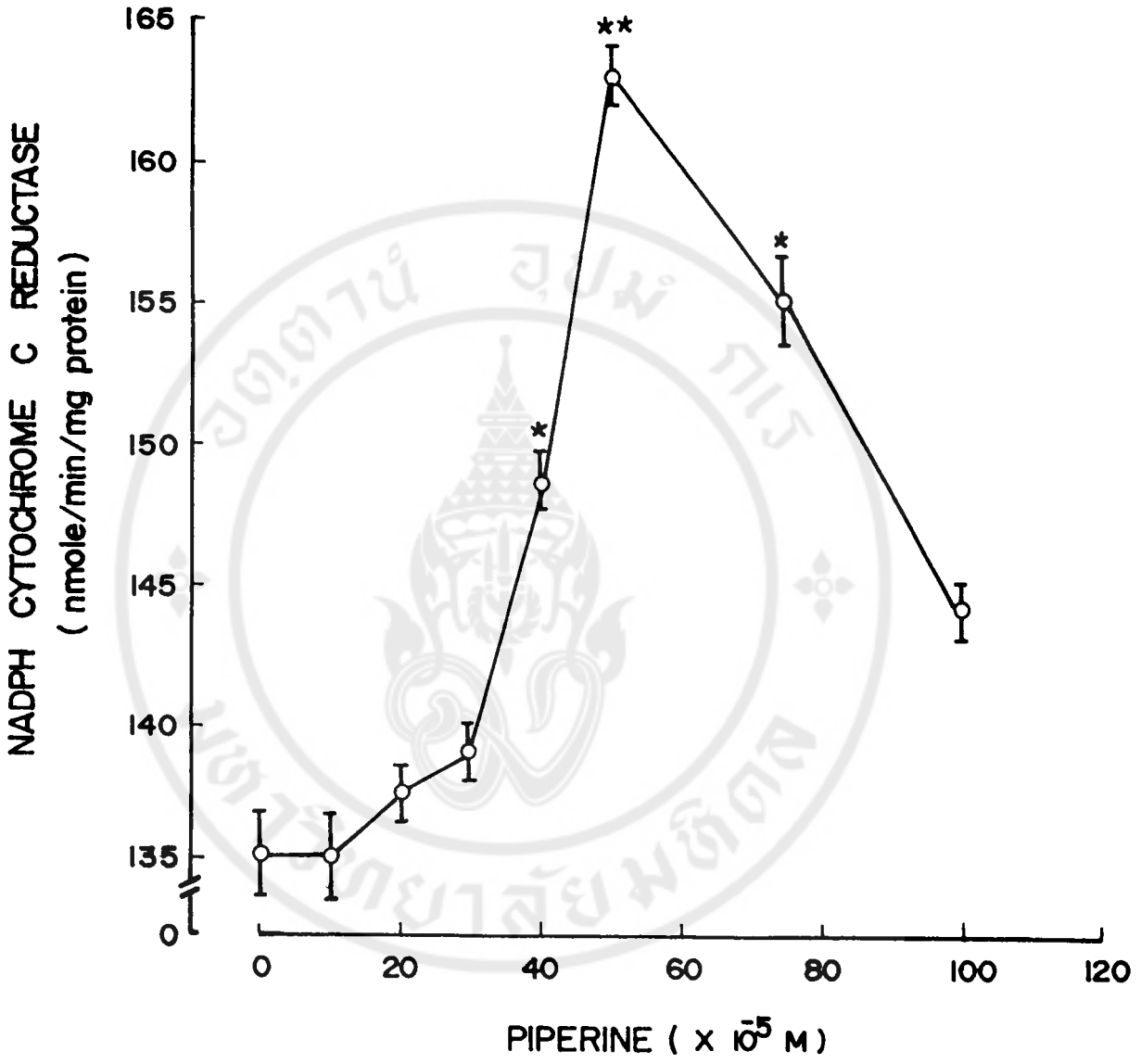

Figure 14. Effect of piperine on CCl_4-induced change of NADPH-cytochrome c reductase activity in rat liver microsomes. Piperine and CCl_4 were added simultaneously and the activity of the enzyme was measured after 30 min of incubation. Each value is mean±SEM, obtained from 4 animals. **P<0.001 when compare between CCl_4 treatment with CCl_4+piperine treatment. *P<0.05 when compare between CCl_4 treatment with CCl_4+piperine treatment.

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CHAPTER V

DISCUSSION

Effect of piperine on CCl_4-induced hepatic injury

Carbon tetrachloride (CCl_4) is known to be toxic to the liver. As the hepatotoxicity of CCl_4 in experimental animals varied in different laboratories, its toxicity under our laboratory conditions was evaluated again. In the present study, the hepatotoxicity was assessed by measuring plasma GPT and GOT levels at 24 h following CCl_4 administration at which the increase of serum enzymes from necrotic or damage hepatocytes are reported to reach maximal level (51,52,53). Administration of various doses of CCl_4 to rats produced a dose-dependent increases in both PGPT and PGOT activities (Table I and Fig. 1). The elevation of PGPT and PGOT activities with the dose of CCl_4 in the present study was in agreement with the earlier report by Korsrud et al., (54) who demonstrated a correlation between the dose of CCl_4 and the extent of membrane damage and other toxicities. In our study, the maximal effect of CCl_4 was observed at a dose of 0.15 ml/kg BW. As the purpose of this study is to investigate the effect of piperine in modifying the CCl_4 toxicity, using of high dose was avoided. Submaximal dose of 0.1 ml/kg BW which produced a considerable degree of toxicity (10 fold increase in the

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enzymes) was employed (Fig. 1). However, this low dose of CCl_4 used in the present study,0.1 ml/kg, BW,lied within a range which was reported to be a subtoxic dose in rat (55). The discrepancy of the elevation of transminase enzyme by low dose of CCl_4 obtained in our study and in the earlier report may be due to the differences in the species of the animals which had different susceptibility to the toxic chemicals (2,3). However,this dose of CCl_4 has been widely employed for evaluation the potentiating action of chemicals on the CCl_4 toxicity (51).

The present study demonstrated that piperine could potentiate hepatotoxic effect of CCl_4 whereas piperine itself did not cause any alteration in plasma transaminase enzymes. The dose of piperine used in this experiment was 100 mg/kg BW, PO, which was chosen according to our earlier study that it neither altered body weight gain nor produced any toxic signs (37). However, this dose level was recently reported to decrease hepatic drug metabolizing enzymes (56). In the present study, administration of piperine at this dose level prior to CCl_4 significantly potentiated CCl_4 heptotoxicity. The maximum potentiation occurred when piperine was given at 4 h prior to CCl_4 at which the activity of PGPT and PGOT enzymes were elevated by approximately 70-80% (Table II and Figure 2). Administration of piperine at 1 and 2 h prior to CCl_4 could not significantly alter the level of PGPT and PGOT. The significant effect was observed

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between 3 and 6 h of pretreatment. Apart from the elevation of plasma transaminase activities, it is apparent that other indices of acute CCl_4 intoxication such as an increased accumulation of hepatic triglycerides and decreased plasma triglycerides (Table II and Fig. 3) were similarly enhanced by piperine pretreatment. The enhancement of CCl_4 hepatotoxicity here was not merely due to the additive effects of piperine and CCl_4 on the liver since piperine itself had no apparent hepatotoxic effect. Therefore, it was suggested that the piperine pretreatment induced a potentiating effect on CCl_4.

Though the dose of piperine used in the present study was based on the information obtained from the previous experiments as mentioned, this dose was actually found to give the highest potentiating effect on CCl_4 in inducing elevation of the transaminase enzymes in plasma and hepatic accumulation of triglycerides (Table III, Figure 4 and 5).

Concerning the mechanism of action of CCl_4, hepatic GPT and GOT are normally localized within the liver cells and they will be released into the plasma when cell membrane is damaged by hepatotoxins. CCl_4 is also considered as a hepatotoxin. It is metabolized to trichloromethyl free radical ($.CCl_3$) by NADPH-cytochrome P-450 electron transport chain in the endoplasmic reticulum. This radical may directly act by covalent binding to membrane proteins and lipids or indirectly act on unsaturated lipids in intracellular membranes which

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ultimately result in lipid peroxidation of the hepatic membrane. The attack of free radical on the membrane would lead to a severe disturbance to the organized structure of the endoplasmic reticulum, to the enzyme activities and hepatic cell necrosis (57). The accumulation of liver triglyceride here was probably due to intracellular membrane function impairment which resulted in an impairment of triglyceride secretion. Triglycerides are usually secreted from the liver into the plasma in the form of VLDL triglycerides. The attack of free radicals intermediate of CCl_4 on the intracellular membrane would actually impaire the protein synthetic function of the cell. A defective of apoprotein synthesis which affect VLDL triglycerides formation and subsequently blocked triglycerides secretion has also been previously reported. In addition, CCl_4 might cause the defect at the level of coupling process to form lipoprotein (25,26). These might be the case of hepatic triglycerides accumulation in our study. In the present study, the potentiating effects of piperine on the increase accumulation of liver triglycerides with a concomitant decrease plasma triglycerides and increase plasma transaminase activities were all dependent upon the dose of piperine (ranging from 50 to 100 mg/kg BW). Though, it was not clear that how piperine potentiated the hepatotoxicity of CCl_4, it was reasonable to speculate that piperine probably interacted with the membrane and resulted in an increased in susceptibility of the cell to

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the toxic action of CCl_4. Alternately, piperine probably accelerated the formation of the highly reactive trichloromethyl radicals. In our study, the potentiating effect of piperine was maximum at the dose of 100 mg/kg BW. The higher dose (150 mg/kg BW) could not produce more effect, in fact, it was decreased. The decrease in the effect at high dose of piperine was not known. It might be due to the biphasic action of the drug as a high dose of piperine has been reported to inhibited the monooxygenase enzymes (58). Since piperine markedly enhanced the effect of CCl_4, time-shift of the peak effect of CCl_4 might be another reason for the reduction of the observed effect. It was also probably due to less amount of piperine absorption as it tended to precipitate at high concentration in the gastrointestinal tract.

The time-course of piperine in producing the effect in animals, depends upon several factors, e.g., solvent used for dissolving the drug, route of administration and the parameters being measured. Pretreatment of piperine by intraperitoneal injection was demonstrated to maximally potentiate pentobarbitone sleeping time at 30 min after administration and the effect was significant till 120 min (38). Meanwhile the oral administration of piperine took a longer time in exerting the effect. Atal and his colleagues (44) reported that an oral administration of piperine in coconut oil produced a maximal inhibition on hepatic arylhydrocarbon hydroxylase (AHH) and UDP-glucuronosyl

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transferase within 1 h and by 6 h they restored to normal values. Likewise, Reen et al., (59) reported a maximal inhibition of pulmonary AHH and 7-ethoxycoumarin deethylase (7E CDE) activity at 1 h after oral administration of piperine in gum acacia and only AHH activity returned to normal value within 4 h. From these data, it seems that piperine had a rapid onset of action, e.g., 0.5 h and 1 h after intraperitoneal and oral administration, respectively. However, in our study the significant potentiating effect of piperine on CCl_4-toxicity was observed between 3 and 6 h after oral administration and the maximal effect was at 4 h. The discrepancy on these time course of responses might be due to the differences in the susceptibility of enzymes and functions being measured. In our study, we measured a leakage of hepatic enzyme into the circulation which was a consequence of membrane damage and it might take time and not be as sensitive as other membranes bound enzymes reported. From the earlier studies on the absorption and distribution of piperine in rat, it has been shown that most of the administered piperine was absorbed (97%) and it was not transformed during absorption by the gut (39). Piperine was reported to be detected in the liver between 0.5 and 24 h after dosing. By intraperitoneal administration, the maximum concentrations of piperine in both serum and liver (2.5% of administered dose) were attained at 3 h after dosage, whereas they were at 6 h after oral administration. In addition, Bhat et al., (39)

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also determined the absorption of piperine *in vitro* by using duodenal segment and reported that about 47-64% of piperine was absorbed by 3 h of incubation with various concentrations of piperine. Therefore, it seems that a considerable amount of piperine in the liver would be attained between 3 and 6 h after administration. This amount of piperine might be required for producing potentiation on the CCl_4-hepatotoxicity. Perhaps, a period of 4 h in our study was mostly desired for the absorption of piperine.

Effect of piperine on CCl_4-stimulated lipid peroxidation

The study on toxicities of CCl_4 has been carried out by several groups of investigators and a number of hypotheses have been proposed to explain the mechanisms of hepatotoxicities-induced by CCl_4. It has been proposed that the homolytic cleavage of CCl_4 which occurs during the metabolism yields the trichloromethyl radical ($.CCl_3$). This free radical can act in two ways. Firstly, it may directly form covalent binding to membrane lipids and proteins resulting in alkylation reaction, and enzyme inactivation. Secondly, it may indirectly interact with membrane unsaturated fatty acids. The CCl_3 radical may abstract a hydrogen atom from a fatty acid, forming chloroform and a lipid radical. The lipid radical may then react with molecular oxygen to initiate lipid peroxidation. The hypothesis that lipid peroxidaton on cellular membrane is a key process responsible for the

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various biochemical, histological and morphological changes observed in carbon tetrachloride intoxication, has been widely accepted (60). However, this hypothesis is still a center of debate. The major point of argument emerge in a number of experiments in which blocking lipid peroxidation was still unsuccessful in inhibiting hepatotoxicity induced by CCl_4 (61). It is apparent that both extent of functional hepatic membrane defects and level of lipid peroxidation are commonly employed to investigate the underlying mechanism of CCl_4-induced hepatic injury. In the present study, the effect of piperine on CCl_4-stimulated lipid peroxidation was also determined. Time-course of CCl_4-stimulated lipid peroxidation *in vivo* has been previously reported (18). It occurs rapidly in the early phase of CCl_4-poisoning and the production of lipid peroxides is reported to get to the peak at about 1-2 h. In our study, we also measured the level of lipid peroxidaton at 1 h after CCl_4-poisoning. The submaximal dose of CCl_4 used here (0.1 mg/kg BW) significantly stimulated the lipid peroxidation in liver. This stimulating effect of CCl_4 was further elevated in the animal which was pretreated with piperine for 4 h (Table IV and Fig. 6). This marked potentiating effect of piperine on the lipid peroxidation was correlated well with the elevation in enzyme PGPT, PGOT and accumulation of triglycerides in the liver after CCl_4-poisoning. From these obtained results, it was likely that the increased

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lipid peroxidation was responsible for the potentiated hepatotoxic effect of piperine in the animals.

The concentration of CCl_4 in the liver which caused an increase in peroxides formed at 1 and 2 h after poisoning was suggested to be of the same magnitude was that which is able to stimulate peroxidation *in vitro* (18). In an attempt to find out whether piperine itself directly act to enhance the CCl_4-stimulated lipid peroxidation, the study was designed to determine the enhancing effect *in vitro* system. CCl_4 is known to have a biphasic influence on lipid peroxidation in the liver both *in vivo* and *in vitro*. At low dose, it stimulated while high dose inhibited (18). To mimic the stimulating action of CCl_4 as observed in our *in vivo* experiment, we explored the dose of CCl_4 in producing stimulation. A dose-dependent stimulation of CCl_4 on lipid peroxidation was observed in the range of $4.7-12.5 \times 10^{-5}$ M. However, when the CCl_4 was added to incubation mixtures containing liver homogenate obtained from the piperine pretreated rat, the potentiating effect of piperine on the lipid peroxidation was rather low ($\leq$7%) (Table VI and Figure 8). This low level of lipid peroxidation in the presence of piperine was not limitted by the capacity of the homogenate to response since the level of lipid peroxidation was higher when the high dose of CCl_4 was added, but the extent of potentiation by piperine was similar, whether low or high doses of CCl_4 were used.

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However, when piperine and CCl_4 were concurrently added to the rat liver homogenate, the potentiating effect on lipid peroxidation remained considerably low, though it was slightly increase (20%). Thus, there was no correlation on the effectiveness of the potentiating effect of piperine on CCl_4 when they were given to animal and when they were added to incubation mixture. The results from *in vitro* exposure to both piperine and CCl_4 were very much lower than that obtained when they were both given to animal. The reason underlying these different interactions between piperine and CCl_4 in producing potentiation was unclear. However, from the experiment when piperine was added at varying time interval, it revealed that a successful potentiation required a concurrent presence of both piperine and CCl_4. The later piperine was added, the lower the effect was obtained. It was possible that the piperine exerted potentiating action by binding to the liver membrane and modifying the membrane to response to CCl_4. In earlier study, piperine was reported to exhibit general affinity for binding microsomal drug metabolizing enzymes *in vitro* and cause non-competitive inhibition (38). The substrate affinity towards enzyme was not affected in the presence of piperine but the enzyme substrate complex became inactive (38). As the degrees of potentiation by piperine on different concentrations of CCl_4 in our study were similar, piperine might non-competitively bind to the

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membrane as previously reported and resulted in a certain degree of membrane alteration.

Effect of piperine on the activity of NADPH-cytochrome c reductase

It is well known that CCl_4 is converted into toxic metabolites during its interaction with the drug metabolizing system. The point of interaction is at the protein components of the endoplasmic reticulum and NADPH-cytochrome c reductase flavoprotein (62, 63). The stimulation of malondialdehyde production due to CCl_4 and endogenous peroxidation were also reported to involve NADPH flavoprotein. The inhibitors of the flavoenzyme was demonstrated to inhibit both peroxidative routes (14). Therefore, the present study designed to examine whether the potentiating effect of piperine on CCl_4-hepatotoxicity associated with the increased metabolic activation of CCl_4 by NADPH-cytochrome c reductase enzyme. CCl_4 has also been reported to exhibit a biphasic effect on hepatic enzyme activity in both *in vivo* and *in vitro*. In our study, administration of low dose of CCl_4 produced a marked stimulation on the activity of NADPH-cytochrome c reductase. This was in agreement with earlier study which demonstrated that low dose of CCl_4 caused an increase in hepatic mixed function oxidase activity (64). An administration of piperine (100 mg/kg BW, PO) at 4 h prior to CCl_4 here was indeed capable of potentiating the stimulating effect of CCl_4 on the NADPH-cytochrome c

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reductase activity. The activity the of enzyme was increased approximately 80% (Table IX and Figure 11) which correlated well with its potentiation on CCl_4-hepatotoxicity (Figure 2) in the similar schedule of treatment. When the schedule of treatment was modified by pretreatment the animal with piperine and later either exposure the hepatic microsome to CCl_4 *in vitro* or to both piperine and CCl_4 *in vitro*, in all cases, the extent of enzyme stimulation correlated well with its potentiation on the lipid peroxidation. These results suggest that potentiating mechanism of piperine associated with the increase in activity of NADPH-cytochrome c reductase which might accelerate formation of the highly reactive metabolite, thereby increasing lipid peroxidation and enhancing its hepatoxicity.

The activity of NADPH-cytochrome c reductase after piperine treatment has been previously studied. Intraperitoneal injection of piperine (40-80 mg/kg) for 3 consecutive days resulted in an increase in enzyme activity but no statistical significance (18). In contrast, recently Dalvi and Dalvi (56) reported that within 1 h after intraperitoneal administration of piperine, NADPH-cytochrome c reductase activity was significantly decreased whereas at 24 h after treatment, the activity was marginally depressed but not significantly different from control. However, in our study, the activity of this enzyme was not altered after oral administration of piperine. No significant different

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was observed among results obtained after 24, 4 h and shortly after exposure to piperine. In fact, the activity of enzyme being obtained after piperine treatment in our study was comparable to that reported by Dalvi and Dalvi (56) at 1 h after treatment, but the value in control animals in that study was variable and higher than the piperine-treated animals, whereas the control values in our study at all conditions were in the same range. Discrepancy of the obtained results among these studies might be due to differences in the route of administration, time points of the study after piperine treatment and strains of the animal used. It is evident from all these studies that, 24 h after piperine treatment, the activity of NADPH-cytochrome c reductase was not significantly altered. Though piperine itself had no effect in our study, in the presence of CCl_4, the activity was markedly enhanced.

Considering the ability of piperine to potentiate the CCl_4-hepatotoxicity, there are several possibilities. Firstly,piperine might enhance the susceptibility of the liver to the hepatoxicity of CCl_4. Secondly, it might stimulate metabolic activation of CCl_4 to form a highly reactive metabolite and lastly it might stimulate lipid peroxidation. According to our results, a correlation between extent of hepatotoxicity, stimulatory level of lipid peroxidation and the increase in the activity of enzyme NADPH-cytochrome c reductase existed.It is possible that piperine directly binds to the liver membrane and

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subsequently renders all those sequence of changes. Though the results obtained from the *in vitro* experiment was lower than those obtained from the *in vivo* study and they could not satisfactorily explain what occurred in animals, the extent of changes lipid peroxidation and enzyme activity in each corresponding schedule of treatment well correlated. It was not clear at present why toxicity of CCl_4 *in vivo* system was much higher than those *in vitro* system. The factors responsible for the differences in these two conditions remained to be established.

Piperine is a compound which contains methylenedioxyphenyl (MDP) as a part of its molecule. Several MDP compounds such as piperonyl butoxide and safrol have been shown to be metabolized by the hepatic mixed function oxidase system and their methylene carbon could induce cytochrome P-450 (18, 27). However, piperine has been reported to exhibit distinct properties, unlike other MDP compound. A comparative study between the effect of safrole and piperine on the drug metabolizing enzyme was carried out by Bhat and Chandrasekhara (18). An *in vivo* treatment of safrol increased liver weight and induced cytochrome P-450 while piperine treatment did not. Binding of safrole and piperine to the reduced cytochrome P-450 *in vitro* also yielded a difference spectrum. It was concluded that piperine was not at all an inducer of hepatic drug metabolizing enzyme or the mixed function oxidase system but it is a specific inhibitor of drug

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metabolism which could differently inhibit different forms of cytochrome P-450 (18). This action of piperine was further supported by the study of Reen and Singh (59). They demonstrated that piperine could discriminate certain cytochrome P-450 forms. The nature of inhibition was found to be non-competitive since the substrates affinity toward the enzyme is not affected but the enzyme substrate complex is rendered inactive in the presence of piperine. Though piperine has been demonstrated to be a potent inhibitor of drug metabolism and markedly inhibited the activity of hepatic cytochrome P-450 (56), its action in modifying the toxicity of CCl_4 could not actually be predicted. The actions of various inhibitors of the microsomal NADPH-cytochrome P-450 electron transport chain have been studied on the stimulatory effect of carbon tetrachloride on malondialdehyde production (62). It has been demonstrated that there was no direct correlation between the effect of inhibition of drug metabolism and the effects on the stimulatory action of carbon tetrachloride. In general, drug metabolism may be strongly inhibited without affecting the CCl_4 effect (62). Conversely, CCl_4 effect on malondialdehyde production can be inhibited by low concentration of free-radical scavengers meanwhile there was no significant depression of drug metabolism (63). All these conclusion derived from the observation that the metabolism of CCl_4 and stimulatory action of CCl_4 on malondialdehyde production did not require the participation of the entire

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NADPH-cytochrome P-450 chain but it required only the proximal region of the NADPH-cytochome P-450 electron-transport chain. The actual site of interaction is possibly with the flavoprotein. Therefore, it is concluded that the potentiating action of piperine on CCl_4-hepatoxicity in our study was not due to its inhibiting action on the drug metabolism but due to its stimulation on NADPH-cytochrome c reductase activity which accelerated the biotransforma-tion of CCl_4 to the highly reactive metabolite which thereby increased lipid peroxidation and enthanced hepatotoxicity.

To our knowledge the results presented in this study constitute the first evidence for the potentiating action of piperine on CCl_4-hepatoxicity. It may provide an additional information for the understanding on the pharmacotherapeutic potentials of piperine and it may have some important impact on occupational safety particularly for the workers who work with CCl_4 and consume piperine.

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CHAPTER VI

CONCLUSION

1. The effect of piperine on CCl_4-induced hepatotoxicity was investigated in rats. The results showed that piperine could potentiate the effect of CCl_4-induced hepatotoxicity, whereas the piperine itself had no hepatotoxic effect.

2. The maximum potentiation occurred when piperine (100 mg/kg BW) was given at 4 h prior to CCl_4 at which the activities of PGOT and PGPT enzymes were elevated by approximately 70-80%

3. The other indices of acute CCl_4 intoxication such as an increased accumulation of hepatic triglycerides and decreased plasma triglycerides levels were similarly enhanced by piperine pretreatment. All these potentiating effects of piperine showed a dose-dependent pattern.

4. Piperine pretreatment at 4 h before CCl_4 administration markedly potentiated CCl_4-stimulated lipid peroxidation and the effect was correlated well with the elevations in PGPT and PGOT activities and accumulation of triglycerides in the liver.

5. The effect of piperine on CCl_4-stimulated lipid peroxidation <u>in vitro</u> was also explored and found to be rather low (approximately 7%). However, when both piperine and CCl_4 were concurrently added to rat liver homogenate, the potentiating effect on lipid peroxidation

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was slightly higher. There was no correlation between the results obtained *in vivo* and *in vitro*.

6. The effect of piperine on CCl_4-stimulated NADPH-cytochrome c reductase activity was also correlated well with its potentiation on CCl_4-stimulated lipid peroxidation in the similar schedule of treatment.

7. The results suggest that the potentiating mechanism of piperine associates with the increase in the activity of NADPH-cytochrome c reductase which accelerates formation of highly reactive metabolite thereby increasing lipid peroxidation and enhancing its hepatotoxicity

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BIBLIOGRAPHY

1. Browning E. Toxicology and metabolisms of industrial solvents. New York : Elsevier Publishing Company, 1965.
2. Fowler, JSL. Chlorinated hydrocarbon toxicity in the fowl and duck. J Comp Pathol 1970; 80: 465-471.
3. Friedman L, Sage J, Blenderman EM. Growth and liver response of chicks and rats to carbon tetrachloride and ethanol. Poultry Sci 1970; 49: 298-309.
4. Dawkins MJR. Carbon tetrachloride poisoning in the liver of new-born rat. J Pathol Bact 1963; 85: 189-196.
5. Reuber MD, and Glover EL. Carbon tetrachloride induced cirrhosis : Effect of age and sex. Arch. Pathol 1968; 85: 275-279.
6. Marchand C, McLean S, Plaa GL. The effect of SKF 525-A on the distribution of carbon tetrachloride in rats. J Pharmacol Exp Ther 1970; 174: 232-238.
7. Nachtomi E. Effect of ethylene dibromide and carbon tetrachloride on lipid peroxidation in rat and chick liver. Exp Mol Pathol 1972; 17: 171-175.
8. Bengmark S, and Olsson E. The effect of sex and of testosterone on toxic liver damage. J Endocrinol 1962; 25: 293-297.

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9. Bernelli-Zazzera A, Bassi M, Comolli R, Luccheli P. Action of testosterone propionate and 4-chloro-testosterone acetate on protein synthesis in vitro. Nature 1958; 182: 663.

10. Tamura V, Tsuchiya T, Harada T, Kuroiwa H. Effect of vitamin E on the carbon tetrachloride poisoned liver of rats. Osaka Diagaku Igaku Zasshi 1958; 10: 1553-1556.

11. Tamura U, Tsuchiya T, Harada T, Kuroiwa H, Kitani T. Effect of vitamin E upon the liver of rats in toxicated with carbon tetrachloride. Med J Osaka Univ 1959; 10: 91-98.

12. Gallagher CH. Protection by anti-oxidants against lethal doses of carbon tetrachloride. Nature 1961; 192: 881-882.

13. Gallagher CH. The effect of antioxidants on poisoning by carbon tetrachloride. Aust J Exp Biol Med Sci 1962; 40: 241-254.

14. Butler TC. Reduction of carbon tetrachloride in vivo and reduction of carbon tetrachloride and chloroform in vitro by tissues and tissue constituents. J Pharmacol Exp Ther 1961; 134: 311.

15. Wirtschafter ZT, Cronyn MW. Free radical mechanism for solvent toxicity. Arch Environ Health 1964b; 9: 186.

Copyright by Mahidol University

16. Bolland JL, Koch KP. The course of autoxidation reactions in polyisoprenes and allied compounds. Part IX. The primary thermal oxidation product of ethyl linoleate. J Chem Soc 1945; 445.

17. McMillian GR, Colvert, JG. Gas phase photo-oxidation Combust Rev 1965; 1: 83.

18. Comporti M, Saccocci C, Diazani MU. Effect of CCl_4 in vitro and in vivo on lipid peroxidation of rat liver homogenates and subcellular fractions. Enzymologia 1965; 29: 185-204.

19. Rouiller CU. Experimental toxic injury of the liver. In: The liver, Vol.II. Edited by Rouiller CU. New York : Academic Press, 1964.

20. Smucker EA, Arcasory, M. Structural and functional changes of endoplasmic recticulum of hepatic parenchymal cells. Internal Rev Exp Pathol 1969; 7: 305.

21. Smucker EA. The biochemical basis of acute liver cell injury. In : Molecular pathology. Edited by Good RA, Day SB, Yunes JJ. Springfield III : Charles & Thomas co., 1975; 490.

22. Aiyar AS, Fatterpaker R, Sreenivasan A. Lipid metabolism in liver injury caused by carbon-tetra-chloride in the rat. Biochem J 1964; 90: 558.

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23. Heimburg M, Weinstein I, Dishman G, Dunkerley A. The action of carbon-tetrachloride on the transport and metabolism of triglycerides and fatty acid by the isolated perfused rat liver and its relationship to the etiology of fatty liver. J Biol Chem 1962; 237: 3623.

24. Poggi M, Paoletti, R. A new insight on carbon tetrachloride effect on triglyceride transport. Biochem Pharmacol 1964; 13: 949.

25. Smucker EA, Benditt EP. Studies on CCl_4 intoxication III. A Subcellular defect in protein synthesis. Biochemistry 1965; 4: 671.

26. Smucker EA, Benditt EP. Carbon tetrachloride poisoning in rats: alteration in ribosome of the liver. Science 1963; 140: 308.

27. Suarez AK, Carbson GP, Fuller GC, Fausto N. Differtial acute effects of phenobarbital and 3-Methylcholanthrene pretreatment on CCl_4-induced hepatotoxicity in rats. Toxico Appl Pharmcol 1972; 23: 171-177.

28. Garner RC, Mclen AEM. Increased-susceptibility to tetrachloride poisoning in the rat after pretreatment with oral phenobarbitone. Biochem Pharmacol 1969; 18: 645-650.

29. Cignoli EV, Castro JA. Effect of inhibitors of drug metabolizing enzymes on carbon tetrachloride hepatotoxicity. Toxicol Appl Pharmacol 1971; 18: 625-637.

Copyright by Mahidol University

30. Orrenius S, Ericsson JLE. Ernter L. Phenobarbital induced synthesis of the microsomal drug-metabolizing enzyme system and its relationship to the proliferation of endoplasmic membranes. A. Morphological and biochemical study. J Cell Biol 1965; 25: 627-639.

31. Chopra RN, Chopra IC. A review of work on Indian medicinal plants. Indian Council of Medical Research, Special report series no. 3 New Delhi 1955; pp:99-107.

32. Kirtikar KR, Basu BD. Indian medicinal plants. 2th ed. Allahabad; Lalit Moham Basu, 1944; 2128-2130.

33. Singh N, Kulshrestha VK, Srivastava RK, Kohli RP. Studies on the analeptic activity of some piper longum alkaloids. J Res Ind Med 1973; 8: 1-9.

34. Neogi NC, Halder RE, Rathe SS. Analysis of central stimulant activity of piper longum. J Res Indian Med 1971; 6: 17-23.

35. Lee EB, Shin KH, Woo WS. Pharmacological study on piperine. Arch Pharmacol Res 1984; 7, 2: 127-132.

36. Casey, RCD. 298 alleged anti-fertility plants of India. Indian J. Med. Sci 1960; 14: 596-600.

37. Piyachaturawat P, Glinsukon T, Toskulkao C. Acute and subacute of piperine in mice, rats and hamsters. Toxicol Lett 1983; 16: 351-359.

Copyright by Mahidol University

38. Mujumdar AM, Dhuley JN, Deshmukh VK, Ramam PH, Thorat SL, Nail SR. Effect of piperine on pentobarbitone induced hyposis in rats. J Exp Biol 1990; 28: 486-487.

39. Bhat BG, Chandrasckhara N. Studies on the metabolism of piperine: absorption, tissue distribution and excretion of urinary conjugates in rats. Toxicology 1986; 40: 83-92.

40. Bhat BG, Chandrasekhara N. Metabolic disposition of piperine in the rat. Toxicology 1987; 44: 99-106.

41. Atal CK, Zutshi U, Rao PG. Scientific evidence on the role of Ayurverdic herbals on bioavailability of drugs. J Ethnopharmacol 1981; 4: 229-232.

42. Zutshi U, Kaul JL. The impact of Ayurverdic herbals on drug bioavailability. India drugs 1982; 19, 12: 476-479.

43. Bano G, Amla V, Raina RK, Zutshi U, Chopra CL. The effect of piperine on pharmacokinetics of phenytoin in healthy volunteers. Planta Med 1987; 53: 568-569.

44. Atal CK, Dubey RK, Singh J. Biochemical basis of enhanced drug bioavailability by piperine. Evidence that piperine is a potent inhibitor of drug metabolism. J Pharmacol Exp Ther 1985; 232: 258-262.

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45. Reitman S, Frankel S. A colorimetric method for determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. Am J Clin Path 1957; 28: 56-63.

46. Mazel P. General principles and procedures for drug metabolism in vitro. In: Fundamentals of drug metabolism and drug dispositon. Edited by La Du BN, Mandel HG, Way EL. New York : Krienger Publishing Company, 1979; 527-582.

47. Lowry OH, Rosebrough NJ, Farr LA, Randall, RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1971;193: 263-275.

48. Biggs HG, Erikson JM, Moorhead WR. Heptane extraction method. Clin Chem 1975; 21: 437.

49. Mendez J, Franklin B, Gahagan H. Simple manual procedure for determination of serum triglycerides. Clin Chem 1975; 21: 768-777.

50. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.

51. Christic GS, Judah JD. Mechanism of action of carbontetrachloride on liver cells. Proc Roy Soc Ser B 1954; 142: 241-257.

52. Pappas NJ. Jn. Source of increased serum aspartate and alanine aminotransferase : cycloheximide effect on carbon tetrachloride hepatotoxicity. Clinica Chim Acta 1986; 9: 181-190.

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53. Labbe G, Descatoric V, Letteron P, Degott C, Tiwel, M. The drug methoxalen, a suicide substrate for cytochrome P-450 decreases the metabolic activation, and prevents the hepatotoxicity of carbontetrachloride in mice. Exp Pharmacol 1987; 36, 6: 907-914.

54. Korssud GO, Grio HC, Mclanghalan JM. Sensitivity of several serum enzymes in detecting carbon tetrachloride induced liver damage in rats. Toxicol Appl Pharmacol 1972; 22: 474-483.

55. Kodanvanti PRS, Joshi UM, Young RA, Bell, AN, Mehendale HM. Role of hypatocellular regeneration in chlordecone potentiated hepatotoxicity of carbontetrachloride. Arch Toxicol 1989; 63: 367-375.

56. Dalvi RR, Dalvi PS. Differences in the effects of piperine and piperonyl butoxide on hepatic drug-metabolizing enzyme system in rats. Drug Chem Pharmacol 1991: 219-229.

57. Comporti M. Biology of disease lipid peroxidation an cellular damage in toxic liver injury. In Acae Pathol 1985; 599-621.

58. Shin KH, Woo WS. Effect of piperine on hepatic microsomal mix-function oxidase system. J Koren Biochem 1985; 18: 9-15.

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59. Reen RK, Singh J. In vitro and in vivo inhibition of pulmonary cytochrome P_{450} activities by piperine, a major ingredient of piperine species. J exp Biol 1991; 29: 568-573.

60. Recknagel RO. Carbon tetrachloride hepatotoxicity. Pharmacol Rev 1967; 19: 145-207.

61. Pilon D, Brodeur J, Plaa GL. Potentiation of CCl_4-induced liver injury by ketonic and ketogenic compounds: Role of CCl_4 dose. Toxico appl Pharmacol 1988; 94: 183-190.

62. Slater TF, Sawyer BC. The stimulatory effects of carbon tetrachloride on peroxidative reactions in rat liver fraction in vitro, interaction sites in the endoplasmic reticulum. Biochem J 1971; 123: 805-814.

63. Powis G. Effect of a single oral dose of methanol, ethanol and propan-2-ol on hepatic microsomal metabolism of foreign compounds in rat. Biochem J 1975; 148: 269-277.

64. Chadwick RW, Copeland MF, Carlson GP, Trela BA, Most BM. Comparison of in vivo and in vitro methods for assesing the effects of carbon tetrachloride on the hepatic drug-metabolizing enzyme system. Toxicol Lett 1988; 42: 309-316.

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20240