EFFECTS OF PIPERINE ON WISTAR RATS

JIRAPORN SUWANA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE (PATHOBIOLOGY)

FACULTY OF GRADUATE STUDIES

MAHIDOL UNIVERSITY

2005

ISBN 974-04-5656-1

Thesis

Entitled

EFFECTS OF PIPERINE ON WISTAR RATS

Miss Jiraporn Suwana
Candidate

Assoc.Prof.Punya Temcharoen,
D.V.M., M.Sc. (Pathobiology)
Major-Advisor

Assist.Prof.Rungtiva Sirimujalin,
D.V.M., Ph.D. (Pathology)
Co-Advisor

Assoc.Prof.Galayanee Doungchawee,
M.Sc. (Pathobiology)
Co-Advisor

Assoc.Prof.Rassmidara Hoonsawat, Ph.D
Dean
Faculty of Graduate Studies

Assoc.Prof.Suda Riengrojpitak, Ph.D
Chair
Master of Science
Programme in Pathobiology
Faculty of Science

Thesis
Entitled

EFFECTS OF PIPERINE ON WISTAR RATS

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Pathobiology)
On
February 17, 2005

Miss Jiraporn Suwana
Candidate

Assist.Prof.Rungtiva Sirimujalin,
D.V.M., Ph.D. (Pathology)
Member

Assoc.Prof.Punya Temcharoen,
D.V.M., M.Sc. (Pathobiology)
Chair

Assist.Prof.Panas Chalermsanyakorn, MD
Member

Assoc.Prof.Galayanee Doungchawee,
M.Sc. (Pathobiology)
Member

Assoc.Prof.Rassmidara Hoonsawat, Ph.D
Dean
Faculty of Graduate Studies
Mahidol University

Prof.Amaret Bhumiratana, Ph.D
Dean
Faculty of Science
Mahidol University

ACKNOWLEDGEMENT

The success of this thesis was attributed to the extensive support and assistance from my benefactors. I would like to express my gratitude to my advisor, Assoc. Prof. Punya Temcharoen for his encouragement and kind advice. I am very much obliged to Assoc. Prof. Galayanee Doungchawee for her continuous guidance, help and facility. I am grateful to Asst. Prof. Dr. Rungtiva Sirimujalin, for her criticism, advice, facility and great patience to revise my thesis writing.

I sincerely thank Asst. Prof. Dr. Panus Chalermsanyakorn for his kind guidance and confirmation of histopathology. I would like to thank Mrs. Pranom Puchadapirom and Mr. Suraphol Kongtim for laboratory assistance.

I would like to express my gratitude to department of pathobiology for valuable acquirements, skills, training and knowledge provision.

I also like to thank all who supported and embolden me in part of the thesis work. This work was supported in part by a scholarship from the Faculty of Graduate Studies, Faculty of Science, Mahidol University.

Finally, I would like to express with deep immense gratitude to my parents and family, who always love, care, sustain and give me faith.

Jiraporn Suwana

EFFECTS OF PIPERINE ON WISTAR RATS.

JIRAPORN SUWANA 4237903 SCPA/M

M.Sc. (PATHOBIOLOGY)

THESIS ADVISORS: PUNYA TEMCHAROEN, M.Sc. (Pathobiology),
GALAYANEE DOUNGCHAWEE, M.Sc. (Pathobiology),
RUNGTIVA SIRIMUJALIN, Ph.D. (Pathology).

ABSTRACT

Piperine (1-piperonyl-piperidine) is the active ingredient of black pepper (*P.-nigrum*) and long pepper (*P. longum*), which has been used in traditional medicine. It is known as a bioenhancer, which increases the bioavailability of drugs and nutrient supplements. It has been noted for its analeptic drug, anti-epilepsy, anti-fertility, and anti-inflammation properties but its immunotoxicity has been rarely reported. The objective of this study is to investigate the general effects of piperine and indicate levels of safe consumption. Rats immunized with rabbit serum proteins were fed piperine at the doses of 5, 10, 25, 50, 100 and 250 mg/kg BW for 7, 14, 30 and 90 days, respectively. The results showed that there were no remarkable effects of piperine at all treated doses on thymus, spleen, liver, adrenal gland and small intestine. Cell proliferation assay by 5'-bromo-2'-deoxy-uridine (BrdU) showed no proliferative change in any cell population of the thymus, spleen and Payer's patches. There was no detectable antibodies response observed by immunoelectrophoresis. However, the rats treated with piperine at the doses of 100 and 250 mg/kg BW showed decreasing in body weight gain and food consumption. Rats treated with piperine at the dose of 100 mg/kg BW were nervous, hyperactive, jumping and running around the cage, in contrast, those treated with piperine at the dose of 250 mg/kg BW showed depressive effects. The pathological findings of the rat's testis fed with piperine at the dose of 250 mg/kgBW showed deformity of the seminiferous tubules with a decrease of spermatozoa while the stomach showed some congestion.

KEY WORDS: PIPERINE/ TOXIC EFFECTS/ GROWTH RATE/
HEMATOLOGY/ IMMUNOLOGY/ HISTOPATHOLOGY.
99 p ISBN 974-04-5656-1

ผลของสารไพเพอรีนต่อหนูขาวสายพันธุ์วิสตาร์ (EFFECTS OF PIPERINE ON WISTAR RATS)

จิราภรณ์ สุวรรณะ 4237903 SCPA/M

วท.ม. (พยาธิชีววิทยา)

คณะกรรมการควบคุมวิทยานิพนธ์: ปัญญา เต็มเจริญ, วท.ม. (พยาธิชีววิทยา), กัลลยานี ดวงฉวี, วท.ม. (พยาธิชีววิทยา), รุ่งทิวา ศิริมุจลินท์, วท.ม. (พยาธิชีววิทยา), Ph.D. (Pathology).

บทคัดย่อ

การศึกษาผลทั่วไปของไพเพอรีนซึ่งเป็นสารหลักในพริกไท มีฤทธิ์กดและกระตุ้นประสาทส่วนกลาง รวมทั้งสามารถต่อต้านการอักเสบ จากการศึกษาหนูขาวเพศผู้ที่ถูกกระตุ้นภูมิคุ้มกันด้วยซีรั่มโปรตีนของกระต่ายหลังป้อนไพเพอรีนที่ความเข้มข้น 5, 10, 25, 50, 100, และ 250 มิลลิกรัมต่อน้ำหนักตัวในระยะเวลา 7, 14, 30 และ 90 วัน ตามลำดับไม่พบการเปลี่ยนแปลงของระบบภูมิคุ้มกันระหว่างกลุ่มที่ได้รับสารไพเพอรีนและกลุ่มควบคุม นอกจากพบว่าหนูขาวที่ได้รับสารไพเพอรีนที่ความเข้มข้น 25 และ 50 มิลลิกรัมต่อน้ำหนักตัวมีการกินอาหารและน้ำหนักตัวเพิ่มขึ้นมากกว่ากลุ่มควบคุม แต่หนูในกลุ่มที่ได้รับไพเพอรีนที่ความเข้มข้น 100 และ 250 มิลลิกรัมต่อน้ำหนักตัวกลับพบว่ากินอาหารน้อยกว่าและมีน้ำหนักตัวลดลง สำหรับพฤติกรรมไม่อยู่นิ่งพบในหนูที่ได้รับไพเพอรีนที่ความเข้มข้น 100 มิลลิกรัมต่อน้ำหนักตัว ต่างจากหนูกลุ่มที่ได้รับสารไพเพอรีนที่ความเข้มข้น 250 มิลลิกรัมต่อน้ำหนักตัวมีอาการเซื่องซึม เมื่อศึกษาพยาธิสภาพของเนื้อเยื่อพบการสร้างอสุจิลดลงและพัฒนาการของอสุจิลดลงในอัณฑะของหนูที่ได้รับไพเพอรีนที่ความเข้มข้น 100 และ250 มิลลิกรัมต่อน้ำหนักตัวเท่านั้น ส่วนอวัยวะอื่นๆ เช่นไทมัส, ม้าม, กระเพาะ, ตับ, ต่อมหมวกไต, ลำไส้เล็ก ไม่พบการเปลี่ยนแปลงที่ชัดเจน เมื่อเทียบกับกลุ่มควบคุม

โดยสรุปไม่พบการเปลี่ยนแปลงใดๆต่อหนูขาวเพศผู้ที่ได้รับสารไพเพรีนที่ความเข้มข้นน้อยกว่า 100 มิลลิกรัมต่อน้ำหนักตัว

99 หน้า ISBN 974-04-5656-1

CONTENTS

LIST OF TABLES

LIST OF TABLES (Cont.)

LIST OF TABLES (Cont.)

LIST OF FIGURES

LIST OF FIGURES (Cont.)

LIST OF FIGURES (Cont.)

LIST OF FIGURES (Cont.)

LIST OF FIGURES (Cont.)

LIST OF ABBREVIATIONS

BrdU	5’-bromo-2’-deoxyuridine
BW	Body weight
IEP	Immunoelectrophoresis.
°C	Degree(s) Celsius
DMH	Dimethylhydrazine
DW	Distilled water
Figs	Figure(s)
g	Gram(s)
hr	Hour(s)
i.g.	Intragastric
i.p.	Intraperitoneal
kg	Kilogram(s)
LD_{50}	Median lethal dose is the statistically derived single dose of a substance that can be expected to cause death in 50% of the tested animals.
M	Molar
MW	Molecular weight
mg	Milligram(s)
min	Minute(s)
ml	Milliliter(s)

LIST OF ABBREVIATIONS (Cont.)

mM	Millimolar
µg	Microgram(s)
µl	Microliter(s)
µmole	Micromole(s)
N	Normal(s)
nmole	Nanomole (s)
nm	Nanometer(s)
O.D.	Optical density (ies)
O/N	Over night
POD	Peroxidase enzyme.
PBS	Phosphate buffer saline
PIP	Piperine
PNP	*p*-Nitrophenol
rpm	Round per minute(s)
S.E.M.	Standard error of mean
TA	*Salmonella* serovar Typhimurium antigens
w/v	Weight by volume.
v/v	Volume by volume

CHAPTER 1
INTRODUCTION

Spices are plant, which have sharp and hot taste. They are also commonly used as relish in many countries. Withally, they also have been largely used for a traditional treatment, especially digestive problems such as absorptive and digestive enhancer [Platel and Srinivasan, 1996]. In the present, many spices have been accepted to use for modern medicine and wildly studied about their chemical, biochemical, biological and toxic properties [Reen, *et al*., 1993; Parmar, *et al*., 1997; Piyachaturawat, Glinsukon and Toskulkao, 1983]. Among spices, pepper is eminent relish and the origin is from India [Chandrasekhara and Srinivasan, 1999]. It belongs to the Piper species (*Piperaceae*) [Singh, 1973] and it is also in quantitative used as folkloric medicine for treatment of asthma, bronchitis, pyrexia, insomnia and digestive problem [Pei, 1983; Atal, Zutshi and Rao, 1981]. The reputation of Piperine species both the black pepper (*Piper nigrum Linn*) and long pepper (*Piper longum Linn*) comes from its pungent taste is caused by the yellow crystalline alkaloid piperine, which presents in the fruits of the pepper. The contents of piperine in *P. nigrum Linn* and *P. longum Linn* are 3-9% and 3-5% (on dry weight basis), respectively [Platel and Srinivasan, 1996]. Piperine (1-piperoyl-piperidine) is a major active component presented in various *Piperaceae spp.* [Atal, Zutshi and Rao, 1981; Mujumdar, *et al*., 1990]. Piperine has many derivative forms, which unequally gives the pungent taste in each form but the most pungent taste form is E, E-(trans-trans-piperine) [Ternes and Krause, 2002]. The biological, pharmacological and biochemical activities of piperine were studied in various aspects both *in vitro* and *in vivo* [Liu and Simon, 1996; Unchern, Saito and Nishiyama, 1998; Izzo, *et al*., 2001]. However, some studies showed that piperine possessed diverse biochemical and pharmacological activity. It had the stimulating and depressing of central nervous system (CNS) activity, which presented in mice [Lee, Shin and Woo, 1984]. In addition, the antipyretic and anti-inflammatory property was investigated in rabbits and rats, respectively [Liu and

Garattini, 1986; Mujumdar, *et al*., 1990]. Biochemically, piperine inhibited oxidative phosphorylation and calcium transportation but it stimulated ATPase activity in isolated rat liver mitochondria [Reanmongkol, *et al.*, 1988]. Piperine demonstrated the biphasic effects on drug metabolizing enzymes with regarded to the dose, route and duration of treatment [Reen *et al.*, 1993; Dalvi and Dalvi, 1991]. Piperine was reported that it protected aflatoxin B1 induced cytotoxicity and micronucleus formation in H411EC3 rat hepatoma cells [Singh, Reen and Wiebel, 1994]. On the contrary, piperine was found to promote the cytotoxic effect of benzo (a) pyrene on V-79 lung fibroblast and also increased in the covalent binding of benzo (a) pyrene to DNA [Chu, Chang, and Wang, 1994]. Furthermore, piperine was reported the adverse effects on the reproductive system by decreasing the weight of the testis of the rats [Malini and Manimaran, 1999], and possesses the anti-fertility effects [Daware, Mujumdar and Ghaskadbi, 2000] and the cytotoxic effects on embryonic rat brain neurons in cell culture [Unchern, Saito and Nishiyama, 1998]. However, it had no genotoxic effect when assessed by Ames test using *Salmonella thyphimurium*, TA 98 and TA 100, micronucleus test, sperm abnormality test and dominant lethal test using Swiss albino mice [Karekar, *et al*., 1996]. In addition, piperine was the non-specific enhancer of drugs absorption such as propanoyl, theophylline, rifampicin, sulphadiazine [Bano, *et al*., 1987; Bano, *et al*., 1991; Zutshi, *et al*., 1985] and nutrient supplements such as Vitamin A, β-carotene or even the Co-enzyme activities such as Co-Q10 [Badmaev, Majeed and Prakash, 2000]. Then, piperine is used as the coordinating supplement for enhancing of drug and other nutrient supplements as described for present day. The acute toxic effects of piperine were studied in various species of animals including mice, rats and hamsters [Piyachaturawat, Glinsukon, and Toskulkao, 1983]. The results demonstrated that the toxic dose of piperine (250mg/kg body weight/day or more) induced respiratory paralysis and dysfunction of many organs and being the cause of death. The histopathological changes were severe hemorrhagic necrosis of the stomach, edema in the gastrointestinal tract, in the urinary bladder and in the adrenal glands [Piyachaturawat, Glinsukon and Toskulkao, 1983]. However, the previous study was carried out for only 7 days while consuming of piperine has been changed both on purpose and duration. Furthermore, no study on the sub-chronic or chronic effects was reported; then, it was interesting to study the

effects of piperine at lower dose within a longer period of time based on the information of previous study [Piyachaturatwat, Glinsukon and Toskulkao, 1983]. Therefore, the main purpose of this study was investigation of the acute and sub-chronic effects of piperine on Wistar rats by using the parameters as followed; physical observation, hematological parameters, pathological changes of target organs (adrenal gland, liver, small intestine, spleen, stomach, testis and thymus), and effect on immune system. The hematological parameters were examined the complete blood counts by using standard method and pathological changes were observed by routine histology technique and examined them under light microscope. Lastingly, effects on immune system were examined as the non-specific immune response in both of cellular immune response (CMIR) and humoral immune response (HMIR). The CMIR effect was indicated by proliferation rate of lymphocytes in the immune organs (thymus, spleen and Payer's patch). The proliferated cells were detected by using 5'-bromo-2'-deoxy-urdine (BrdU) incorporation method. BrdU is a thymidine analog, which incorporated into DNA at S phase of the cell cycle and it was recognized by a monoclonal mouse anti-bromodeoxyuridine antibody. HMIR was indicated by antibody presentation, which was detected by immunoelectrophoresis method.

The objectives of this study:

1. To investigate acute oral repeated doses toxicity of piperine in young adult rats.
2. To investigate short term repeated doses toxicity of piperine in young adult rats and mature rats.
3. To investigate sub-chronic toxicity of piperine in young adult rats.

For each experiment, these parameters including: hematology, cell mediated immune response (BrdU cell proliferation assays), immunoelectrophoresis and the histopathological changes of target organs were examined.

CHAPTER 2
LITERATURE REVIEW

2.1 OVERVIEW OF PIPERINE

Piperine (1-piperoyl piperidine) is the major active pungent constituent in various species of *Piper species* (*Piperaceae*) [Johri and Zutshi, 1992; Finar, 1975]. Among these, the black pepper (*Piper nigrum Linn*) and long pepper (*Piper longum Linn*) are well known and widely used as relish by the large number of people all over the world. They are also used as ingredient in the folkloric medicine for treatment of gastrointestinal tract disorder since ancient time [Pei, 1983]. The piperine contents in *P.nigrum Linn* and *P. longum Linn* are 3-9% and 3-5% (on dry weight basis), respectively [Platel and Srinivasan, 2001]. It can be isolated from the oleoresin of the pepper by extraction from the powdered fruit of the plant by dichloromethane or alcohol and purified in crystalline powder [Bhat and Chandrasekhara, 1985].

Chemical name: The chemical name of piperine is (*E, E*)-1-[5-(1, 3-benzodioxol-5-yl)-1-oxo-2, 4-pentadienyl] piperidine.

Molecular weight: 285.34

Molecular formula: $C_{17}H_{19}NO_3$

Percent of organic component: C=71.55%, H=6.71%, N=4.91%, and O=16.82%.

PIPERINE STRUCTURE

Piperine structure (figure-1) consists of three important components including methylenedioxyphenyl (MDP) ring, side chain with conjugated double bonds and a basic piperidine moiety attached through a carbonylamide linkage to side chain [Finar, 1975; MERCK, 2000].

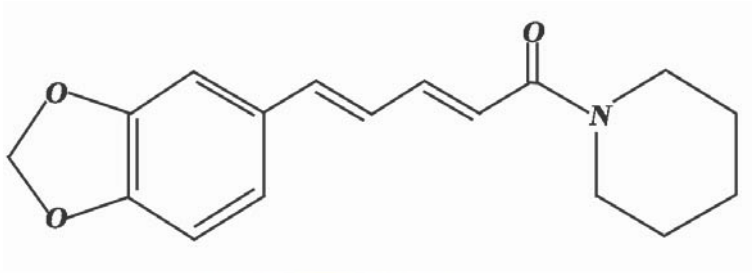

Figure-1: The chemical structure of piperine ($C_{17}H_{19}NO_3$).

2.1.1 METABOLISM OF PIPERINE

It has been reported that after giving piperine to the male albino rats, regardless of the dose and route of administration, about 97 % of piperine was absorbed in the gastrointestinal tracts [Bhat and Chandrasekhara, 1986]. Only three percents were excreted as piperine in feces but no free form of piperine was excreted in the urine. The piperine transformation in duodenal segment is higher than jejunal and ileal segments and there were no transformed piperine during absorption by the small intestine [Bhat and Chandrasekhara, 1986].

Piperine is biotransformed in the liver by the hepatic cytochrome P-450 dependent monooxygenase [Bhat and Chandrasekhara, 1987]. It is biotransformed to piperic acid, piperonylic acid, piperonyl alcohol, piperonal and vanillic acid and their conjugated. The probable reactions of piperine metabolism are found 3 reactions, which are demethylenation, glucuronidation and sulphation of piperine. After administration of the piperine to rats with regardless to the route of administration, trace to small amount of piperine could be detected in serum, spleen, kidney and liver from 30 minutes and none of it was detected in anywhere after 24 hours [Bhat and Chandrasekhara, 1987]. It indicated that all absorbed piperine was biotransformed and excreted in urine in metabolic form [Bhat and Chandrasekhara, 1986]. It was speculated that piperine was absorbed into portal vein, which was first hydrolyzed to piperic acid and piperidine mainly in the liver, and then the former was transformed to piperonylic acid after successive oxidation of the side chain [Bhat and Chandrasekhara, 1987]. The oxidative cleavage of side chains of piperine may also leads to formation of piperonal. Subsequently, the methylenedioxypyronyl (MDP) aldehyde, piperonal could undergo oxidation to piperonylic acid and/or reduction to piperonyl alcohol. Another principal route of piperine metabolism was through

cleavage of the methylenedioxy group. The major metabolite would be catechols, which were then *O*- methylated to methoxyphenols, which only vanillic acid had been identified. The proposed of the metabolic pathway of piperine was shown in figure 2 [Bhat and Chandrasekhara, 1987].

The metabolism of piperine in a group of human volunteers was studied [Shoba *et al*., 1998]. Some of the metabolites of piperine included the 5-(3, 4 - dihydroxyphenyl) valeric acid piperidine and its derivative were hydroxylated at position 4 of the piperidine ring, which were detected in the urine of most of the volunteers while 15 % of the tested persons could be detected 5-(3, 4 - dihydroxyphenyl-2, 4 pentadienoic acid piperidine) in their urine [Shoba, *et al*., 1998].

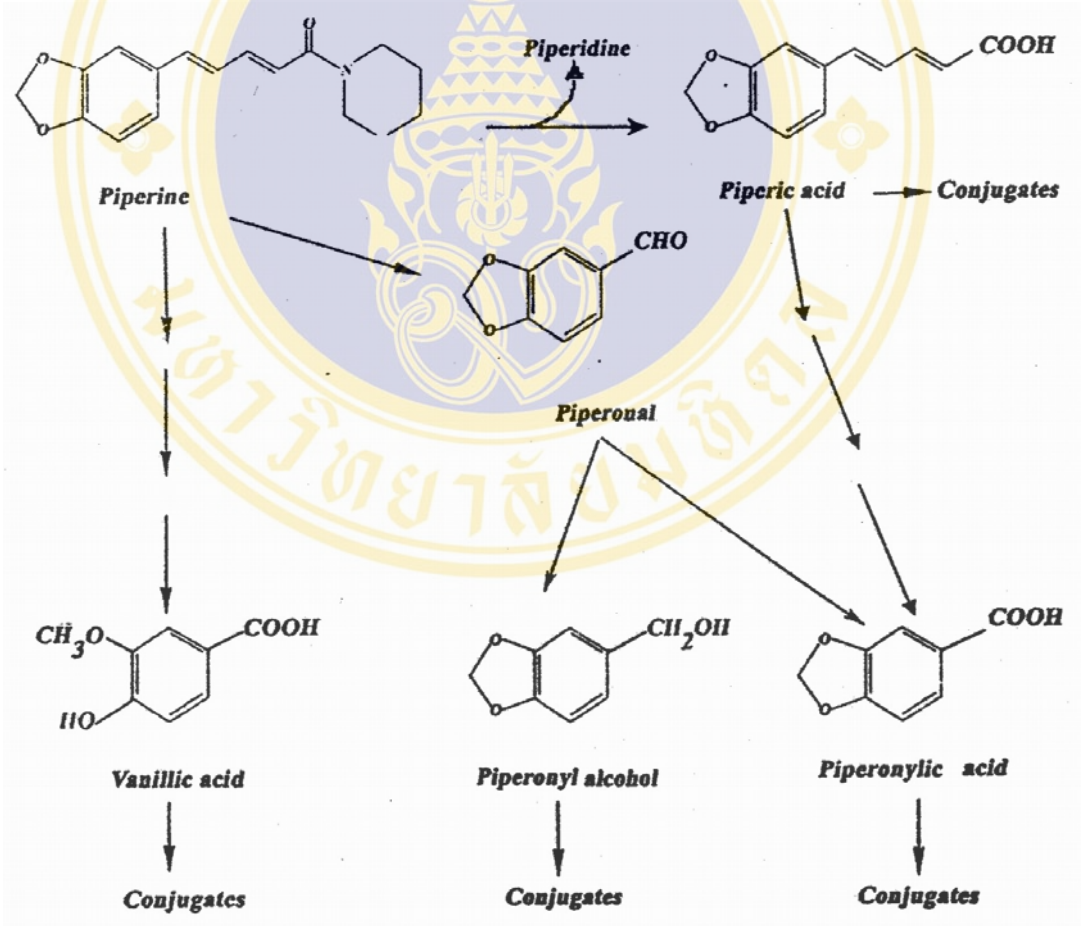

Figure 2: The proposed metabolic pathway of piperine biotransformation in albino rats [Bhat 1987]

2.1.2 EFFECTS OF PIPERINE ON DRUG METABOLIZING ENZYMES

The effect of piperine on the drug metabolizing enzymes has been studied extensively both *in vitro* and *in vivo* [Atal, Dubey and Singh, 1985; Reen and Singh, 1991] Piperine showed to inhibit the activity of the enzymes arylhydrocarbon hydroxylase (AHH), ethylmorphine-N-demethylase, 7-ethoxycoumarin-O-deethylase (7ECDE) and 3-hydroxy-benzo (a) pyrene glucuronidase in rat post mitochondrial supernatant *in vitr*o in a dose dependent manner. The inhibitory effect of piperine on these reactions in postmitochondrial supernatant from 3-methylcholanthrene and phenobarbital treated rats was similar to the control. These studies indicated that piperine was a non-specific inhibitor of drug metabolizing enzymes [Atal, Dubey and Singh, 1985]. A single oral administration of piperine (125 mg/kg body weight) in rats strongly inhibited the hepatic arylhydrocarbon hydroxylase (AHH) and UDP-glucuronyltransferase activities [Atal, Dubey and Singh, 1985]. The maximal inhibition of hepatic arylhydrocarbon hydroxylase (AHH) of rats observed within 1 hour and by 6 hours after administration, the enzymatic activity returned to the normal control value [Atal, Dubey and Singh, 1985]. Pretreatment with piperine exhibited prolonged hexobarbital sleeping time and zoxazolamine paralysis time in mice at half the dose of SKF -525 A. These results exhibited that piperine was a potent inhibitor of drug metabolism [Atal, Dubey and Singh, 1985]. Later, Singh and his colleagues demonstrated that piperine modified the rate of glucuronidation in isolated epithelial cells of the guinea-pig small intestine by lowering the endogenous UDP glucoronic acid content and also by inhibiting the transferase activity [Singh, Dubey and Atal, 1986]. In 1991, Reen and Singh studied the effects of piperine on pulmonary cytochrome P-450 activities both *in vitro* and *in vivo* [Reen and Singh, 1991]. They reported that piperine caused differential inhibition of cytochrome P-450 which affected on the activity of AHH and 7ECDE. Piperine at 100 µM inhibited 50 % of AHH and 7ECDE activity which was the dose dependent when compared to control and 3-methycholanthrene (3MC) treated rats. Whereas, it caused strong inhibition at lower concentration (35% at 10 µM) and relatively much lesser inhibition with further increased in piperine concentration in guinea pig [Reen and Singh, 1991]. *In vivo* study, rat received piperine at the dose of 25 mg/kg body weight exhibited a maximal

inhibition of AHH and 7ECDE at 1 hour, while only AHH returned to normal values within 4 hours. However, daily treatment of piperine (15 mg/kg body weight) to the rats for 7 days, 7ECDE was consistently inhibited while AHH showed faster recovery [Reen and Singh, 1991]. In addition, piperine was a potent inhibitor of UDP-glucose dehydrogenase (UDP-GDH) in dose related manner in both liver and small intestinal tissues, which involved in the conjugated double bonds of the molecule [Singh, Dubey and Atal, 1986]. Furthermore, piperine showed the various effects in liver and small intestine glucuronidation in rats. The glucuronidation decreased after obtained piperine at 10 μM in intestinal cells whereas in the liver, glucuronidation was decrease when obtained at 50-100 μM. These results suggested that piperine had lesser effects on liver glucuronidation than rat intestine. [Reen, *et al*., 1993]. After piperine orally fed to the rat at the dose of 100 mg/kg body weight caused an increase in hepatic microsomal cytochrome P-450 and cytochrome b_5, NADPH-cytochrome c reductase, benzphetamine N-demethylase, aminopyrine N- demethylase and aniline hydroxylase within 24 hours after treatment. Rats were given piperine intraperitoneally (10 mg/kg body weight) had no effect on the activities of the aforementioned enzymes, while at the doses of 800 mg/kg and 100 mg/kg (i.g. and i.p.) respectively, had significantly decrease in the activities of the mentioned enzymes except cytochrome b_5 and NADP-cytochrome c reductase [Dalvi and Dalvi, 1991]. Piperine had affected on the liver microsomal P450 in rats treated with piperine at the dose of 1.4 mmol/kg orally for three consecutive days, resulting in biphasic effects on the expression of liver microsomal P450. These results demonstrated that piperine treatment suppressed cytochrome P4502E1 expression while it enhanced cytochrome P4502B and P4501A expression. However, piperine had no effect on hepatic microsomal epoxide hydrolase (mEH) and glutathione S-transferase (GST) expression [Kang, *et al*., 1994].

2.1.3 PHARMACOLOGICAL PROPERTIES OF PIPERINE

Piperine as well as black pepper and long pepper have been shown to have broad range of pharmacological activities. Many papers demonstrated that they had the influence on the central nervous system (CNS) [Pei, 1983; D'Hooge *et al*., 1996], gastrointestinal tract [Johri and Zutshi, 1992]. Piperine and its fruits also

possess substantial anti-inflammatory and antiseptic properties [Mujumdar, *et al*., 1990].

Effect on the CNS and conductivity

Immediate response of excitation and convulsion was observed in all animal species such as mice, rats, and hamsters after piperine treatment [Piyachaturawat, Glinsukon, and Toskulkao, 1983; Pei, *et al*., 1980]. Piperine belongs to the chemical family of cinnamamides, which are natural occurring compounds that have potential uses as anticonvulsant and analeptic drugs [Pei, 1983 and D'Hooge, *et al*., 1996]. It stimulated and depressed central nervous system (CNS). This compound suppressed convulsion by stimulating the release of serotonin (5-HT), which was neurotransmitter from the cerebral cortex [Mori, Kabuto and Pei, 1985], and catecholamine from the adrenal gland [Kawada, *et al*., 1988]. In addition, piperine interacted with the seratonergic system and depleted substance P in spinal cord; a neuromuscular transmission and sensory receptors [Takaki, *et al*., 1990].

Analeptic effect of piperine

Piperine was effective as nalorphine, which was commonly used to counteract morphine intoxication. Morphine was often used to treat chronic and incurable pain. It caused respiratory depression as complication when increased dosage to maintain a long-term analgesic effect. Piperine administered intravenously at the dose of 5 mg/kg reversed the respiratory depression within 10-20 minutes after administration. Therefore, piperine was used as the antidote of morphine over dosed in patients [Singh, 1973].

Effect of piperine on the gastrointestinal tract

Piperine induced the alterations in membrane dynamics and permeation characteristics, along with induction in the synthesis of proteins associated with cytoskeleton function, resulting in an increase in the small intestine absorptive surface, thus assisting efficient permeation through the epithelial barrier [Khajuria, Zutshi and Bedi, 1998]. In addition, it reduced intestinal motility, both *in vitro* and *in vivo* [Takaki, *et al*., 1990; Izzo, *et al*., 2001] and it also decreased in the fluid accumulation in the small intestine, which induced by castor oil in dose-dependent [Capasso, *et al*., 2002]. Otherwise, it stimulated digestive enzyme including pancreatic lipase and bile

secretion in the gastrointestinal tract [Johri and Zutshi, 1992]. They were also increasing blood supply and hydrochloric secretion [Ononiwu, Ibeneme and Ebong, 2002].

Anti-inflammatory and anti-allergic effects of piperine

The crude extract and purified agent of *Piperaceae* spp. showed the inhibitory properties against cycloxygenase (COX-1), which played an important role of prostaglandin formation [Vane, 1971]. Prostaglandin is the relevant mediators in the development of inflammatory diseases such as rheumatism. The work provided evidence that extracts of several *Piper* species acted as cycloxygenase antagonist [Vane, 1971]. In addition, it only was not inhibit cycloxygenase activity but also inhibited 5-lipoxygenase (5-Lox) [Mujumdar, *et al*., 1990]. 5-Lox is the key enzyme in the metabolism of arachidonic acid and it has responsibility in the formation of proinflammatory leukotrienes which played an important role in the induction of asthma. [Mujumdar, *et al*., 1990].

2.1.4 TOXICOLOGICAL EFFECTS OF PIPERINE

Acute and sub-acute studies.

Acute effect of piperine was studied in many animals, which were rats, mice, and hamsters. The LD_{50} value for a single i.v., i.p., s.c., i.g. and i.m. administration of piperine to adult male mice were 15.1, 43, 200, 330 and 400 mg/kg body weight, respectively. Regard to the sex and route of administration, the results showed increasing of i.p LD_{50} value were 60 and 132 mg/kg body weight in female and weanling male mice, respectively. However, the animals fed with piperine at the lethal dose were died within 3-7 minutes and the cause of death was respiratory paralysis. There were hemorrhagic necrosis and edema found in gastrointestinal tract, urinary bladder, and adrenal gland in sub-acute toxicity studies of rats fed with piperine at the dose of 500 mg/kg body weight. The rats died within 1-3 days after treatment and the cause of death must be related to multiple organ failure. [Piyachaturawat, Glinsukon and Toskulkao, 1983].

Acute reproductive effect.

The effects of piperine on the reproductive system have been studied in various animal models [Piyachaturawat, *et al*., 1991; Piyachaturawat and Pholpramool, 1997; Malini and Manimaran, 1999]. Upon piperine administered to albino rats at a dose of 170 mg/kgBW by orally (i.g.) or 85 mg/kgBW intraperitoneally (i.p.) resulting in antifertility effect; which were similarly found in mice and hamsters [Piyachaturawat, *et al*., 1991]. The results showed that piperine caused reduction in both of fertilization and spermatogenesis. It inhibited the acrosome reaction by decreasing calcium influx into sperm but had no effect on sperm motility. Moreover, piperine at the dose of 10 mg/kg body weight, p.o., also caused a marked increase in serum gonadotropins and decrease in intratesticular testosterone concentration [Malini and Manimaran, 1999].

Mutagenic and carcinogenic activity.

The mutagenic and carcinogenic activities of pepper were reported that piperine had non-mutagenic activity by using *Salmonella thyphimurium* strains TA 98 and TA 100 (Ames test) micronucleus test, sperm shape abnormality test and dominant lethal test using Swiss albino mice [Karekar, *et al*., 1996]. In the Ames test, piperine at the dose range of 0.005-10 μmole/plate did not induce his^+revertant, with or without metabolic activation indicating its non-mutagenic property. In the bone marrow micronucleus test in mice, piperine at the doses of 10 and 20 mg/kg body weight showed that piperine at the given dose did not induce micronucleus formation [Karekar, *et al*., 1996].

2.2 BASIC TOXICOLOGY [Hayes, 1994]

Toxicology is the study of the adverse effects induced by chemical agents on living organism. In addition, there are various toxic levels occurring in variable organ, a type of cell, or some of specific biochemical activities in the body.

Toxic effects are primarily of two general types, which are specific organ effects and systemic effects.

It can be studied by using many testing methods, which divide into three major types.

1. Acute exposure is defined as exposure to a chemical for less than 24 hour and usually refers to a single administration, or repeated exposures may be given within a 24 hour period for some slightly toxic or practically non-toxic chemicals.

2. Repeated exposures divided into three categories: sub-acute, sub-chronic, and chronic. Sub-acute exposure refers to repeated exposure to a chemical for 1 month or less, sub-chronic for 1 to 3 months and chronic for more than 3 months. These three categories of repeated exposure can be by any route, but most often they occur by the oral route, with the chemical added directly to the diet.

The common routes of administration in both acute and sub-chronic toxicity studies are dietary, oral, dermal, and inhalation. The dosage is the most important and critical factor in determining if a substance will be an acute or a chronic toxicant.

These studies include physical and clinical observations. The physical observation is routinely evaluated during the treatment phase of them. Each animal should be observed twice daily for overt signs of toxicity, moribundity, and mortality. These changes include the skin, fur, respiratory function, autonomic, central nervous system, somatomotor function, general behavior and body weight. Body weight is the indicators of the condition of an animal if it is monitored frequently and carefully during a study. It should be measured and recorded at least once per week. Body weight loss was possibly due to decreasing feed consumption, water consumption, disease or specific toxic effects. Feed consumption generally is measured and recorded once per week in rodent studies but the accurate measurement of feed consumption is important for studies in which the test material is administered in the diet. In addition, clinical pathology changes are indicators of general health and toxicity and must be assessed before termination of the study. In rodents, clinical pathology determinations usually are performed in each group includes hematology (hematocrit, hemoglobin, erythrocyte count, total leukocyte count, and differential leukocyte) and clinical chemistry such as liver function enzyme. Histopathology inspects the morphological changes or the damage of tissue or target organ, which is performed by histochemistry method and examined under light microscope [Hayes, 1994]. However, toxic effect or toxicity is complex with many influencing factors. The target organs effect may be varied depending on dosage and route of exposure but

dosage is the most important. Many xenobiotics distribute in the body and often affect only specific target organs. Toxicity can be result from adverse cellular, biochemical, or macromolecular changes. However, some of xenobiotics may also act indirectly by modification of an essential biochemical function, interference with nutrition and alteration of a physiological mechanism.

The toxicity of a substance depend many factors, which described below.

1. Dosage is especially dose-time relationship.

2. Exposure route is important in determining toxicity. Some chemicals show different effects, which the exposure route are difference. They may be highly toxic by one route but not by others. Two major reasons are differences in absorption and distribution within the body.

3. Species, toxic responses can vary substantially depending on the species. The difference animal species are attributable to difference in metabolism results different in the tolerance, resistance or susceptibility to toxic substances. Others may be due to anatomical or physiological differences. Based on studying of some substances examine their LD_{50} in various species. The result is shown unequal LD_{50} of the same tested substances between animal species.

4. Age, some substances have more effects on young animals than adult animals. It depends on type of substances and their properties.

5. Sex, some substances have interaction with sex hormone.

6. The ability to be absorbed is essential for systemic toxicity to occur. Some chemicals are readily absorbed and others poorly absorbed. The metabolism of substances is showing no harmful effects when it is the innate form but their metabolite may damage to the body.

7. Excretion, some substances can get rid them out of the body in both of innate chemical agents and their metabolite. However, some kinds of them remaining because they unable to excrete of the body, which may cause damage to the body.

2.3 IMMUNOLOGY

There are many contaminants in the environment including air pollution, pathogens in soil, and water. However, living organisms have mechanism to protect themselves from foreign things, so called immune system. The immune system is the defense mechanism of living organisms purposing to prevent, destroy, and remove the foreign materials. Immunology is the study of the immune response. There are two types of immunity divided by specific response including non-specific (or innate) and specific (or acquire) immunity but both of them are work together.

Non-specific immunity is the first line of defense that plays a role against any foreign materials and composes of several different types of mechanism including physical, chemical, and cell immunity. All details are shown in Table-1.

TABLE-1: The defense mechanisms of non-specific immune response.

Mechanism	Examples
Physical barriers	♦ Skin ♦ Mucous membranes
Chemical barriers	♦ Acidity in stomach ♦ Lysozyme in secretions (tears, saliva) ♦ Complement in plasma ♦ Interferons (IFNs)
Cells	♦ Phagocytes ♦ Natural killer cells (NK-cells) ♦ Eosinophils ♦ Basophils

Physical barrier, skin is the first barriers to protect the non-self materials such as bacteria and virus. Lactate in sweat is the pH conditions, which a few of pathogens can tolerate and survive in this condition. Whereas, food might be contaminated with pathogens, mucous membrane locates in many places such as respiratory, gastrointestinal tract, and urinary system. Mucus produce from these membranes are

sticky and effectively to trap micro-organisms and particular matters. Some of them are destroyed by acidity in the stomach.

Chemical barrier, there are some groups of proteins, which detect in high level over normal range showing the signs of attack. Complement is proteins in plasma and it always present when activated cause lysis bacteria. It is activate direct by bacteria contact or indirect by antibodies, which produce from specific immune response but it have to bound to immunogen. Lysozyme in secretions killed pathogens by hydrolysing their cell walls caused osmotic-lysis. Finally, interferons (IFNs) are produced from virus infected cells sending stimulated signal to non-infected cells produce protein to inhibit vial replication.

Cells activities, there are four types of cell working on non-specific immune response. Phagocytes: neutrophillic mononuclear cells (neutrophils, eosinophils, and basophils) and monocytes. Both of them ingest and kill pathogens, especially bacteria and parasites by two mechanisms which are toxic oxygen metabolite production and lysozomal phagocytic vacuole. However, virus infection is killed by NK cells.

Specific (acquire) immune response is high specificity but it can only be acquire response when it is activated against individual types of organism or macromolecules, so called immunogen that is the things can activate specific immune response. At the first time of contact, it has immunological memory for that immunogen and response is produce more reactive level and rapid response in the second time of the same immunogen while non-specific always respond the same level. Antigen is molecule combines with specific antibody but it does not necessarily be immunogen. Antibody is the protein producing from HMIR, which combines with specific antigen. However, immunogen can activate both of HMIR and CMIR but depending on individual immunogen, one type usually predominates. There are two kinds of individual organism or macromolecules response depending on their characteristics. The protein production is antibody involving the humoral immunity (HMI), while the cellular cytotoxicity is cell-mediated immunity (CMI). Both of HMI and CMI are involving to lymphocytes, which is T-lymphocytes and B-lymphocytes. Humoral immunity (HMI) is the response of activated B-cells enhancing by T-cells to stimulate B-cells differentiate into plasma cells that secrete copious quantities of

antibody. However, stimulated B-cells also form memory B-cells that do not secrete antibody. Then, antibody combines with specific antigen forming the antigen-antibody. Then, antibody combines with specific antigen forming the antigen-antibody complex, which activate complement fixation and then phagocytic occurred.

Cellular cytotoxicity (CMI) is the T-cells response. It recognize foreign *peptides* bound to major histocompatibility proteins (MHC) via the T-cell receptor Two types of T-cells are

1. T-helper cells (T_H) express CD4 (Cluster of Differentiation 4) on surface ($CD4^+$). It recognizes class II MHC in complex with *foreign* peptides on antigen presenting cells, leading to activation of T_H cell. Activation causes release of cytokines that activate B-cells, macrophages, T_c cells.
2. T-cytotoxic cells (T_C) express CD8 (Cluster of Differentiation 8) on surface ($CD8^+$). This cell recognizes class I HMC/foreign peptides on almost any cell, leading to formation of a cytotoxic T lymphocyte (CTL).

In addition, excessive exposure of lymphoid tissues to chemicals or drugs may alter the delicate balance between cells of the immune system, and possibly results in undesirable effects on the immune system, namely immunotoxic substances. Immunotoxicology is the study of undesirable or adverse effects on the immune system inducing by inorganic or organic substances. The functional immune alteration is detected by the assay with regard to the general immunotoxicology testing guideline [Farang and Abo-Zieal, 1999]. Briefly, recommendations for the inclusion of immunotoxicity evaluations in animal models suggest such evaluations be conducted in rodents. There are trial panel of assay to identify immunotoxicity substances including the measurement of (1) altered lymphoid organ weights or histology; (2) quantitative changes in cellularity of lymphoid tissue, peripheral blood leukocytes and/or bone marrow; (3) impairment of cell function at the effectors or regulatory level. The primary immunotoxicity screening panel was shown in Table-1 referring to study the response effect of the specific immune types of the tests. This

table covers a large number of *in vivo* and *in vitro* procedures for measurement of changes in various indicators of immunotoxic effects [Kuby, 1997; Hayes, 1994].

TABLE-2: Assays commonly employed to assess immune function in experimental animals and humans.

Tier 1	
Parameter	**Testing**
Hematology	▪ Leukocyte counts and cell differential count
Weights	▪ Body, spleen, thymus, kidney, liver
Histology	▪ Spleen, thymus, lymph node cellularity
Humeral immunity	▪ Plaque forming cells (PFC) to T-cell independent antigens
Cell-mediated immunity	▪ Lymphocyte proliferation

CHAPTER 3
MATERIALS AND METHODS

A. ANIMALS.

The young adult male Wistar rats, 4-5 weeks old, weighing 100-120 g and mature male Wistar rats, 8-10 weeks old, weighing 140 -180 g were used in these experiments. All rats were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom, Thailand. The animals were kept in the hanging stainless steel cages, one animal/cage and housed in the controlled condition at room temperature of 24 ± 1^{o}C, relative humidity of 60 ± 2% with an automatic light cycle period of 12 hours light (06.00 am. to 06.00 pm.) and 12 hours dark (06.00 pm. to 06.00 am). They were fed with rodent chow diet (C.P. mice Food, Pokphand Animal Feed Co. Ltd., Bangkok) and tap water *ad libitum*. The animals were allowed to acclimatize for 1 week in this condition prior to start the experiment. They were fasted for 18-20 hours but allowed free access to water before sacrificed.

B. GENERAL EXPERIMENTAL PROTOCOL

1. PREPARATION OF PIPERINE SUSPENSION.

Piperine (suspended in corn oil) at the doses of 5, 10, 25, 50, 100, and 250 mg/kg body weight were freshly prepared before giving to the animals via gastric tube. The final volume of piperine suspension for each dose administering the animal was adjusted to 10 ml/kg body weight.

2. EXPERIMENTAL PROTOCOL.

THE TOXIC EFFECTS OF PIPERINE ON THE MALE WISTAR RATS.

The objective of this experiment was to investigate the toxic effect of piperine on the target organs after administration various doses of piperine at various time intervals.

EXPERIMENT I: ACUTE ORAL TOXICITY.

A. Acute oral repeated dose toxicity test in young adult rat: 7 days.

The groups of 32 young adult male Wistar rats, 4-5 weeks old, were randomly divided into 4 groups, 8 animals for each group.

Group1: The animals were received corn oil in the volume of 10 ml/kg body weight orally once daily for 7 days.

Group2-4: The animals were received piperine orally at the doses of 10,100,and 250 mg/kg body weight respectively, once daily for 7 days

B. Acute oral repeated dose toxicity test in young adult rat: 14 days.

The groups of 32 young adult male Wistar rats, 4-5 weeks old, were randomly divided into 4 groups, 8 animals for each group.

Group1: The animals were received corn oil in the volume of 10 ml/kg body weight orally once daily for 14 days.

Group2-4: The animals were received piperine orally at the doses of 10,100, and 250 mg/kg body weight respectively, once daily for 14 days.

EXPERIMENT II: SHORT TERM ORAL REPEATED DOSE TOXICITY TEST: 30 DAYS.

A. The young adult male Wistar rats.

The groups of 32 young adult male Wistar rats, 4-5 weeks old, were randomly divided into 4 groups, 8 animals for each group.

Group1: The animals were received corn oil in the volume of 10 ml/kg body weight orally once daily for 30 days.

Group2-4: The animals were received piperine orally at the doses of 10,100, and 250 mg/kg body weight respectively, once daily for 30 days.

B. The mature male Wistar rats.

The groups of 32 mature male Wistar rats, 9 weeks old, were randomly divided into 4 groups, 8 animals for each group.

Group1: The animals were received corn oil in the volume of 10 ml/kg body weight orally once daily for 30 days.

Group2-4: The animals were received piperine orally at the doses of 5, 25, and 50 mg/kg body weight respectively, once daily for 30 days.

EXPERIMENT III: SUBCHRONIC ORAL TOXICITY TEST: 90 DAYS.

The groups of 32 young adult male Wistar rats, 4-5 weeks old, were randomly divided into 4 groups, 8 animals for each group.

Group1: The animals were received corn oil in the volume of 10 ml/kg body weight orally once daily for 90 days.

Group2-4: The animals were received piperine orally at the doses of 10,100, and 250 mg/kg body weight respectively, once daily for 90 days.

Through out the experiment, clinical sign of rats were observed while body weight, food consumption and mortality were recorded daily. Animals were fasted overnight (18 – 20 hours) and injected intraperitoneally with BrdU solution at the dose of 100 mg/kg body weight prior to sacrificed for two hours for studying lymphoid cell proliferation. Combination of ketamine at the dose of 0.2 mg/kg body weight and xylasine at the dose 0.5 mg/kg body weight were used as anesthetic drug. Blood samples were collected from the aorta at abdominal part and divided into two parts, one was clotted blood and another was anti-coagulated blood. Anti-coagulated blood was used for examination of hematological parameters including hemoglobin (Hb-test) and hematocrit (Hct-test) values, total red blood cell number (RBC's count), total white blood cell number (WBC's count), and leukocyte differentiation. Clotted blood was centrifuged and collected serum that it was used for immunoelectrophoresis. The lymphoid organs including thymus, spleen, and Payer's patch were removed and weighed, fixed in 10% neutral buffered formalin for studying histopathological changes and cell proliferation. The other major organs (heart, lung, liver, kidneys, adrenal glands, urinary bladder, and testis) were removed, weighed and fixed in 10% neutral buffered formalin for the histopathological examination.

C EXPERIMENTAL METHOD

HEMATOLOGICAL STUDY

Blood samples were collected from the aorta at abdominal part and divided into two parts. Approximately, 2 ml of blood samples were transferred into the test tube containing the anticoagulant (EDTA; 1.5 mg/ml of blood [Hosti, 2000] and mixed well. The hematological parameters including hemoglobin (Hb), hematocrit (Hct) values, erythrocyte counts, total leukocyte counts, and leukocyte differentiation were determined within 24 hours by standard clinical hematology laboratory procedure [Maxwell, 1967]. The remaining of blood samples were transferred into the centrifuge tube, 5 ml. and centrifuged at 3000 rpm for 10 minutes. Following centrifugation, plasma was removed with pasture pipette and kept frozen at –80 ^{o}C until subsequent determination. All procedures were given in details in Appendix I.

HISTOPATHOLOGICAL STUDY.

After necropsy, the major organs included thymus, spleen, Peyer's patch, heart, lung, liver, kidneys, adrenal glands, urinary bladder, and testis were removed. They were weighed and fixed in 10% neutral buffered formalin for 24 - 48 hours. Tissue samples were processed according to standard method of tissue preparation for histopathological study. The procedure of this method was shown in Appendix IV. Tissues were stained with hematoxylin and eosin and examined under the light microscopy for pathological changes.

STUDY OF IMMUNE RESPONSE

1) CELL IMMUNE RESPONSE BY BrdU INCORPORATION.

5-bromo-2 – deoxyuridine (BrdU), a halogenated nucleotide analogue of thymidine was used to incorporate into DNA during s-phase of the cell cycle to study cell proliferation [Hammers, *et al*., 2002]. BrdU is widely used for the advantage to H^3-thymidine incorporation in term of no risk of radioactive exposure and also studying the kinetics of cell proliferation. Furthermore, BrdU revealed equivalent specificity and sensitivity as the auto-radiographic detection. The incorporation of BrdU can be determined by immunocytochemistry or immunohistochemistry. In the present study, lymphoid tissues including thymus,

spleen, and Payer's patch from experimental animals were determined after antigen stimulation with rabbit serum.

1.1) BrdU preparation

Freshly prepared BrdU at the dose of 100 mg/kg body weight by dissolving in 0.9% normal saline solution (250 mg/ml) at 56^0C was injected intraperitoneally into each animals at least 2 hours prior to sacrifice.

1.2) Immunohistochemistry

BrdU incorporated cells were detected by specific antibody (mouse monoclonal antibody against BrdU) and determined as describe in Appendix V (Harms, *et al*., 1986). In brief, the tissue sections on PLL-coated slides were treated with 250 ml. of 10 μM., 0.01 M. sodium citrate buffer, pH 6.0 as antigen retrieval under a microwave exposure for 3 minutes at medium power, followed by cooling at room temperature for 30 minutes [Shi, Cote, and Taylor, 1997; Matsuura and Suzuki, 1997]. Thereafter, they were immersed in 2 N HCL for 25 minutes to unfold DNA and washed several changes with distilled water. Before immuno staining endogenous peroxidase activity of tissue was eliminated with 3% H_2O_2 for15 minutes, several changes of distilled water and PBS, pH 7.4, three times for 5 minutes. Non-specific background was blocked with 3% bovine serum albumin for 30 minutes. All steps were performed in a humidity chamber. Primary antibody of mouse anti-BrdU (Sigma, USA) prepared as 1:75 dilution was applied on the sections and incubated for 1 hr at room temperature and 4^oC overnight (RT). After washing , biotinylated rabbit anti mouse IgG (DAKO, CA) was diluted at 1:400 dilution, added to the sections and were incubated for 30 minutes. Then 3, 3'-diaminobenzidine (DAB) was applied and incubated for 5 minutes before stop with distilled water. The reaction was induced by using horseradish peroxidase conjugated avidin for 45 minutes and visualized after incubation with substrate 3, 3'-diaminobenzidine (DAB). The sections were counterstained with hematoxylin and dehydrated with serially graded ethanol, and xylene (according to Appendix IV). The positive BrdU or incorporated cells were then evaluated in comparison with control tissue sections. The results were determined by random samplings and counting them under 20 high power field of a microscope (40x objective lens).

2. IMMUNOELECTROPHORESIS (IEP)

Immunoelectrophoresis (IEP) is a qualitative method, which used to detect the presence or absence of antibody in serum [Jacobs, 1996]. IEP consists of two steps, the electrophoretic separation of antigens and immuno diffusion. The antigens placed in a well were separated according to their net charges. Then, specific antibodies were put into a parallel trough and let them diffused until precipitation bands formed as seen in the figure3.

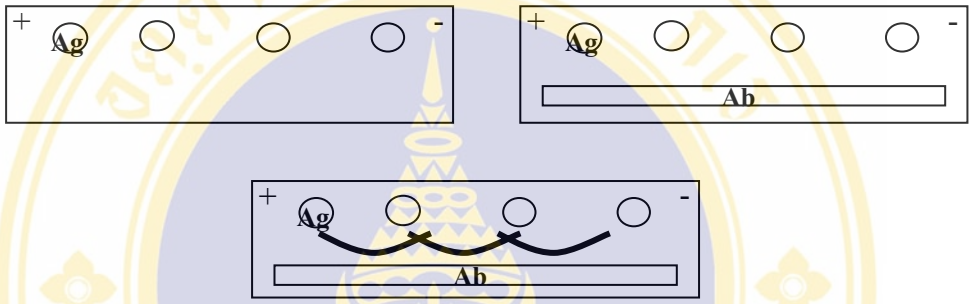

Figure-3: Diagram shows precipitation lines by immunoelectrophoresis.

D. STATISTICAL METHODS

Results represent by mean $\pm$ standard error of means ($X \pm$ SEM). The data obtained from experimental groups and control group were analyzed by one-way analysis of variance (ANOVA). If the data showed applicable and significant value it was further analyzed by Scheffe's test used to determine the statistical significance. A *p*-value less than 0.05 was considered significant.

CHAPTER 4
RESULTS

EXPERIMENT I: ACUTE ORAL TOXICITY.

A. Acute oral repeated dose toxicity test in young adult rats: 7 days.

1.1) Clinical signs.

No apparent clinical signs were detected in rats receiving piperine at the dose of 10 mg/kg body weight (BW) while rats received piperine at the dose of 100 mg/kg body weight (BW) showed hyperactivities such as running and jumping around the cage and at the dose of 250 mg/kg body weight (BW) showed depression, drowsy and sleepiness. Those signs developed within 10 minutes after piperine administration and most of them recovered within 24 hours after piperine treatment. No piperine treated animals died during the experiment.

1.2) Body weight and food consumption.

The growth curves of the rats fed with piperine at various doses for 7 days were shown in figure 4. There were no significant changes in the average body weight among rats receiving corn oil and the piperine at the doses of 10 mg/kg BW. The body weight gain, relative total food consumption and food conversion efficiency (%) were showed in Table-3 and Table-4. The body weight gain of rats fed with piperine at the doses of 100 and 250 mg/kgBW were 24.88 ± 3.39* g and 13.32 ± 3.01* g, respectively. The food consumption of the rats fed with piperine at the doses of 100 and 250 mg/kgBW were 121.17±5.96*g and 101.67 ± 2.38* g, respectively. There were lower statistical significant ($P \leq 0.05$) in both of body weight gain and food consumption when compared to control group. In addition, the summary chart included the percentage of the body weight gain and average food consumption of the piperine administered groups compared with control group was shown in figure 5. It showed that the percentage of body weight gain and food consumption were decreased in all of piperine treated rats but at the doses of 100 and 250 mg/kgBW were reduced 45% and 70% of body weight gain, 10% and 25% of food consumption, respectively. It was lower significantly ($*P \leq 0.05$) when compared to control.

TABLE-3: Changes in body weight of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 7 days.

Doses of piperine (mg/kg BW)	Body weight (g)		
	Day-0	Day-7	Gain
0	139.3 ± 2.46	182.73 ± 2.52	43.43 ± 1.11
10	142.22 ±2.75	183.60 ± 4.1	41.38 ± 2.01
100	147.36 ± 2.53	172.25 ± 3.40	24.88 ± 3.39*
250	153.76 ± 1.77	167.08 ± 4.90	13.32 ± 3.01*

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

TABLE-4: Changes in daily food consumption of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 7 days.

Doses of piperine (mg/kg BW)	Body weight gain (g)	Relative total food consumption (g)	Food conversion efficiency (g%)
0	43.43 ± 1.11	132.83 ± 1.28	35.83 ± 1.82
10	41.38 ± 2.01	129.33 ± 3.58	32.33 ± 2.12
100	34.88 ± 3.39*	121.17 ± 5.96*	29.83 ± 2.83*
250	13.32 ± 3.01*	101.67 ± 2.38*	13.17 ± 3.13*

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

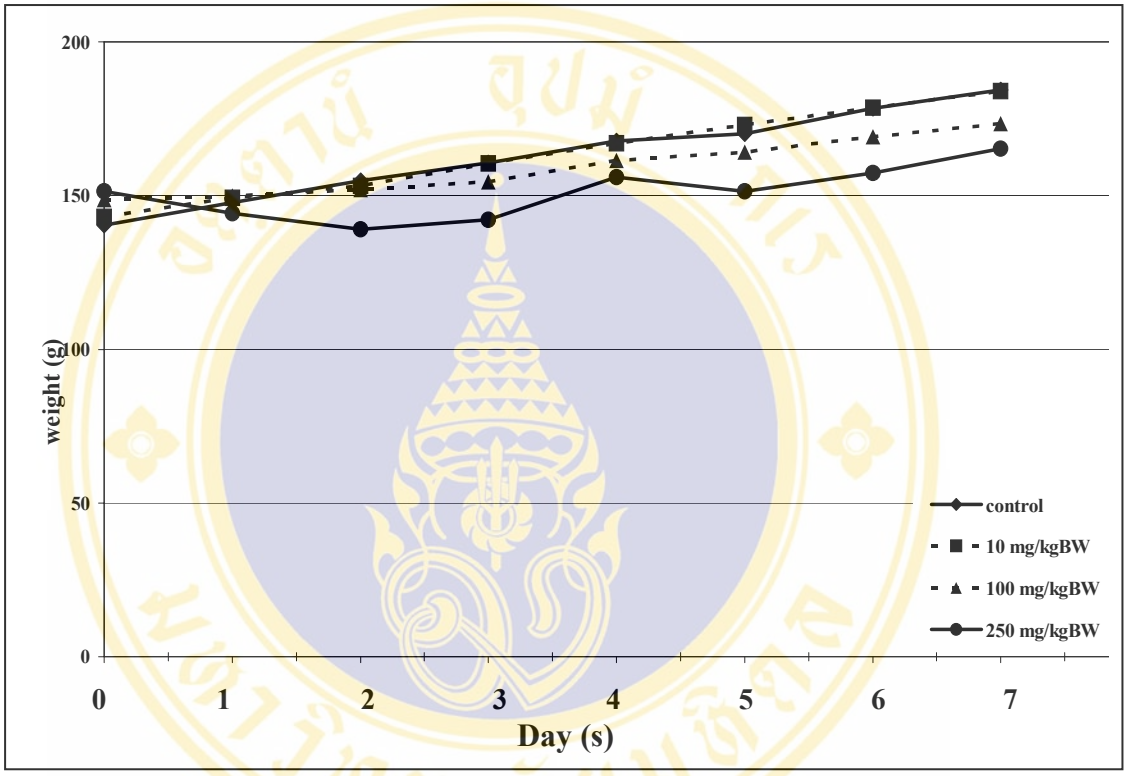

Figure 4: Graph showing growth of young adult rats fed with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 7 days.

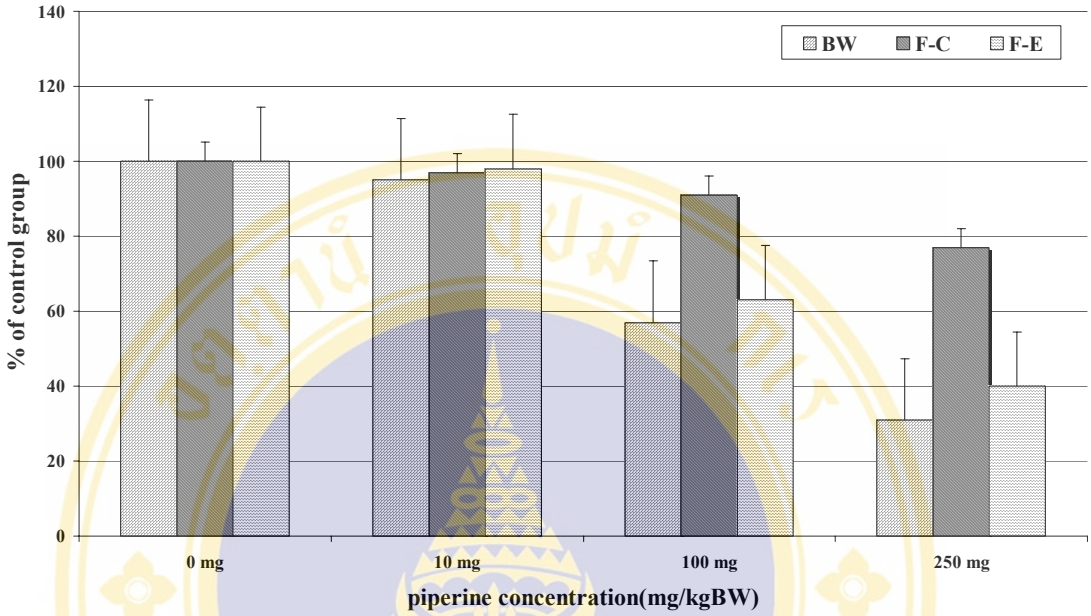

Figure 5: Histogram showing summary of the effects of piperine administration at the doses of 0, 10, 100 and 250 mg/kgBW to Wistar rats for 7 days on body weight gain, food consumption (F-C) and food conversion (F-E). The data presented as percentage of BW, F-C and F-E obtained from the experimental animals.

1.3) Hematological examination.

Data of hematological parameters measured in rats fed piperine at various doses (0, 10, 100 and 250 mg/kg BW) for 7 days were presented in Table-5. Feeding piperine at mentioned doses caused no significant alteration in white blood cells, red blood cells, amount of hemoglobin or levels of hematocrit between the piperine treated groups and the control group. However, there were statistically significant variations ($*P \leq 0.05$) in the decreasing of the percentage of neutrophils and increasing of the percentage of lymphocytes when compared to control group.

Table-5: Hematological parameters of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 7 days.

Parameters	Piperine (mg/kg BW/day)			
	0	10	100	250
Hb (g/dl)	11.9 ± 0.17	11.45 ± 0.50	12.1 ± 0.40	11.3 ± 0.50
Hct (%)	46.5 ± 0.80	47.0 ± 1.60	47.5 ± 1.40	45.8 ± 1.20
RBC (x 10^6/mm^3)	7.6 ± 0.30	7.6 ± 0.40	8.1 ± 0.40	6.7 ± 0.20
WBC (x 10^3/mm^3)	3.4 ± 0.33	3.1 ± 0.37	4.1 ± 0.27	2.82 ± 0.27
Neutrophils (%)	78.8 ± 2.5	76.7 ± 3.5	51.8 ± 3.3*	57.6 ± 4.4*
Eosinophils (%)	0	1.5 ± 0.05	0	0
Basophils (%)	0	0	0	0
Monocytes (%)	4.0 ± 0.03	0.8 ± 0.04	0.7 ±0.04	0
Lymphocytes (%)	18.3 ± 2.5	17.3 ± 3.3	41.3 ± 3.0*	40.3 ± 4.0*
Morphology	N	N	N	N

Hb: Hemoglobin, Hct: Hematocrit, RBC: Erythrocyte counts, WBC: Leukocyte counts, N: normal.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

.

1.4) Relative organ weight.

The major organs including liver, spleen and thymus were removed and weighed. The relative organ weight was calculated by the formula presented in the appendix VI. The results of the organ weights and the relative organ weights were shown in Table-6. No significant difference in organ weights and relative organ weights were found between the piperine treated rats and the control.

TABLE-6: Changes in organ weight of the rat administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 7 days.

Organ (g) (g%)	Piperine (mg/kg BW/day)			
	0	10	100	250
Liver	6.56 ± 0.19 (3.6±0.1)	6.7 ± 0.17 (3.7±0.1)	6.1 ± 0.15 (3.5±0.1)	6.0 ± 0.1 (3.6±0.1)
Spleen	0.60 ±0.01 (0.34±0.01)	0.62 ± 0.02 (0.34±0.01)	0.62 ± 0.01 (0.36±0.01)	0.56 ± 0.03 (0.33±0.01)
Thymus	0.63 ± 0.01 (0.35±0.01)	0.65 ± 0.02 (0.36±0.01)	0.6 ± 0.02 (0.36±0.005)	0.59 ± 0.04 (0.35±0.01)

Note: The relative organ weights were shown in the parentheses.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

1.5) Humeral immune response study

Determination of antibody response after immunized rats with rabbit normal serum was determined in experimental animals by using electrophoresis method. There was no significant difference in antibody response between control group and treated groups. Therefore, piperine has no any stimulatory effect on the antibody induction in rats after fed with piperine for 7 days.

1.6) Pathological findings.

No remarkable changes were observed in gross appearances of target organs including small intestine, thymus, spleen, and liver both in control and piperine treated rats.

A. Small intestine

The histology of small intestine of the rats in control group showed normal structure. The mucosa was thrown into transverse fold covered with villi, the finger like or leaf like structure. The villus had a connective tissue of the lamina propria as a core and cover by simple columnar epithelium which is continuous with the crypt (figure 8). Goblet cells were scattered among the cell lining of the villi. There were few intraepithelial mononuclear cells and few lymphocytes and plasma cells in the lamina propria. The aggregated lymphatic nodules or Payer's patches could be seen in the lamina propria. No remarkable histopathological changes were observed in the small intestine among all of the piperine treated rats when compared with the control.

B. Thymus

The histology of thymus of the rats in control group looked normal both in cell morphology and density and organ architecture. It was lobulated in shape and enclosed by a thin connective tissue capsule. This connective tissue penetrated into the organ as interlobular septa and subdividing it into numerous incomplete lobules. Thymus was divided into two parts, a deeply basophilic outer cortex which was composed of densely packed lymphocytes, and inner eosinophillic medulla which was composed of loosely packed lymphocytes and other cells types. The histology of the thymus of the piperine treated rats looked the same as seen in the control group.

C. Spleen

The histology of spleen in the control rat looked normal. It was enclosed with a thick capsule, which extended into the parenchyma as trabeculae. Its parenchyma was composed of white pulp, a lymphoid aggregation and red pulp, a highly vascular tissue. There were also no changes in the spleen of the rats receiving piperine at various doses.

D. Testis.

The histology of the testis of the control rat showed the intact basement membrane of seminiferous tubules, spermatogenic cells, Leydig's cells, and sertori cells. The seminiferous tubules showed various stages of spermatogenic cells including spermatogonia, primary spermatocyte, secondary spermatocyte, spermatid, and abundant spermatozoa in the lumen. In contrast, the testis of the rats receiving piperine at the dose of 250 mg/kg body weight showed thin layers of spermatogenic cells and decreased in the density of the spermatozoa in the lumen (figure-6). However, there were no changes in the testis of the rats receiving piperine at the doses of 10 and 100mg/kg body weight, respectively.

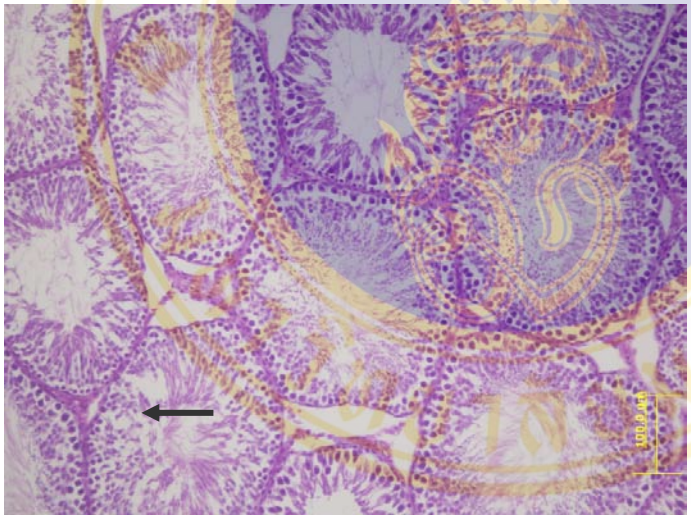

Figure-6: Histopathology of the testis of the rat treated with piperine at the dose of 250 mg/kgBW, 7 days, shows disorganizing of germ cells (arrow). (10X), H&E stain.

E. Adrenal gland

The histology of the adrenal glands of all rats both in the control and all piperine treated groups showed normal appearance in both of the adrenal cortex and medulla zones. No hemorrhage and degenerative necrosis were seen.

F. Stomach

The stomach of the rats both in the control and piperine treated groups were normal both in gross appearance and microscopic finding. The mucosa was intact. No ulceration and erosion were seen. The submucosa was also normal appearance. No hemorrhage and edema were observed.

G. Liver

The liver was normal both in gross appearance and microscopic appearance. It was lobulated and normal architecture including hepatocytes which showed normal distribution and density of them was normal pack. No hemorrhage or necrosis area was found. Liver size was also normal then no remarkable effect on liver of piperine treated rats when compared to control group.

B. Acute oral repeated dose toxicity test in weanling rat: 14 days

1.1) Clinical signs

None of the rats died during the experiment and the clinical signs observed in piperine treated rats at the dose of 100 mg/kgBW, were salivation, hyperactivities while the rats fed with piperine at the dose of 250 mg/kgBW showed dropping, depression, and sleepiness after piperine feeding.

1.2) Body weight and food consumption

The growth curve of rats fed with piperine at various doses graphically shown in figure 7. The results showed that the average body weight of rats fed with piperine at the dose of 250 mg/kgBW were decreased in day-3 of treatment, finally, their weight increased at day-7of treatment the until end of the experiment.

The body weight and the amount of food consumption were shown in Table-7 and Table-8, respectively. The weight gain and food consumption were 51.67 ± 6.14* and 258.50 ± 6.77* in rats receiving piperine at the dose of 100 mg/kg BW, then 36.67 ± 11.48* g and 219.70 ± 8.80* g in rats receiving piperine at the dose of 250 mg/kgBW. Therefore, both of piperine mentioned groups showed decreasing significant difference ($P \leq 0.05$) in body weight gain and food consumption when compared with control group. The percentage of the body weight gain and averaging food consumption compared between piperine treated groups and control group showed in figure 8. It showed that the percentage of body weight gain and food consumption of piperine treated rats at the dose of 250 mg/kgBW was reduced 40% and 15%, respectively. It was significant difference ($*P \leq 0.05$) when compared to the control (100%).

TABLE-7: Changes in body weight of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 14 days.

Doses of piperine (mg/kg BW)	Body weight (g)		
	Day-0	Day-14	Gain
0	155.93 ± 2.46	212.01 ± 3.90	61.50 ± 9.45
10	157.75 ± 1.06	217.82 ± 4.08	65.33 ± 8.21
100	165.78 ± 1.67	212.50 ± 4.06	51.67 ± 6.14
250	173.16 ± 1.22	206.00 ± 4.06	36.67 ± 11.48*

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

TABLE-8: Changes in daily food consumption of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 14 days.

Doses of piperine (mg/kg BW)	Body weight gain (g)	Relative total food consumption (g)	Food conversion efficiency (g%)
0	61.50 ± 9.45	262.67 ± 3.00	23.67± 3.76
10	65.33 ± 8.21	254.14 ± 2.02	25.50 ± 3.13
100	51.67 ± 6.14	258.50 ± 6.77	20.17 ± 2.87
250	36.67 ± 11.48*	219.70 ± 8.80*	17.17 ± 5.29*

Values are means ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

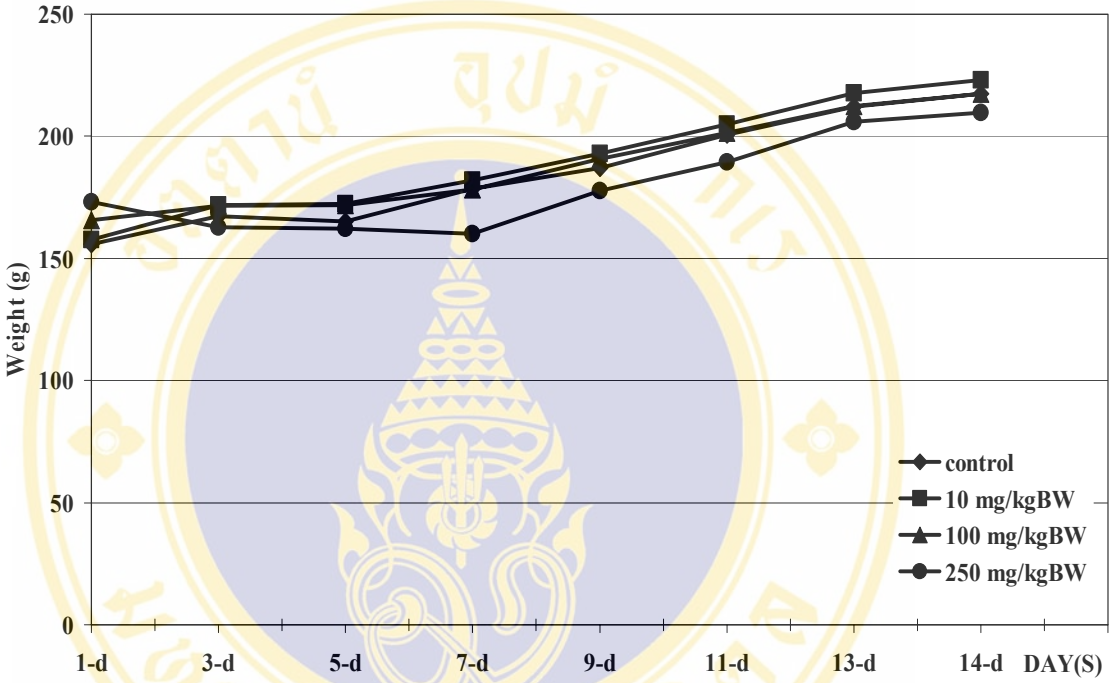

Figure 7: Graph showing growth of young adult rats fed with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 14 days.

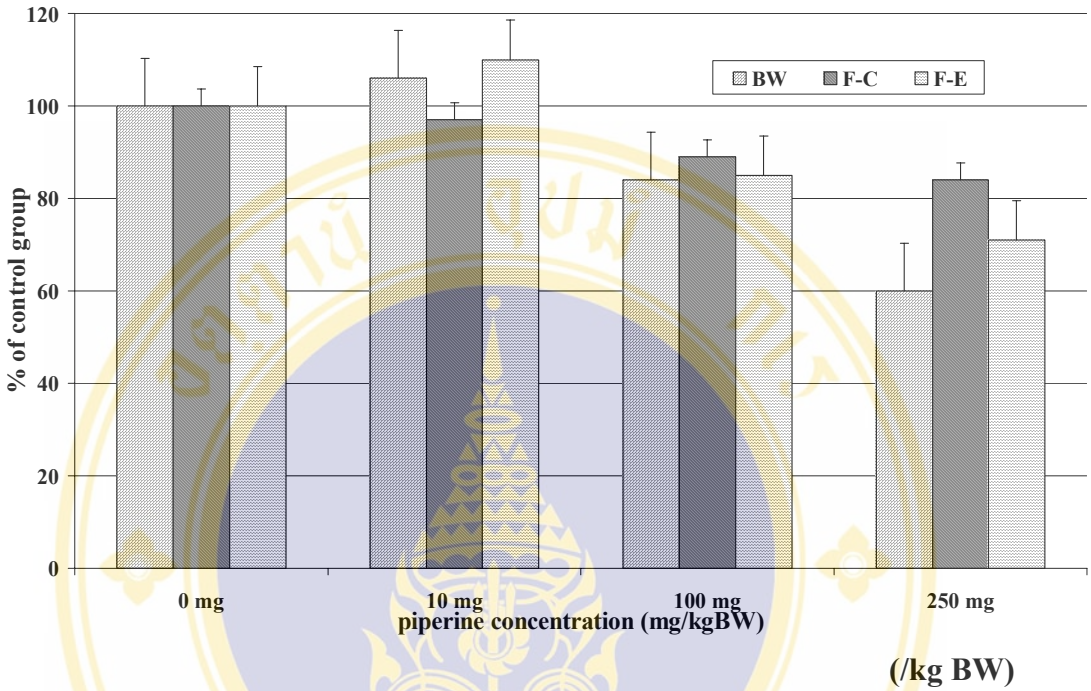

Figure 8: Histogram showing summary of the effects of piperine administration at the doses of 0, 10, 100 and 250 mg/kgBW to Wistar rats for 14 days on body weight gain, food consumption (F-C) and food conversion (F-E). The data presented as percentage of BW, F-C and F-E obtained from the experimental animals.

1.3) Hematological examination.

The hematological values of rats fed with piperine at various doses (0, 10, 100 and 250 mg/kg BW) for 14 days were showed in Table-9. There were no piperine effects on white blood cells, red blood cells, amount of hemoglobin, levels of hematocrit or cell differentiation. Therefore, they had no statistically significant variations ($*P \leq 0.05$) in the rats receiving piperine groups when compared to control group.

Table-9: Hematological parameters of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 14 days.

Parameters	**Piperine (mg/kg BW/day)**			
	0	10	100	250
Hb (g/dl)	14.6 ± 1.1	14.7 ± 0.5	14.7 ± 0.7	14.4 ± 0.8
Hct (%)	46.1 ± 2.3	47.0 ± 1.6	47.5 ± 1.4	47.5 ± 1.7
RBC (x 10^6/mm^3)	6.7 ± 0.4	6.7 ± 0.5	6.7 ± 0.3	6.9 ± 0.2
WBC (x 10^3/mm^3)	3.5 ± 0.3	3.6 ± 0.12	3.2 ± 0.1	3.56 ± 0.14
Neutrophils (%)	60.3 ± 2.40	59.3 ± 4.50	56.0 ± 4.80	63.5 ± 1.9
Eosinophils (%)	3.0 ± 0.08	4.5 ± 0.07	0	0
Basophils (%)	0	0	0	0
Monocytes (%)	1.70 ± 0.03	0.80 ± 0.02	0	0
Lymphocytes (%)	36.8 ± 4.2	34.0 ± 4.3	41.2 ± 4.8	32.3 ± 2.3
Morphology	N	N	N	N

Hb: Hemoglobin, Hct: Hematocrit, RBC: Erythrocyte counts, WBC: Leukocyte counts, N: normal.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

1.4) Relative organ weight.

The result of the organs weight and the relative organ weight were shown in Table-10. The relative organ weight was presented as percentage of body weight for the treated groups, which were nearly equal to the control group. No significant difference ($P \leq 0.05$) was detected. In addition, the relative organ weights were calculated according to the formula in the Appendix VI.

TABLE-10: Changes in organ weight of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 14 days.

Organ (mg) (g%)	**Piperine (mg/kg BW/day)**			
	0	10	100	250
Liver	8.2 ± 0.2 (3.7 ± 0.16)	7.90± 0.5 (3.5 ± 0.16)	5.74 ± 0.4 (3.4 ± 0.11)	7.6 ± 0.3 (3.6 ± 0.11)
Spleen	0.61± 0.03 (0.28± 0.01)	1.61 ± 0.02 (0.27± 0.01)	0.58 ± 0.03 (0.28 ± 0.01)	0.58 ± 0.01 (0.28 ± 0.01)
Thymus	0.61± 0.02 (0.28 ± 0.01)	0.67 ± 0.05 (0.30 ± 0.01)	0.57 ± 0.04 (0.26±0.005)	0.57± 0.04 (0.27± 0.01)

Note: The relative organ weight showed in the parentheses.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

1.5) Humeral immune response study.

The antibody response of experimental animals immunizing with normal rabbit serum were determined by using electrophoresis method. The results showed that piperine had no remarkable effects on antibody response in rats after fed with piperine at various doses for 14 days.

1.6) Pathological examination

Even though piperine treated rats at the dose of 250 mg/kg body weight were shown deformation in both of cell structure and arrangement of cells lining of testis. Microscopic was shown the disorganized and lost of cell to cell lining contact and decreased in cell population at various stages of spermatogenic series; consecutively, the amount of spermatozoa was decreased.

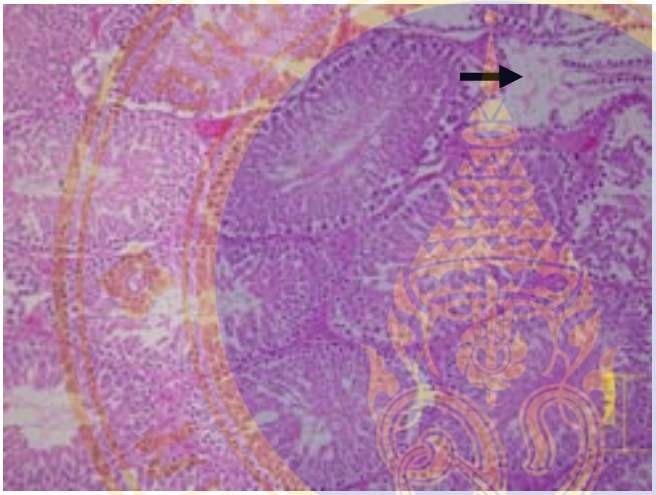

Figure-9: Histopathology of testis of rat fed with piperine at the dose of 250 mg/kgBW for 14 days shows structural deformation and lost of cell to cell contacts (arrow). (10X), H&E stain.

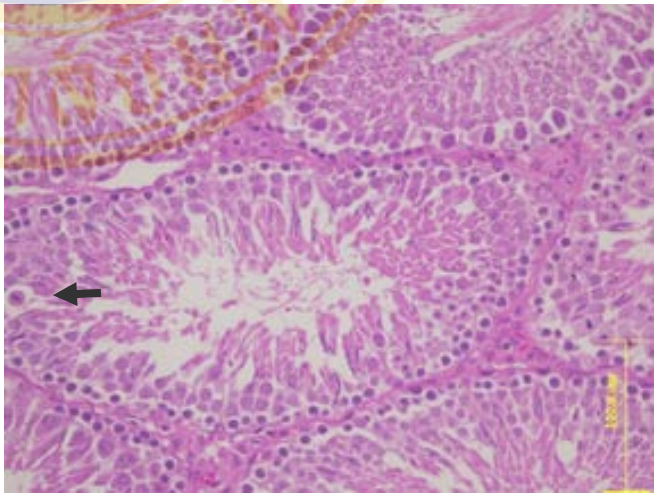

Figure-10: Histopathology of testis of rat treated with piperine at the dose of 250 mg/kgBW for 14 days, shows disorganized spermatogenesis (arrow). (20X), H&E stain.

In addition, piperine at all given doses to the rats had no remarkable effects on histopathological changes of the other organs including liver, thymus, adrenal gland, small intestine and stomach.

EXPERIMENT II: SHORT TERM ORAL REPEATED DOSE TOXICITY TEST FOR 30 DAYS.

A. The young adult male Wistar rat.

1.1) Clinical signs.

The rats received piperine at the dose of 100 were shown hyperactive while those received piperine at the dose of 250 mg/kg BW were shown depression, drowsy, then laid down at the bottom of the cages. This mentioned behavior presented within 5 minutes after piperine feeding.

1.2) Body weight and food consumption

The growth curve was shown in figure 11. There were no significant difference ($P \leq 0.05$) of the average body weight among rats receiving corn oil and the piperine at the dose of 10 mg/kgBW. Whereas, the body weight gain of rats fed with piperine at the doses of 100 and 250 mg/kgBW decreasing at day-5 after piperine administration. Therefore, body weight gain was lower significant difference ($P \leq 0.05$) when compared to control group at the end of the experiment.

Body weight and daily mean consumption of rats were shown in Table-11 and Table-12. The body weight gain of piperine treated rats at the doses of 100 and 250 mg/kg body weight were 133.43 ± 3.54 and 129.17 ± 2.79; respectively, which decreased statistically differences when compared to control group (166.28 ± 2.06). For food consumption, at the dose of 100 mg/kg body weight was 518.83 ± 6.91 and at the dose of 250 mg/kg body weight was 500.33 ± 6.12, then it decreased statistically differences when compared to control (586.67 ± 8.75) as well. In addition, the chart in figure 12 was shown the percentage of the body weight gain and average food consumption of the rats treated with piperine at various doses compared with the control group. It showed percentage of body weight gain, food consumption decreased in all of piperine treated rats but at the doses of 100 and 250 mg/kgBW were reduced 20% and 10% of body weight gain, 25% and 15% of food consumption, respectively. Therefore, it was lower significant difference ($*P \leq 0.05$) when compared to control (100% of food consumption and body weight gain).

TABLE-11: Changes in body weight of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 30 days.

Piperine dosages (mg/kg BW)	Body weight (g)		
	Initiation	Final	Gain
0	139.30 ± 2.41	305.58 ± 8.44	166.28 ± 2.06
10	142.21± 2.75	299.45 ± 9.26	141.83 ± 3.29
100	148.43 ± 2.14	281.86 ± 9.63	133.43 ± 3.54*
250	159.13 ± 3.37	288.32 ± 4.89	129.17 ± 2.79*

Values are mean ± SEM of 6 rats/group,

* $P \leq 0.05$ showed significant difference from control group.

TABLE-12: Changes in daily food consumption of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 30 days.

Dose of piperine (mg/kg BW/day)	Body weight gain (g)	Relative total food consumption (g)	Food conversion efficiency (g%)
0	166.28 ± 2.06	586.67± 8.75	28.27 ± 0.95
10	141.83 ± 3.29	541.17± 8.72	26.06 ± 3.75
100	133.43 ± 3.54*	518.83± 6.91*	25.67 ± 1.50
250	129.17 ± 2.79*	500.33± 6.12*	23.20 ± 0.70

Values are mean ± SEM of 6 rats/group,

* $P \leq 0.05$ showed significant difference from control group.

.

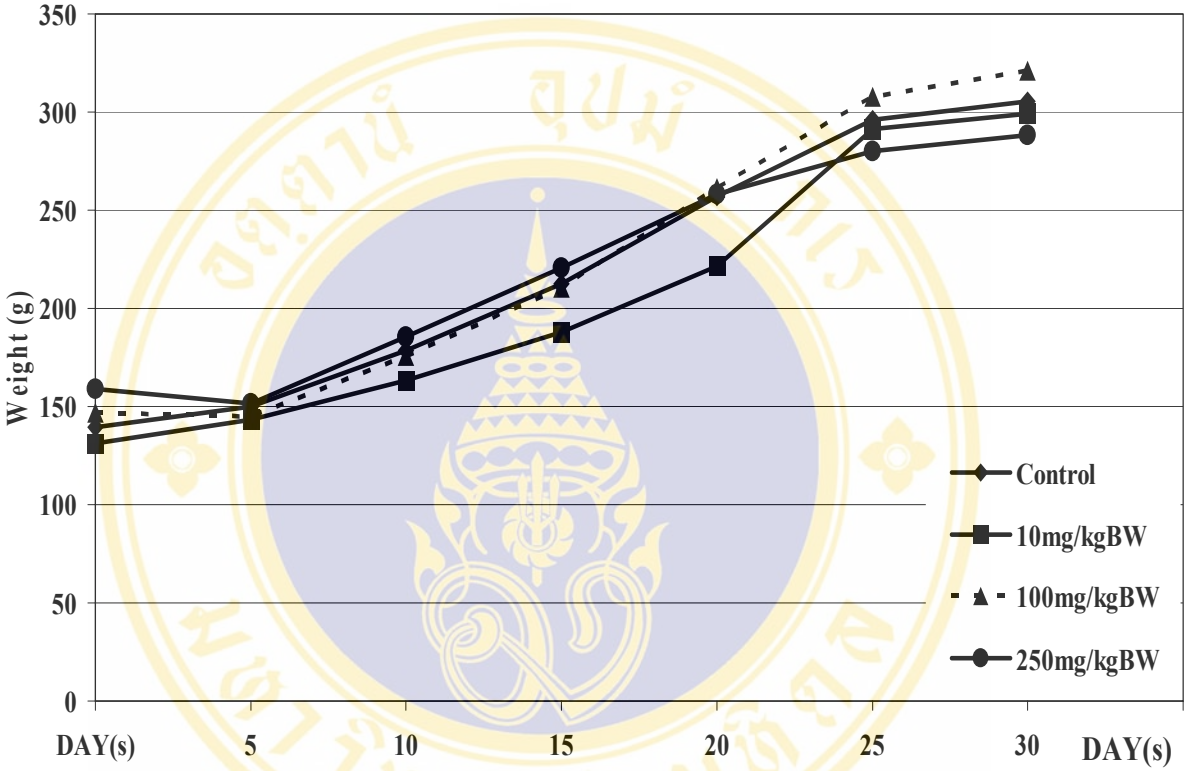

Figure 11: Graph showing growth curves of young adult rats fed with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 30 days.

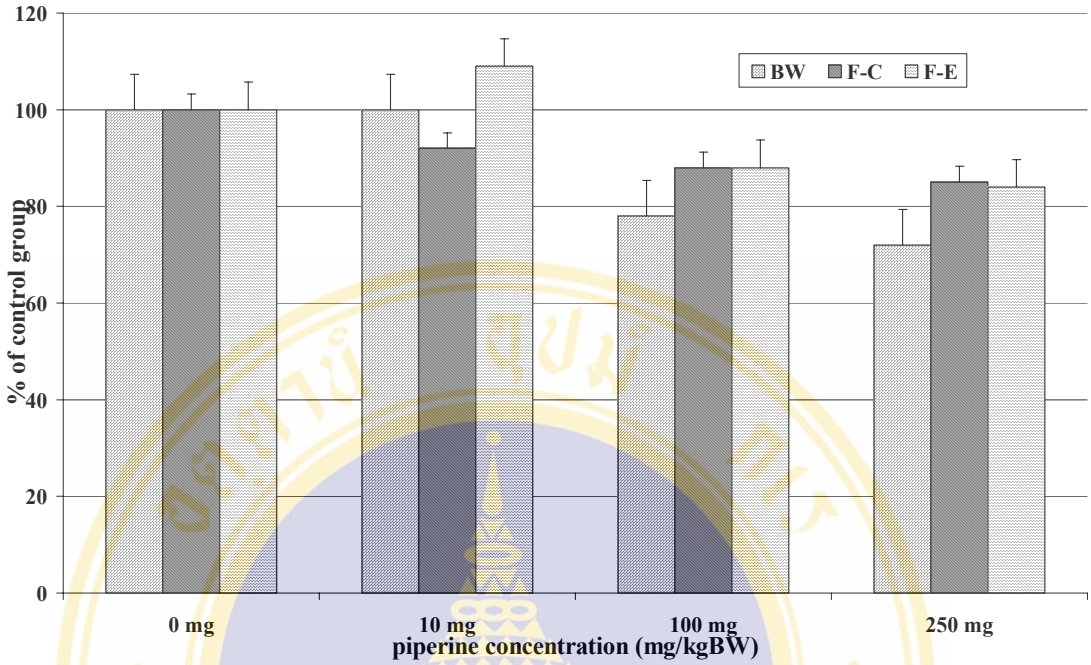

Figure 12: Histogram showing summary of the effects of piperine administration at the doses of 0, 10, 100 and 250 mg/kgBW to Wistar rats for 30 days on body weight gain, food consumption (F-C) and food conversion (F-E). The data presented as percentage of BW, F-C and F-E obtained from the experimental animals.

1.3) Hematological examination.

The hematological values of rats fed with piperine at the doses of 0, 10, 100 and 250 mg/kgBW were shown in Table-13. By variance analysis, they had no significant difference ($P \leq 0.05$) in hematological parameters including hemoglobin, hematocrit, erythrocyte counts, leukocyte counts, and differential leukocyte counts (neutrophils, eosinophils, basophils, monocytes, and lymphocytes). Therefore, piperine with given doses had no effects on hematological parameters.

Table-13: Hematological parameters of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 30 days.

Parameters	Piperine (mg/kg BW/day)			
	0	10	100	250
Hb (g/dl)	14.6 ± 2.3	14.7± 0.5	14.7 ± 0.7	14.4 ± 0.8
Hct (%)	46.1 ± 1.3	46.0 ± 1.5	47.5 ± 1.4	46.7 ± 2.1
RBC (x 10^6/mm^3)	6.9 ± 0.5	6.7 ± 1.2	6.5 ± 0.3	6.9 ± 0.2
WBC (x 10^3/mm^3)	3.3 ± 0.8	3.1± 0.37	3.1 ± 0.21	53/5 ± 0.27
Neutrophils (%)	66.0 ± 4.40	55.0 ± 3.2	71.8 ± 2.60	7931 ± 2.2
Eosinophils (%)	0	0	0	0
Basophils (%)	1.0 ± 0.03	0.8 ± 0.04	0.5 ± 0.04	0
Monocytes (%)	1.70 ± 0.03	0.8 ± 0.02	0	0
Lymphocytes (%)	30.7 ± 4.4	43.2 ± 3.4	27.7 ± 2.8	27.6 ± 2.4
Morphology	N	N	N	N

Hb: Hemoglobin, Hct: Hematocrit, RBC: Erythrocyte counts, WBC: Leukocyte counts, N: normal.

Values are mean $\pm$ SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

1.4) Relative organ weight.

The relative organ weight was calculated by using the formula as presented in the appendix VI. The result of the organ weights and the relative organ weights showed in Table-14. There were no significant differences on the relative organ weights in rats administered with piperine at the doses of 10, 100 and 250 mg/kg BW/days for 30 days when compared to the control.

TABLE-14: Changes in organ weight of the rat administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 30 days.

Organ (mg) **(g%)**	**Piperine (mg/kg BW/day)**			
	0	10	100	250
Liver	10.6± 0.3 (3.47 ± 0.1)	10.7± 0.2 (3.43± 0.2)	10.8 ± 0.2 (3.43 ± 0.1)	10.1 ± 0.3 (3.5 ± 0.16)
Spleen	0.78± 0.02 (0.25 ± 0.01)	0.75 ± 0.02 (0.25 ± 0.01)	0.79 ± 0.03 (0.24 ± 0.01)	0.73 ± 0.04 (0.24 ± 0.01)
Thymus	0.54± 0.02 (0.17 ± 0.01)	0.57 ± 0.02 (0.18 ± 0.01)	0.57 ± 0.04 (0.17±0.005)	0.53± 0.03 (0.18 ± 0.01)

Note: The relative organ weights were shown in the parentheses.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

1.5) Humeral immune response study.

After immunization, the antibody response of rats fed with piperine at various doses (10, 100, and 250 mg/kg body weight) was determined by using electrophoresis method. It showed no different antibody response pattern between piperine treated groups and control group. Therefore, piperine had no effect on the antibody production of rats fed with piperine in this experiment.

1.6) Histopathological examination

In this experiment, the major organs were examined including thymus, spleen, liver, adrenal gland, small intestine, stomach, and testis. No histological changes were detected in target organs except the seminiferous tubule and stomach, which were described below.

A. Testis.

The results showed abnormal in both of organ architecture and cell morphology. There were disorganized of cell lining, lost in cell contacts, inconsequently, decreased the amounts of spermatozoa. All histopathological changes were found in five of six rats treated with piperine at the dose of 250 mg/kgBW.

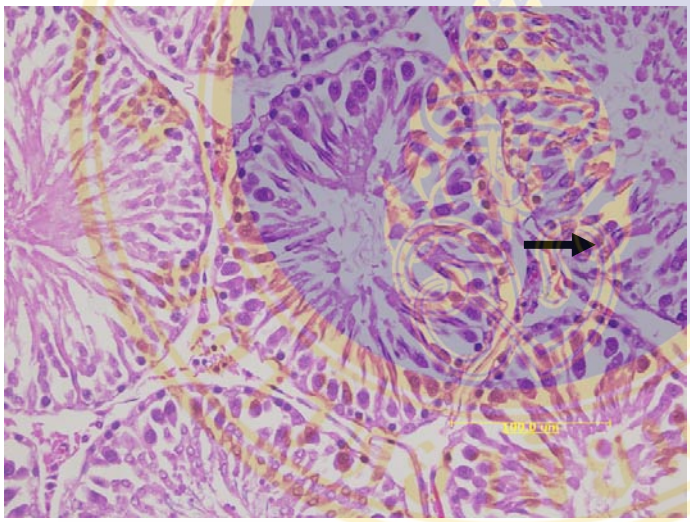

Figure-13: Histopathology of testis of the rat fed with piperine at the dose of 250 mg/kgBW for 30 days shows lost of cell to cell contacts and disorganizing of spermatogenesis (arrow). (10X), H&E stain.

F. Stomach

Even though one of six rats fed with piperine at the dose of 250 mg/kgBW was found congestion in the glandular part as presented below. However, no remarkable changes were observed.

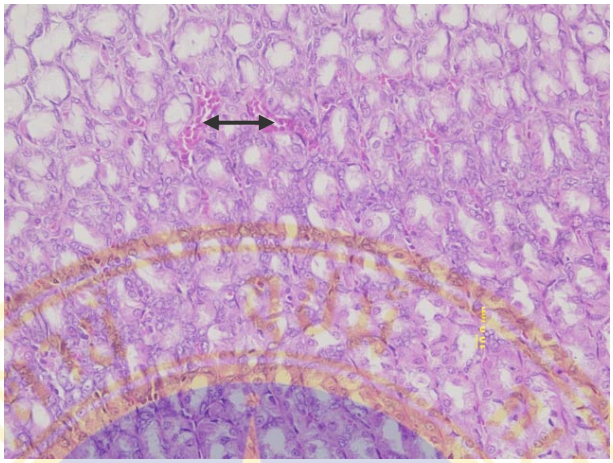

Figure 14: This micrograph shows congestion (arrow) of the stomach of rat treated with piperine (250 mg/kgBW), 30 days. (4X), H&E stain.

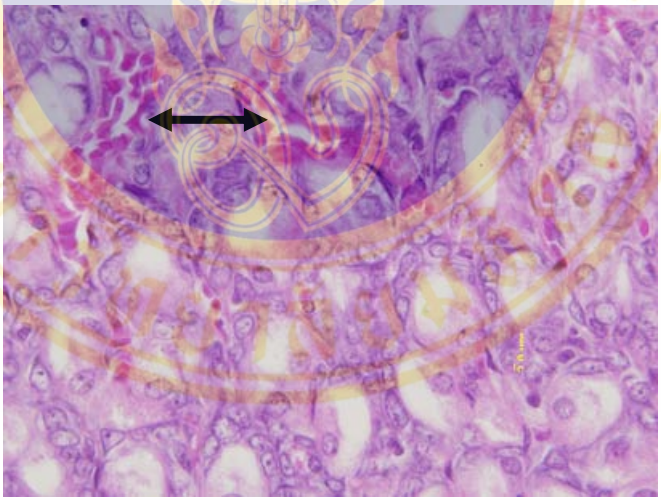

Figure 15: The micrograph shows congestion (arrow) of the stomach of piperine treated rat (250 mg/kgBW) for 30 day, at a higher magnification. (20X), H&E stain.

1.7) Cell proliferation assay.

The results of BrdU incorporation (positive cells) were shown in Table-15. Slight difference of BrdU positive cells was noticed but there were no statistical significance of BrdU positive cells among rats administered variably with piperine compared to control animals.

Table-15: The BrdU incorporated cells of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 30 days.

Piperine (mg/kg BW)	Thymus		C/Tx		Spleen		C/Tx		Payer's patches		C/Tx	
	Before	After	B	A	Before	After	B	A	Before	After	B	A
0	71 ± 1.7	78±2.4	1	1	28±1.5	35±1.7	1	1	58 ± 2.1	64±1.9	1	1
10	57 ± 2.5	62±2.8	0.8	0.8	32± 1.9	30±2.3	1.1	0.9	59 ± 1.8	61±2.2	1.0	0.9
100	69 ± 1.9	80±4.4	1.0	1.0	30± 1.7	33±2.4	1.1	0.9	64 ± 2.4	62±1.9	1.1	1.0
250	56 ± 2.7	67±4.4	0.8	0.8	38± 2.1	40±2.4	1.3	1.2	79± 2.6	89±4.9	1.4	1.4

C/Tx; control/piperine administration ratio, B; before immunization, A; after immunization.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

B. The mature male Wistar rat.

. 1.1) Clinical signs.

None of clinical signs such as sensitive to noise, startle, depression, or weakness was detected in the rats received piperine at the doses of 5, 25 and 50 mg/kg body weight. In addition, no piperine treated animals died during the experiment.

1.2) Body weight and food consumption.

The growth curves of rats fed with piperine at the doses of 5, 25, and 50 mg/kgBW were shown in figure 16. There was no remarkable changes in the weight gain between piperine treated groups and control of treatment. The body weight, body weight gain, relative total food consumption and food conversion efficiency (%) were summarized in Table-16 and Table-17. The body weight gain was 168.17 ± 4.73 in control group and 150.83 ± 12.59, 165.16 ± 4.08 and 162.00 ± 1.93 in piperine at the doses of 5, 25 and 50 mg/kgBW, respectively.

The average total food consumption in the control group was 473.17 ± 16.59 in the control group and 485.00 ± 32.66, 500.0 ± 21.72, and 538.33 ± 15.63 in piperine at the doses of 5, 25 and 50 mg/kgBW, respectively. In addition, the chart of all parameters as mentioned in the figure 17 included the percentage of treated groups compared with control calculating by the formula as presented in the appendix VI. It showed percentage of body weight gain, food consumption of piperine treated rats showed different values in the percentage of body weight gain, food consumption and food efficiency but they had no significant difference ($*P \geq 0.05$) when compared with control group.

TABLE -16: Changes in body weight of the adult rats administered with piperine at the doses of 0, 5, 25 and 50 mg/kgBW for 30 days.

Doses of piperine (mg/kg BW)	Body weight (g)		
	Day-0	Day-30t	Body weight gain
0	237.83 ± 1.16	406.00 ± 5.43	168.17 ± 4.73
5	242.33 ± 1.35	393.16 ± 2.82	150.83 ± 12.59
25	238.33 ± 1.70	403.50 ± 3.98	165.16 ± 4.08
50	240.66 ± 1.33	402.66 ± 2.70	162.00 ± 1.93

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

TABLE-17: Changes in food consumption of the adult rats administered with piperine at the doses of 0, 5, 25 and 50 mg/kgBW for 30 days.

Doses of piperine (mg/kg BW)	Body weight gain (g)	Relative total food consumption (g)	Food conversion efficiency (%)
0	168.17 ± 4.73	473.17 ± 16.96	35.83 ± 0.95
5	150.83 ± 12.59	485.00 ± 32.66	31.83 ± 3.75
25	165.16 ± 4.08	500.00 ± 21.72	33.33 ± 1.15
50	162.00 ± 1.93	538.33 ± 15.63	29.83 ± 0.70

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

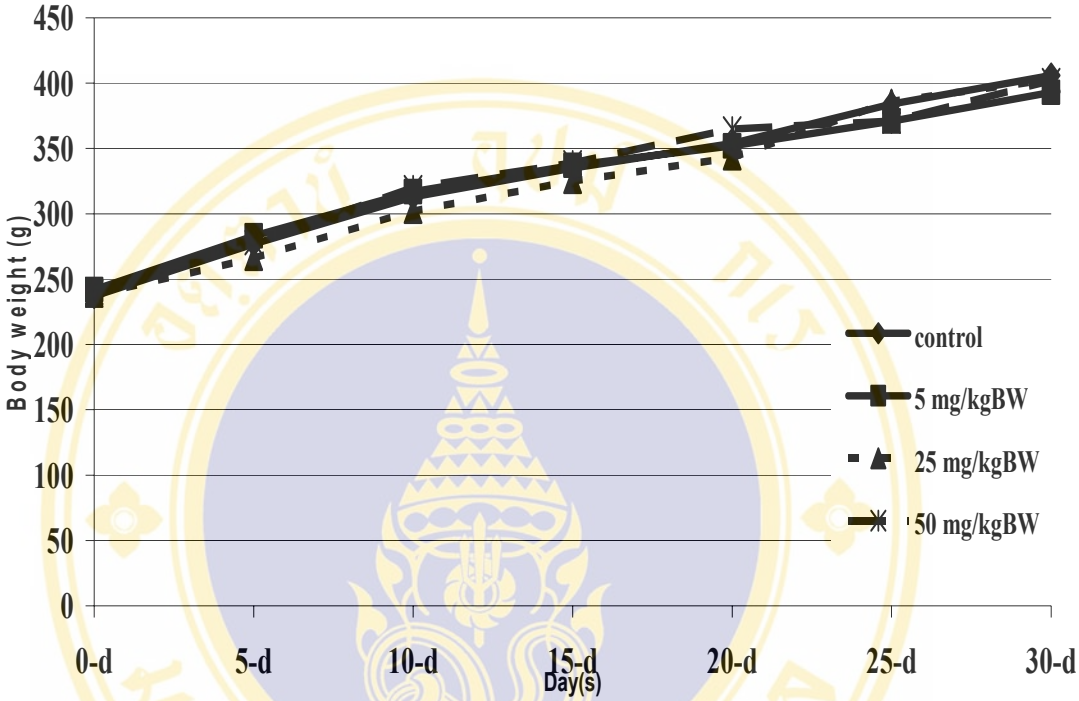

Figure 16: Graph showing growth of adult rats fed with piperine at the doses of 0, 5, 25 and 50 mg/kgBW for 30 days.

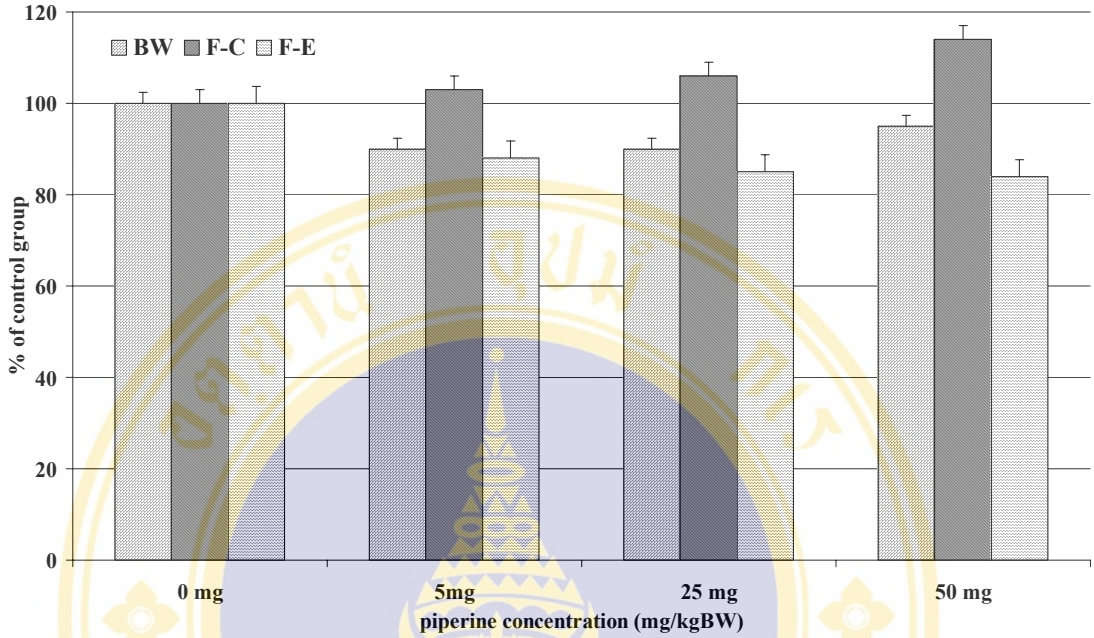

Figure 17: Histogram showing summary of the effects of piperine administration at the doses of 0, 5, 25 and 50 mg/kgBW to Wistar rats for 30 days on body weight gain, food consumption (F-C) and food conversion (F-E). The data presented as percentage of BW, F-C and F-E obtained from the experimental animals.

1.3) Hematological examination.

The results of hematological values of adult rats receiving piperine at the doses of 0, 5, 25 and 50 mg/kgBW were shown in Table 4. Feeding piperine at mentioned doses caused no significant alteration ($P \leq 0.05$) in white blood cells count, red blood cells count, amount of hemoglobin, levels of hematocrit or leukocyte differentiation (neutrophils, eosinophils, basophils, monocytes and lymphocytes) between the piperine treated groups and the control of treatment.

Table-18: Hematological parameters of the adult rats administered with piperine at the doses of 0, 5, 25 and 50 mg/kgBW for 30 days.

Parameters	Piperine (mg/kg BW/day)			
	0	5	25	50
Hb (g/dl)	44.3 ±0.42	45.20 ± 0.60	46.5± 0.42	46.3 ± 0.49
Hct (%)	14.6 ± 0.30	15.2 ± 3.31	16.1 ± 0.37	15.8 ± 0.19
RBC (x 10^6/mm^3)	7.0 ± 0.31	6.9 ± 0.21	7.3 ± 0.21	7.15± 0.20
WBC (x 10^3/mm^3)	3.0 ± 0.24	3.0 ± 0.37	3.1 ± 0.14	2.92 ± 0.15
Neutrophils (%)	28.3 ± 6.21	24.5 ± 4.8	35.8 ± 9.26	24.17 ± 3.3
Eosinophils (%)	0	0	0	0
Basophils (%)	0	0	0	0
Monocytes (%)	1.5 ± 0.05	1.2 ± 0.03	1.1 ±0.04	1.80 ± 0.03
Lymphocytes (%)	67.2 ± 12.8	73.83 ± 4.9	62.6 ± 9.6	73.2 ± 3.6
Morphology	N	N	N	N

Hb: Hemoglobin, Hct: Hematocrit, RBC: Erythrocyte counts, WBC: Leukocyte counts, N: normal.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

1.4) Relative organ weight.

The result of the organs weight and the organs relative weight were shown in Table 4-C. The organ weight was presented as percentage of body weight for the treated groups, which were nearly equal to the control of treatment. They had no significance difference was detected; in addition, the relative organ weights were calculated according to the formula in the Appendix VI

TABLE-19: Changes in organ weight of the adult rats administered with piperine at the doses of 0, 5, 25 and 50 mg/kgBW for 30 days.

Organ (g)	Piperine (mg/kg BW/day)			
	0	5	25	50
Liver	12.46± 0.69 (3.02 ± 0.12)	12.9± 0.63 (3.33± 0.1)	11.43± 0.6 (3.20 ± 0.16)	12.1 ± 0.36 (2.8 ± 0.18)
Spleen	0.94± 0.01 (0.23± 0.01)	0.85 ± 0.06 (0.23 ± 0.03)	0.91 ± 0.07 (0.23 ± 0.02)	0.86 ± 0.07 (0.23 ± 0.01)
Thymus	0.75± 0.05 (0.12± 0.01)	0.7 ± 0.01 (0.2 ± 0.01)	0.69 ± 0.05 (0.17 ± 0.02)	0.59± 0.04 (0.17± 0.18)

Note: The relative organs weight showed in the parentheses.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

1.5) Humeral immune response assay.

Determination of antibody response after immunized with heterogenous proteins from rabbit normal serum was determined in experimental animals by using electrophoresis method. There was no significant difference of antibody response between control group and treated groups. Therefore, piperine had no remarkable effect on antibody response between adult rats administered piperine at the doses of 5, 25 and 50 mg/kg BW for 30 days.

1.6) Histopathological examination

The results of histological changes were shown normal morphology in all target organs except in the seminiferous tubule. At the dose of 50 mg/kgBW showed a few disorganizing of germ cells but none of tubular destruction was found.

The small intestine demonstrated normal gross appearances. Microscopically, the small intestine composed of three distinct layers including mucosae, muscularis and serosa layers. The mucosa was thrown into transverse fold covered with the villi, finger like projection. Lamina propria acted as a core of villi and they were lined with simple columnar epithelium. In addition, it also had goblets cells scattered among the cell lining and villi and few lymphocytes were observed. There was aggregation of lymphocyte in lamina propria, which was called Payer's Patches (figure-18). The muscularis possessing an inner circular and an outer longitudinal layer of smooth muscle arranged in coarse bundles. The outer layer is serosa. Therefore, piperine had no remarkable effect on the histological changes of small intestine.

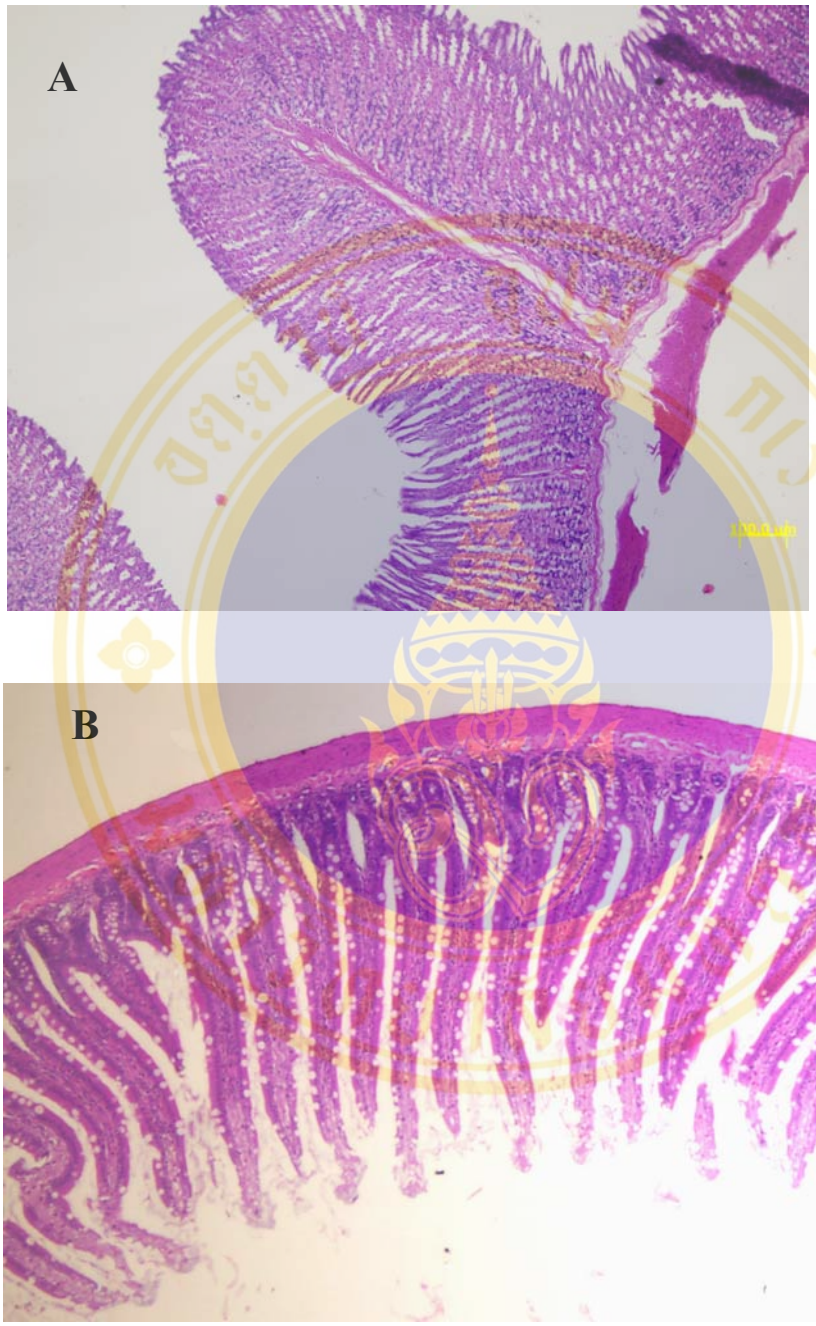

Figure 18: Histopathology of the small intestine derived from the control rat (A) and rat fed with piperine at the dose of 50 mg/kgBW for 30 days (B) shows three layers; mucosa, muscularis and serosa. The mucosal folds show villous structure. (4X), H&E stain.

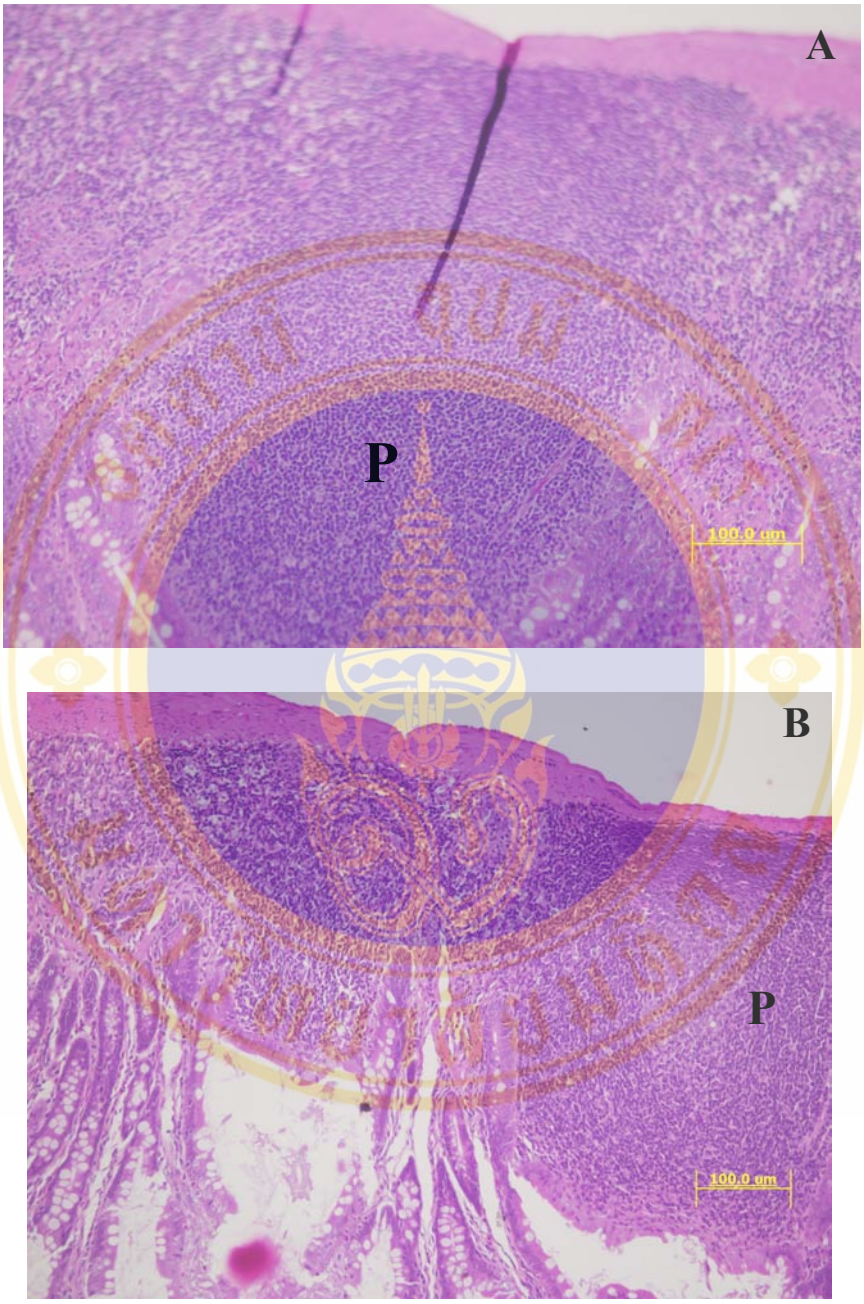

Figure 19: Histology of Payer's patch (P) compared between control rat (A), (10X) and rat treated with piperine at the dose of 50 mg/kgBW, 30 days (B) shows the aggregation of lymphocytes in the small intestinal mucosa, (4X). H&E stain.

Thymus

In the control rats, thymus had thin layer of capsule and had lobulated shape. It was composed of two zones, which were outer basophilic; cortex and inner eosinophillic area; so called medulla. In addition, treated rats looked similar to normal control. Therefore, piperine had no remarkable effect on the histology of thymus in this experiment.

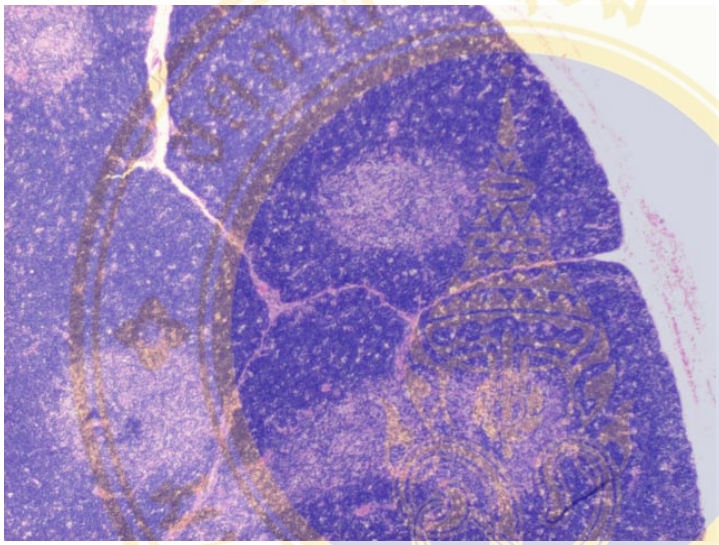

Figure-20: Microscopically, thymus of the rat fed piperine at the dose of 50 mg/kgBW for 30 days. It shows the eosinophillic inner zone and highly lobulated pattern. 4X, H&E.

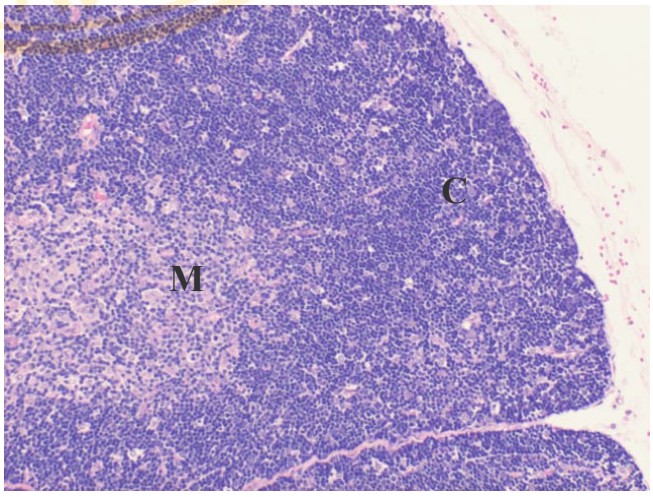

Figure-21: Histology of thymus has two zones which are outer basopillic, cortex (C) and inner eosinophillic, medulla (M).(10X), H&E stain.

Spleen

Spleen was normal both in control and tested groups by gross appearances. Microscopically, it also had normal architecture and it was covered with thin capsule that it protruded into parenchyma as trabeculae. The parenchymal part composed of white pulp, which was prominent with lymphoid aggregation and vascular part, so called red pulp. None of histopathological changes were observed, so piperine had no effect on spleen in this experiment

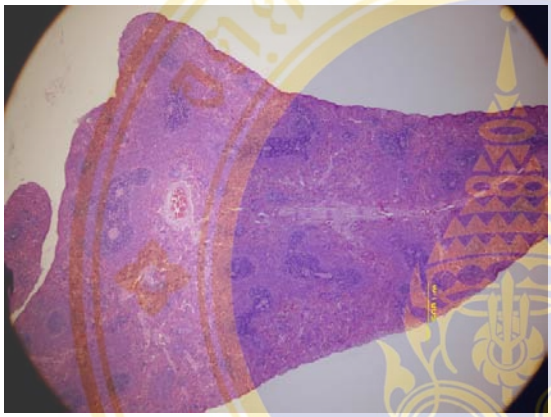

Figure-22: Microscopically, spleen of the rat fed with piperine at the dose of 50 mg/kgBW, 30 days shows thin capsule and discrete of nodules composing of white pulp and red pulp. (4X), H&E stain.

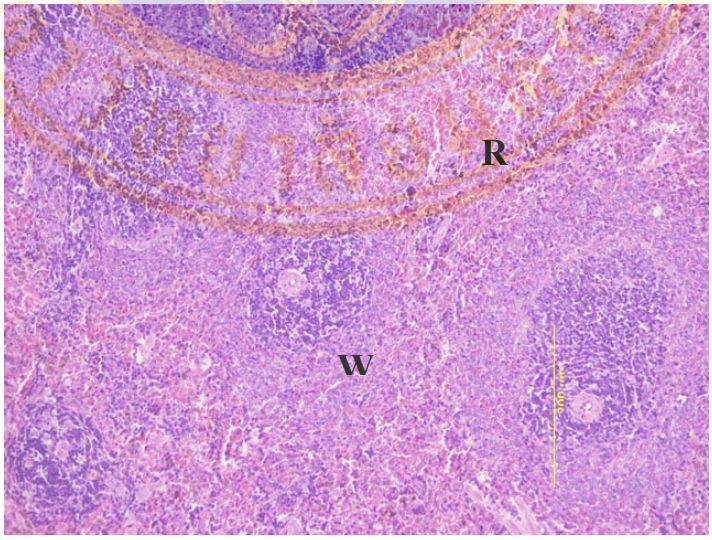

Figure-23: Histology of spleen of the rat fed with piperine at the dose of 50 mg/kgBW shows lymphoid aggregation into follicles, white pulp (W) and the red matrix consisting of vascular tissue, red pulp(R). (10X), H&E.

Adrenal gland

Adrenal gland showed normal cells and structure in both of the control group and treated groups, which included adrenal cortex and adrenal medulla. None of hemorrhage, inflammation or necrosis was observed. Therefore, piperine had no remarkable effect on the histology of adrenal gland.

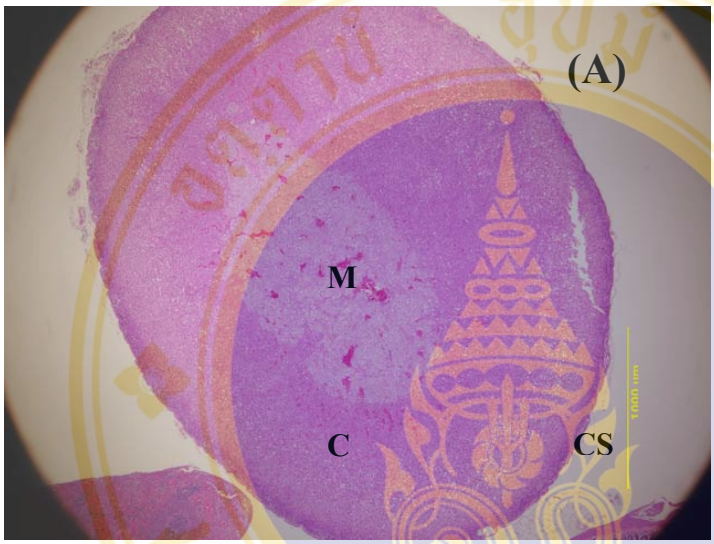

Figure 24-A: Microscopically, the adrenal gland of piperine treated rat at the dose of 50 mg/kgBW, 30 days shows normal architecture, which composed of capsule (CS), cortex (C) and medulla (M). (4X), H&E stain.

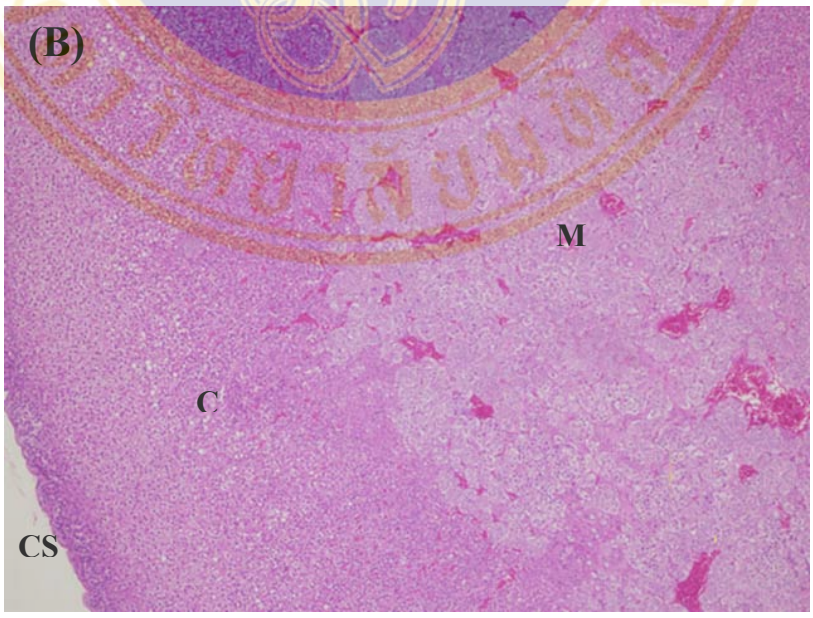

Figure 24-B: The micrograph of adrenal gland of rat at the dose of 50 mg/kgBW, 30 days shows deep blue outer membrane, capsule (CS), cortex (C) and the center with pale eosinophillic, medulla (M). (10X), H&E stain.

Stomach

The stomach remains histological in normal. The outer membrane was muscularis mucosae which had the next layer as mucosa composing of prominent base of the gland (figure-25). The congestion or hemorrhage was not presented; therefore, there are no remarkable histological changes of stomach in the rats fed with piperine at the doses of 0, 5, 25 and 50 mg/kg BW for 30 days.

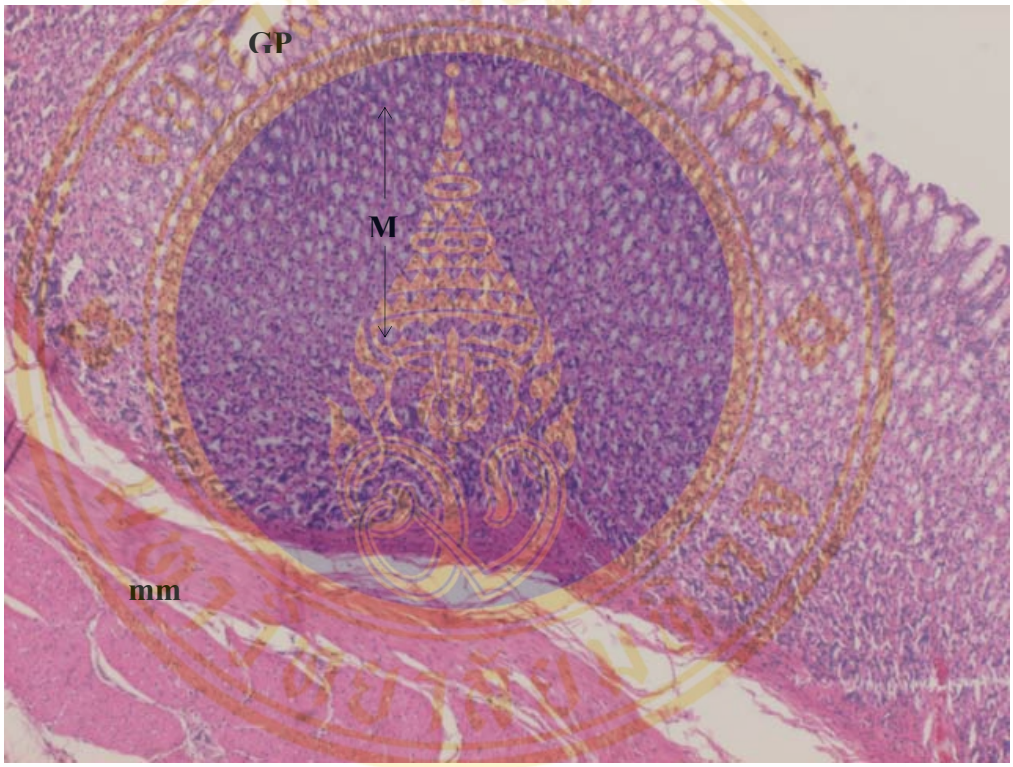

Figure-25: Microscopically, the body of the stomach of the rat fed with piperine at the dose of 50 mg/kgBW for 30 days shows the mucosa (M) consists of gastric glands and fold of the muscularis mucosae (mm) which is open into lumen by gastric pits (GP). (4X), H&E stain.

Testis

The seminiferous tubules showed normal cells morphology and a few disorganization of germ cells but they had no tubular destruction were found. Therefore, it had no remarkable in pathological changes of seminiferous tubules.

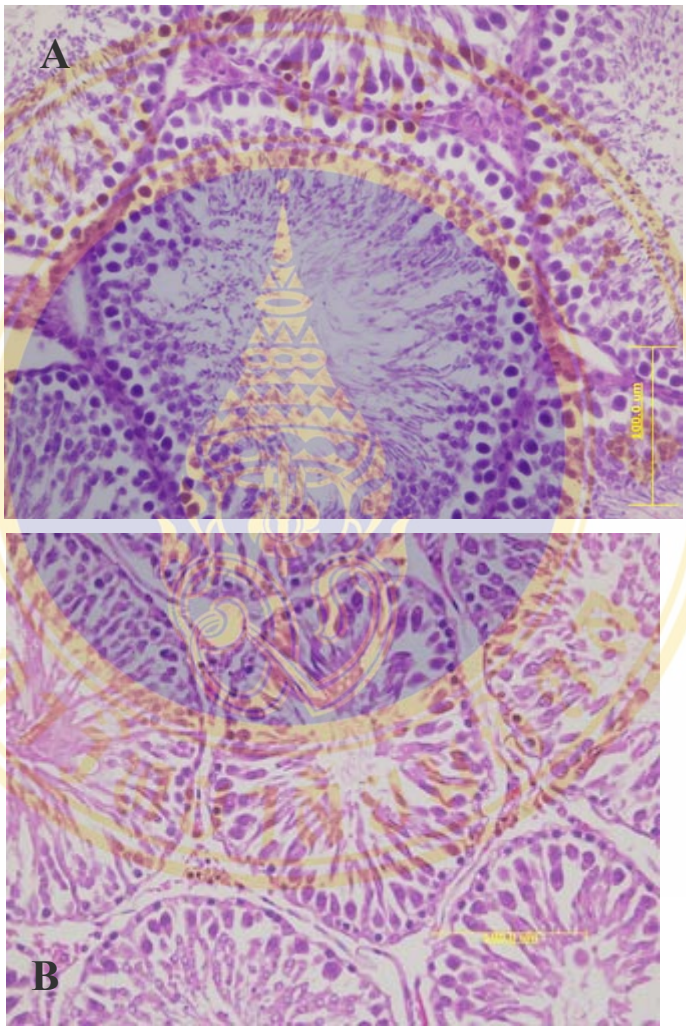

Figure-26: The micrograph of the testis of the control rat (A) shows normal cell morphology (20X) compared with rat fed with piperine at the dose of 50 mg/kgBW, 30 days (B) shows a few germ cells disorganization but no tubular destruction was observed. (10X), H&E stain.

Liver

Liver showed normal gross appearance and microscopic examination (figure- 27) were showing normal architectures including normal density and distribution of hepatocytes. None of pathological changes such as hemorrhage and necrosis were found.

Figure 27-A: The micrograph of the liver of the rat fed with piperine at the dose of 50 mg/kgBW, 30 days shows thin capsule and normal distribution of hepatocytes. (4X), H & E stain.

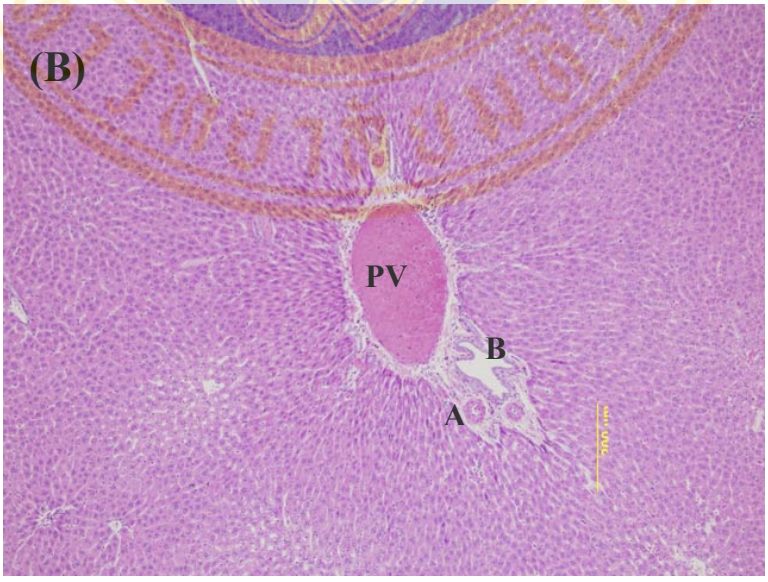

Figure 27-B: Histology of the liver of the rat fed with piperine at the dose of 50 mg/kgBW shows normal hepatocytes, portal vein (PV), artery (A) and bile duct (B). (10X), H & E stain.

1.7) Cell proliferation assay. (BrdU cell incorporation)

The results of BrdU incorporation (positive cells) were showing in Table-20. Slight difference of BrdU positive cells was noticed but there were no statistical significance of BrdU positive cells among rats administered variably with piperine as compared to control animals.

Table-20: The BrdU incorporated cells of the rats administered with piperine at the doses of 0, 5, 25 and 50 mg/kgBW for 30 days.

Piperine (mg/kg BW)	Thymus		C/Tx		Spleen		C/Tx		Small intestine		C/Tx	
	Before	After	B	A	Before	After	B	A	Before	After	B	A
0	67±2.5	72±3.1	1	1	47±0.9	53±2.7	1	1	52±1.8	59±2.3	1	1
5	72±2.4	73±3.2	1.07	1.01	43±1.4	50±3.8	0.9	0.9	63±1.9	58±2.5	1.2	1.0
25	61±0.6	70±1.0	1	0.97	53±1.4	55±3.2	1.0	1.0	51±1.3	56±0.9	1.0	0.9
50	71±3.1	75±3.7	1.05	1.04	59±2.4	63±3.5	1.2	1.2	65±1.2	70±2.2	1.2	1.2

C/Tx; control/treatment ratio, B; before immunization, A; after immunization.

Values are mean $\pm$ SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

EXPERIMENT III: SUBCHRONIC ORAL TOXICITY TEST 90 DAYS.

1.1) Clinical signs.

The clinical signs showed hyperactive activities such as running and jumping around their cages at the dose of 100 mg/kgBW feeding, while depression, drowsy and lied on the bottom of the cages was found at the dose of 250 mg/kgBW. However, none of rats died during the experiment.

1.2) Body weight gain and food consumption

The growth curve of rats fed with piperine at the dose of 100 and 250 mg/kgBW showed a pattern of decreasing among the first and the second week. Then, the weight increased until end of experiment.

The body weight gain and food consumption were shown in Table -21 and Table-22. The mean consumption of rats chow (daily consumption per cage/1 rat) was 1718.00 ± 43.35 g in the control group, 1585.6± 105.18 g and 1347.3 ± 121.81 g in the group of animal fed with piperine at the doses of 100, and 250 mg/kg body weight, respectively. The body weight gain was 305.35 ± 10.7 g in control group, 250.83± 4.36* g and 242.42 ± 7.58* g in the group of animal fed with piperine at the doses of 100 and 250 mg/kg body weight. In both of the body weight gain and food consumption were significant difference among treated groups and control group. In addition, figure-29 showed the data presented as percentage of body weight gain, food consumption, and food conversion efficiency of rats treated with piperine groups compared with control group. It showed percentage of body weight gain, food consumption decreased in all of piperine treated rats but at the doses of 100 and 250 mg/kgBW were reduced 35% and 10% of body weight gain, 43% and 20% of food consumption, respectively. It was significant difference (*$P \leq 0.05$) when compared to control (100% of body weight gain and 100% of food consumption).

TABLE -21: Changes in body weight of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 90 days.

Doses of piperine (mg/kg BW)	Body weight (g)		
	Day-0	Day-90	Body weight gain
0	129.20 ± 2.84	434.55 ± 3.02	305.35 ± 10.7
10	133.83 ± 1.53	421.70 ± 2.93	287.86 ± 9.81
100	138.51 ± 3.91	389.35 ± 5.99	250.83± 4.36*
250	131.26 ± 5.26	373.68 ± 1.58	242.42± 7.58*

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

TABLE-22: Changes in daily food consumption of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 90 days.

Doses of piperine (mg/kg BW)	Body weight gain (g)	Relative total food consumption (g)	Food conversion efficiency (g%)
0	305.35 ± 10.7	1718.00 ± 43.35	17.83 ± 0.75
10	287.86 ± 9.81	1660.83 ± 41.05	17.33 ± 0.33
100	250.83± 4.36*	1585.6± 105.18*	15.50 ± 0.72
250	242.42± 7.58*	1347.3± 121.81*	18.67 ± 1.56

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

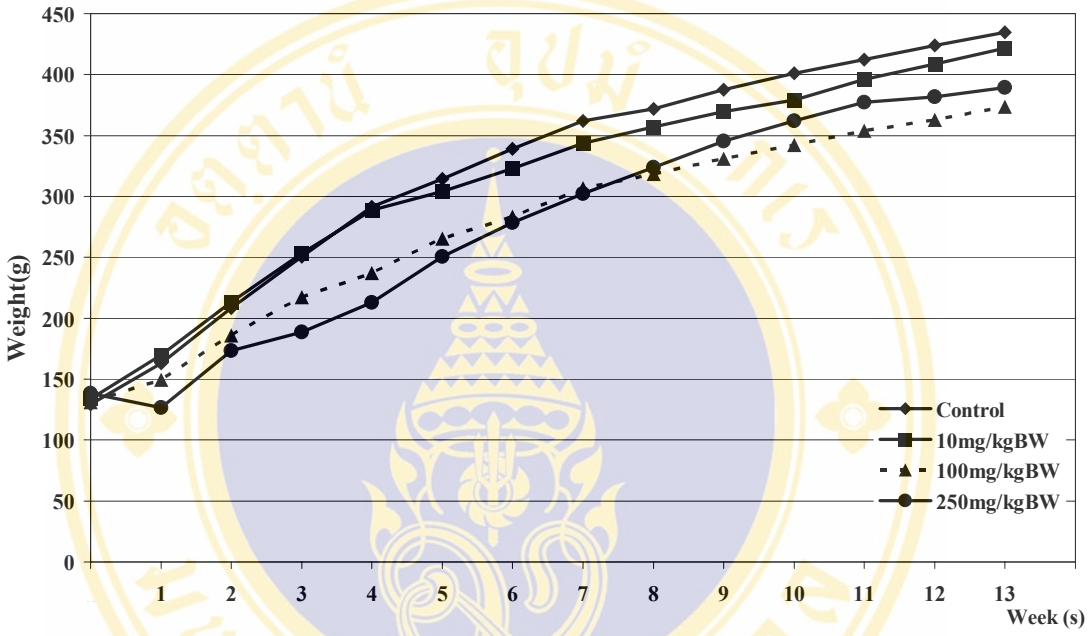

Figure 28: Graph showing growth of young adult rats fed with piperine at the doses of 0, 10, 100 and 250 mg/kgBW. for 90 days.

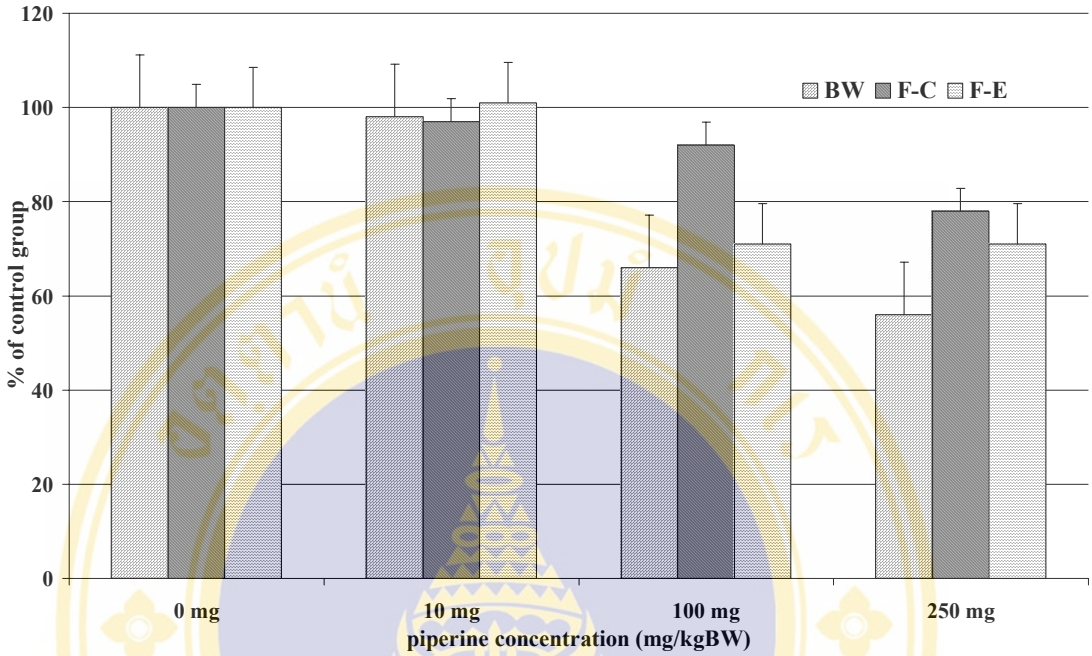

Figure 29: Histogram showing summary of the effects of piperine administration at the doses of 0, 10, 100 and 250 mg/kgBW to Wistar rats for 90 days on body weight gain, food consumption (F-C) and food conversion (F-E). The data presented as percentage of BW, F-C and F-E obtained from the experimental animals.

1.3) Hematological examination.

The results of complete blood counts included hemoglobin, hematocrit, erythrocytes' count, leukocytes' count, and leukocyte differentiation were shown in Table-23. By variance analysis, they showed slightly differences within groups of treated rats but no statistically significance between piperine treated groups and control group.

Table-23: Hematological parameters of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 90 days.

Parameters	Piperine (mg/kg BW/day)			
	0	10	100	250
Hb (g/dl)	49.0 ± 1.3	50.2±0.67	44.8 ± 3.5	50.3 ± 0.2
Hct (%)	13.8 ± 1.0	14.2 ± 0.9	14.8 ± 0.8	15.8 ± 0.3
RBC (x 10^6/mm^3)	7.4 ± 2.6	7.9 ± 0.17	7.7 ± 0.3	7.6 ± 0.3
WBC (x 10^3/mm^3)	3.8±0.03	3.3±0.14	3.7±0.31	3.6±0.22
Neutrophils (%)	63.3±1.9	64.5±1.9	59.2±0.7	62.3±1.7
Eosinophils (%)	0.5+0.03	0.3+0.02	0	0
Basophils (%)	1.3±0.06	2.5±0.1	2.2±0.02	0.6±0.02
Monocytes (%)	1±0.03	1.3±0.02	1.2±0.04	1.7±0.03
Lymphocytes (%)	32.8±2.2	31.3±2.7	37.2±2.3	34.8±1.3
Morphology	N	N	N	N

Hb: Hemoglobin, Hct: Hematocrit, RBC: Erythrocyte counts, WBC: Leukocyte counts, N: normal.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

1.4) Relative organ weight.

The results of organs weight were shown in Table-24, data was in the same range and it had no significant difference between treated groups and control group. In addition, the relative organ weight was the number represented in the parentheses, which calculated from the formula as presented in the appendix VI.

TABLE-24: Changes in organ weight of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 90 days.

Organ (mg) (g%)	Piperine (mg/kg BW/days)			
	0	10	100	250
Liver	15.2 ± 0.56 (3.58 ± 0.08)	14.3± 0.62 (3.51± 0.06)	13.3 ± 0.4 (3.63± 0.04)	13.5± 0.61 (3.4 ± 0.04)
Spleen	0.78± 0.02 (0.19± 0.01)	0.75 ± 0.02 (0.19± 0.01)	0.79 ± 0.03 (0.18 ± 0.01)	0.73 ± 0.03 (0.20 ± 0.01)
Thymus	0.57± 0.02 (0.15 ± 0.01)	0.55 ± 0.03 (0.13±0.005)	0.57 ± 0.02 (0.17±0.004)	0.54± 0.01 (0.15±0.004)

Note: The relative organs weight was shown in the parentheses.

Values are mean ± SEM of 6 rats/group,

*$P < 0.05$; significant difference from control group.

1.5) Humeral immune response assay.

Determination of antibody response after 0.01 M injections of heterogenous proteins from rabbit normal serum was determined in experimental animals by using electrophoresis method. There was no significant difference of antibody response between control group and treated groups. Therefore, piperine has no any stimulatory effect on the antibody induction in rats after fed with piperine for 90 days.

1.6) Histopathological examination

The histopathological examination of the liver, the stomach, the adrenal gland, the thymus, the spleen, and Payer's patch, revealed normal in cell morphology except in testis. The seminiferous tubules, spermatogenic cells, leydig's cells and sertori cells showed normal architecture in the control group. Lumen was filled with spermatozoa and also normal in spermatogenic series development. Whereas, experimental rats fed with piperine at the doses of 250 mg/kgBW showed deformity of basement membrane, lost of cell-to-cell contacting and also abnormal spermatozoa production (figure 30).

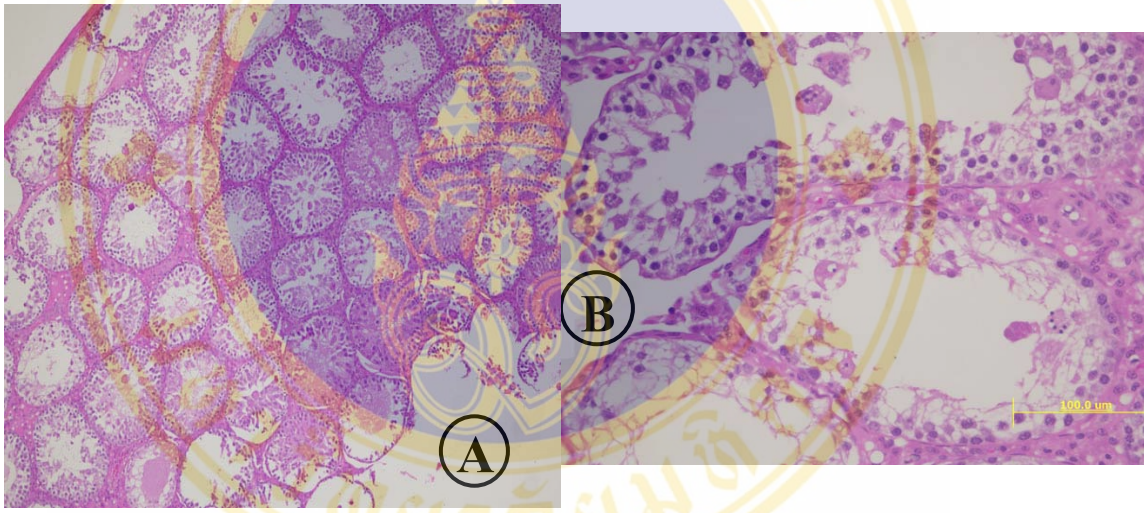

Figure-30: Histopathology of the testis of the rat fed with piperine at the dose of 250 mg/kgBW for 90 days (A) shows the deformity of seminiferous tubule,4X with higher magnification (B). 10X, H&E stain.

1.2.2 Cell proliferation assay. (BrdU incorporated cell)

The results of cell proliferation assay were shown in Table-25. No remarkable differences were observed by calculating the number of cells positive as brown color under 20 high power fields (40X) under light microscope. Data showed no statistically different between experimental rats administered with any doses of 10, 100 and 250 mg/kgBW of 90 days treatment and control group. Thus, piperine gave no remarkable effects on cellular immune response. The BrdU positive cells were presented in the figure 31.

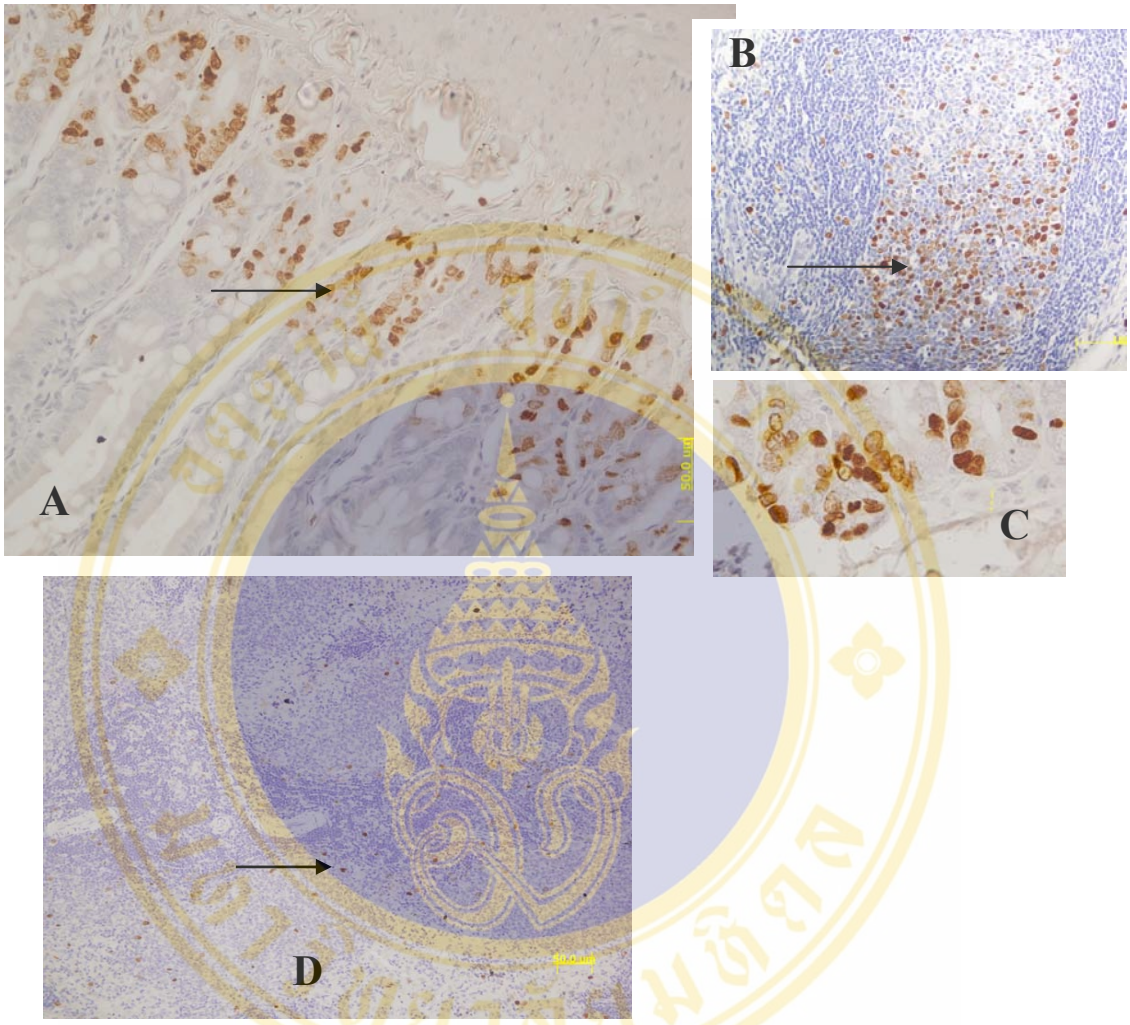

Figure-31: Histology of the small intestine of the rat fed with piperine at the dose of 250 mg/kgBW for 90 days (A) shows BrdU positive cells, 10X. The BrdU positive cells presented as brown color cells (arrow); Payer's Patches (B), 4X and C is BrdU positive cells in the small intestine with the higher magnification (20X) and BrdU positive cells in spleen (D), 4X, H & E stain.

Table-25: The BrdU incorporated cells of the rats administered with piperine at the doses of 0, 10, 100 and 250 mg/kgBW for 90 days.

Piperine (mg/kg BW)	Thymus		C/Tx		Spleen		C/Tx		Small intestine		C/Tx	
	Before	After	B	A	Before	After	B	A	Before	After	B	A
0	89±3.9	107±4.8	1	1	58±1.4	71±2.6	1	1	65±2.4	74±3.1	1	1
10	101±2.6	94 ± 4.2	0.8	1.1	50±2.3	62±4.7	0.9	0.9	75±1.8	81±3.3	1.1	1.1
100	83 ± 1.8	95 ± 4.2	1.1	1.1	48±1.7	61±4.9	0.8	0.9	63±2.6	78±1.8	1.0	1.0
250	96 ± 3.3	93 ± 2.7	0.9	1.1	47±3.1	68±4.4	0.8	0.9	83±3.7	92±2.5	1.2	1.2

C/Tx; control/treatment ratio, B; before immunization, A; after immunization.

Values are mean ± SEM of 6 rats/group,

*$P \leq 0.05$ showed significant difference from control group.

CHAPTER 5
DISCUSSION

Piperine is well-known relish around the world but it is used on various purposes in the present day. Piperines are widely used as nutrient supplements, which are mostly taken with some drugs or vitamins. It was reported that piperine could act as a bioenhancer which was able to increase the bioavailability of many drug [Atal, Zutshi, and Rao, 1981] such as theophylline [Bano, *et al*., 1991], rifampicin [Zutshi, *et al*., 1985], beta-lactam antibiotic [Hiwale, Dhuley, and Naik, 2002] and vitamins [Badmaev, Majeed, and Prakash, 1999]. This property is possibly caused by its drug-metabolism inhibiting activity leading to such drugs remain in the body longer [Atal, Dubey, and Singh, 1985]. In the present study, rats immunized with normal rabbit protein were orally fed with piperine at the doses of 0, 5, 10, 25, 50, 100 and 250 mg/kgBW with in 7, 14, 30, and 90 days. The results showed that piperine affected the behavioral responses, body weight gain and food consumption regardless period of treatment. Both acute and sub-chronic treated groups have been found behavioral responses in two characteristics at different dosages. The rats fed with piperine at the dose of 100 mg/kgBW were over stimulated such as running and jumping around their cages and being sensitive to the noise, which were developed within 2 minutes after piperine administration. On the other hand, at the dose of 250 mg/kgBW, the rats performance were depression, weakness of their legs after being received piperine and finally, lay down at the bottom of the cages. In addition, they were sensitive to noise. The possibility cause of conflicting results between two doses (100 and 250 mg/kgBW) might be piperine affected the central nervous system (CNS) in both stimulation and depression centers. Consistent with the studies of acute effects, piperine had central stimulating activity, caused the loss of body weight gain and reduced food consumption [Piyachaturawat, Glinsukon, and Toskulkao, 1983; Singh, 1973]. Moreover, piperines belong to a chemical family of cinnamides, which are naturally occurring compounds that have a potential of anticonvulsant [Liu, and Garattini, 1986] and analeptic function [Pei, 1983and D'Hooge, *et al*., 1996]. Piperine

is able to suppress convulsion by stimulating the release of serotonin (5-HT) from cerebral cortex [Mori, Kabuto, and Pei, 1985] and that of catecholamine from the adrenal gland [Kawada, *et al*., 1988]. Due to the release of 5-HT, a portion is taken up by presynaptic serotonergic neurons in a manner similar to that of the reuptake of norepinephrine. Norepinephrine along with catecholamine exhibits peripheral nervous system excitatory and inhibitory effects as well as actions in the CNS. In addition, piperine also increased the body weight gain at the low dosage, 25 mg/kgBW. However, after concentrations of piperine were risen up to 100 and 250 mg/kgBW, respectively, the weight of rats decreased in dose-dependent pattern. On this aspect, the increased body weight gain in the low dosage might due to properties of piperine as a bioenhancer [Atal, Zutshi, and Rao, 1981; Badmaev, Majeed, and Prakash, 2000]. Piperine can also induce alterations in membrane dynamics and permeation characteristics, along with induction in the synthesis of proteins associated with cytoskeletal function, resulting in an increase in the small intestine absorptive surface, thus this also facilitated the efficient permeation through the epithelial barrier [Khajuria, Thusu, and Zutshi, 2002]. Furthermore, it enhanced the gastrointestinal tract secretion [Capasso, *et al*., 2002] and pancreatic digestive enzymes [Platel and Srinivasan, 2000], so the rats could have more food consumption resulting in their increased body weight gain. According to the growth curves during the experimental diet periods, it is evident that piperine could delay the progress of the growth rates with regardless to ages or periods of treatment. The significant reducing in the body weight of the rats receiving piperine at the doses of 100 and 250 mg/kgBW are consistent with the results of the report in previous studies [Piyachaturawat, Glinsukon, and Toskulkao, 1983]. This could be the irritating effects of piperine due to the impact of reducing the consumed diet. Based on evidence that the oral irritation was induced by rinses with capsicum oleoresin and piperine, constituents of red and black pepper, [Dessirer, *et al*., 1999] respectively [Lawless, 1984], the oral irritation and gastrointestinal tract irritation [Piyachaturawat, Glinsukon, and Toskulkao, 1983]. Consistent with the previous studies, the oral and gastrointestinal tract irritation could be induced by capsicum oleoresin and piperine, constituents of red and black pepper rinsing, respectively. Therefore, the piperine treated rats might not have appropriate food consumption and finally, they lost their weight.

The histopathological study was assessed in this experiment with regard to piperine concentration and target organs as in the previous study [Piyachaturawat, Glinsukon, and Toskulkao, 1983]. The target organs included thymus, liver, stomach, small intestine, adrenal gland, and testis were examined. There were no piperine effects on these target organs except on testis and stomach. Piperine caused severe damage to the seminiferous tubules and decreased in spermatocytes and spermatids. It was reported that piperine enables to reduce the intratesticular testosterone [Malini and Manimaran, 1999]. Additionally, piperine is a potently non-specific inhibitor of NADPH-dependent cytochrome P_{450} catalysed reaction [Reen and Singh, 1991; Reen, Wieblel, and Singh, 1996]. As cytochrome P_{450} is involved in cholesterol side chain cleavage and 17, 21-hydroxylations in the steroidogenic pathway [Miller, 1988], one might expect this compound interfere with steroidogenic activity. It is well circumstanced that androgens are the major regulators of growth, structure and functions of accessory sex organs [Orgebin-Crist, Eller, and Danzo, 1983; Mooradian, Morley, and Korenman, 1987]. Generally, the intratesticular testosterone concentration is 50 times higher than in peripheral blood serum [Turner, 1991] and it is widely accepted that a level of this order is required for spermatogenesis [Roberts, *et al*., 1991]. The principal action of testosterone is to facilitate the maturation of round to elongated spermatids during spermiogenesis. [Zirkin and Robaire, 1989; O'Donell, McLachlan, and Wreford, 1994]. Furthermore, testosterone acts to stimulate the spermatid binding to sertoli cells specifically at the transition from stages VII to VIII of the seminiferous epithelium [Cameron, Muffly, and Nazian, 1993]. Therefore, possible causes as mentioned above may be linked to the observed disruption of spermatogenesis in piperine treated rats at the doses of 100 and 250 mg/kgBW in this study.

Piperine can induce the hemorrhage of stomach, which has been reported in the previous study [Piyachaturawat, Glinsukon, and Toskulkao 1983], on the contrary, the histopathological change of stomach in this study was congestions in the body part of the stomach which found in 1 0f 6 rats and then it had no statistical significance. These uncorrelated results might be caused by different used of solvents between previous and present study. In the previous studies, 95% ethanol was employed as the piperine solvent, while the present one, corn oil was used instead. Ethanol can also

have antagonistic or additive effects on drugs with which it shares metabolic pathways. Ethanol stimulates increased microsomal oxidation then, the acute ingestion of ethanol, there may be competition for these metabolic pathways, which may slow metabolism of drugs and, in turn, produce transient elevations of drug level. It was demonstrated that ethanol induces hemorrhagic gastric ulceration [Laszlo, *et al*., 1989], which was used to create experimental mucosal erosions in animals [Tarmawski, *et al*., 1988 and Del Valle, 1999]. In addition, pre-exposure for 2 hours to 12% (v/v) ethanol reduced membrane fluidity at the lipid-water interface due to (cells adjust) their membrane permeability change. [Silverira, *et al*., 2003] resulting in an increased permeability or absorption of an agent. Presumably, piperine is a mucosal irritating agent and ethanol also induces the hemorrhagic erosion. Then, one possible cause is the drug interaction between piperine and ethanol to produce hemorrhage of stomach as in previous study. Therefore, the different solvents may be the cause of the uncorrelated results between the present and previous study.

In summary, piperine at the doses of 100 and 250 mg/kgBW had the effects on the body weight, food consumption and testis of male Wistar rats in the present study, but it had no effects on behavioral responses, body weight, food consumption, hematological parameters, cell proliferation, antibody responses and histopathological findings while the dose of piperine lower than these

BIBLIOGRAPHY

Ammon, H.P.T. and Wahl, M.A. (1999). Pharmacology of *Curcuma longa*. *Planta Med., 57,* 1-7.

Animal care and use committee of Berkeley laboratory animal (2003). Guidelines for anesthesia and analgesia in rodents and rabbits. Office of Laboratory Animals Care, Berkeley Press, 1-1.

Atal, C.K., Dubey, R.K. and Singh, J. (1985). Biochemical basis of enhanced drug bioavailability by piperine: Evidence that piperine is a potent inhibitor of drug metabolism. *J. Pharmacol. Exp.Ther., 232*(1), 258-262.

Atal, C.K., Zutshi, U. and Rao, P.G. (1981). Scientific evidence on the role of Ayurvedic herbals on bioavailability of drugs. *J. Ethnopharmacol., 4*(2), 229-232.

Badmaev, V., Majeed, M. and Norkus, E.P. (1999). Piperine, an alkaloid derived from black pepper increases serum response of beta-carotene during 14-days of oral beta-carotene supplementation. *Nutr. Res., 19* (3), 381-388.

Badmaev, V., Majeed, M. and Prakash, L. (2000). Piperine derived from black pepper increases the plasma levels of coenzyme q10 following oral supplementation. *J. Nutr. Biochem.,11*(2), 109-113.

Bai, Y.F. and Xu, H. (2000). Protective action of piperine against experimental gastric ulcer. *Acta. Pharmacol. Sin., 21*(4), 357-359.

Bano, G., Amla, V., Raina, R.K., Zutshi, U. and Chopra, C.L. (1987). The effect of piperine on pharmacokinetics of phenytoin in healthy volunteers. *Planta Med., 53*(6), 568-569.

Bano, G., Raina, R.K., Zutshi, U., Bedi, K.L., Johri, R.K. and Sharma, S.C. (1991). Effect of piperine on bioavailability and pharmacokinetics of propranolol and theophylline in healthy volunteers. *Eur. J. Clin. Pharmacol., 41*(6), 615-617.

Bhat, B.G. and Chandrasekhara, N. (1985). Determination of piperine in biological tissues by thin-layer chromatography and ultraviolet absorption densitometry. *J. Chromatogr., 338*(1), 259-263.

Bhat, B.G. and Chandrasekhara, N. (1986). Studies on the metabolism of piperine: absorption, tissue distribution and excretion of urinary conjugates in rats. *Toxicology, 40*(1), 83-92.

Bhat, B.G. and Chandrasekhara, N. (1987). Metabolic disposition of piperine in the rat. *Toxicology, 44*(1), 99-106.

Cameron, D.F., Muffly, K.E. and Nazian, S.J. (1993). Reduced testosterone during puberty results in a midspermiogenic lesion. Proceedings of the Society for *Experimental Biology and Medicine, 202*, 457-464.

Capasso, R., Izzo, A.A, Borrelli, F., Russo, A., Sautebin, L., Pinto, A., Capasso, F. and Mascolo, N. (2002). Effect of piperine, the active ingredient of black pepper, on intestinal secretion in mice. *Life Sciences, 71*, 2311-2317.

Carson, F.L. (1997). Histotechnology. (pp. s232-234). Chicago: ASCP.

Chandrasekhara, N. and Srinivasan, K. (1999). Health effects of spices in trends in food science and technology global perspective. Mysore *Association of Food Scientists and Technologists*, 725-737.

Chi, R.S. and Cote, T.R. (1997). Antigen retrieval immunohistochemistry, past, present and future. *J. Histochem. & Cytochem., 45*(3), 327-343.

Chu, C.Y., Chang, J.P. and Wang, C.J. (1994). Modulatory effect of piperine on benzo[a]pyrene cytotoxicity and DNA adduct formation in V-79 lung fibroblast cells. Food Chem. *Toxicol., 32*(4), 373-377.

Dalvi, R.R. and Dalvi, P.S. (1991). Differences in the effects of piperine and piperonyl butoxide on hepatic drug-metabolizing enzyme system in rats. *Drug Chem. Toxicol., 14* (1-2), 219-229.

Davis, L. and Kuttan, G. (2000). Immunomodulatory activity of *Withania sominifera. J. Ethanopharmacol., 71*, 193–200.

Daware, M.B., Mujumdar, A.M. and Ghaskadbi, S. (2000). Reproductive toxicity of piperine in Swiss albino mice. *Planta Med., 66*(3), 231-236.

del Valle, J. (1999). CCK receptor trafficking: a novel paradigm of travel focus on "regulation of lateral mobility and cellular trafficking of the CCK receptor by a partial agonist". *Am. J. Physiol., 276*, 537-538.

Dessirier, J.M., Nguyen, N., Sieffermann, J.M., Carstens, E. and O'Mahony, M. (1999). Oral irritant properties of piperine and nicotine: psychophysical evidence for asymmetrical desensitization effects. *Chem Senses., 24*(4), 405-413.

D'Hooge, R., Pei, Y.Q., Raes, A., Lebrun, P., van Bogaert, P.P. and de Deyn, P.P. (1996). Anticonvulsant activity of piperine on seizures induced by excitatory amino acid receptor agonists. *Arzneimittelforschung, 46*(6), 557-560.

Duke, J.A. (1985). CRC Handbook of Medicinal Herbs (pp. 232-234). Boca Raton, Fla.: CRC Press

Farang, S.E. and Abo-Zieal, M. (1999). Degradation of the natural mutagenic compound safrole in spices by cooking and irradiation. National Center for radiation research and technology. *Immunotoxicity Testing Guidance*. USA: FDA reviewers.

Finar, I.L. (1975). Stereochemistry and the chemistry of natural products. *Organic Chemistry* (5th ed.). London: Longman's UK publication, ELBS.

Fox, J.G. (1998). Principles of anesthesia and analgesia. (pp. 1-10).*Laboratory Animal Medicine*

Ghoshal, S., Prasad, B.N. and Lakshmi, V. (1996). Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica in vitro* and *in vivo*. *J. Ethnopharmacol., 50*(3), 167-170.

Gupta, S.K., Bansal, P., Bhardwaj, R.K. and Velpandian, T. (2000). Comparative anti-nociceptive, anti-inflammatory and toxicity profile of nimesulide vs nimesulide and piperine combination. *Pharmacol. Res., 41*(6), 657-662.

Hammers, H.J., Saballus, M., Sheikzadeh, S. and Schlenke, P. (2002). Introduction of a novel proliferation assay for pharmacological studies allowing the combination of BrdU detection and phenotyping. *J. Immunological Methods, 264*, 89-93.

Harms, G.V., Goor, H., Koudstaal, J., de Ley, L. and Hardonk, M.J. (1986). Immunohistochemical demonstration of DNA-incorporated 5-bromo-2-deoxy-uridine in frozen and plastic embedded sections. *Histochem., 85*(2), 139-143.

Hayes, A.W. (1994). Acute toxicity and Eye Irritancy. *Principles and Methods of Toxicology* (3th ed.). New York: Raven press.

Hiwale, A.R., Dhuley, J.N. and Naik, S. (2002). Effect of co-administration of piperine on pharmacokinetics of beta-lactam antibiotics in rats. *Indian J. Exp. Biol., 40* (3), 277-281.

Hosti, J. (2000). Measurement of prothrombin time (PT) in EDTA plasma with combined thromboplastin reagents. *Clin. Chem., 46*(1), 1844 – 1846.

Houwen, B. (2000). Blood film preparation and staining procedures. *Laboratory Hematology* (vol. 6, pp. 1-7). Carden Jennings Publishing Co., Ltd.

Houwen, B. (2001). The differential cell count. *Laboratory Hematology* (vol. 7, pp. 89-100). Carden Jennings Publishing Co., Ltd.

IBIDI group. (2004). Coating procedures for the ibidi µ-slide product family. *IBIDI Integrated BioDiagnostics* (1). IBIDI research group.

Izzo, A.A., Capasso, R., Pinto, L., Di Carlo, G., Mascolo, N. and Capasso, F. (2001). Effect of vanilloid drugs on gastrointestinal transit in mice. *Br. J. Pharmacol., 132*(7), 1411-1416.

Jacobs, D.S. (1996). Laboratory Test Handbook 4 th edition, 312-314.

Jamwal, D.S. and Singh, J. (1993). Effects of piperine on enzyme activities and bioenergetic functions in isolated rat liver mitochondria. *J. Biochem. Toxicol., 8*(4), 167-174.

Jecker, P., Beuleke, A., Dressendorfer, I., Pabst, R. and Westermann, J. (1997). Long term oral application of 5-bromo-2'-deoxyuridine does not reliably label proliferating immune cells in the LEW rat. *J. Histochem. & Cytochem., 45*(3), 393-401.

Johri, R.K. and Zutshi, U. (1992). An Ayurvedic formulation 'Trikatu' and its constituents. *J. Ethnopharmacol., 37*(2), 85-91.

Kacmar, P., Pistl, J. and Mikula, I. (1990). Immunotoxicology and veterinary medicine. *ACTA VET. BRNO., 68*, 57-79.

Kang, M.H., Won, S.M., Park, S.S., Kim, S.G., Novak, R.F. and Kim, N.D. (1994). Piperine effects on the expression of P4502E1, P4502B and P4501A in rat. *Xenobiotica, 24*(12), 1195-1204.

Karekar, V.R., Mujumdar, A.M., Joshi, S.S., Dhuley, J., Shinde, S.L. and Ghaskadbi, S. (1996). Assessment of genotoxic effect of piperine using *Salmonella*

typhimurium and somatic and germ cells of Swiss albino mice. *Arzneimittelforschung, 46*(10), 972-975.

Kawada, T., Sakabe, S., Watanabe, T., Yamamoto, M. and Iwai, K. (1988). Some pungent principles of spices cause the adrenal medulla to secrete catecholamine in anesthetized rats. *Proc. Soc. Exp. Biol. Med., 188*(2), 229-233.

Khajuria, A., Thusu, N. and Zutshi, U. (2002). Piperine modulate permeability characteristics of intestine by inducing alterations in membrane dynamics: influence on brush border membrane fluidity, ultrastructure and enzyme kinetics. *Phytomedicine, 9*(3), 224-231.

Khajuria, A., Zutshi, U. and Bedi, K.L. (1998). Permeability characteristics of piperine on oral absorption an active alkaloid from peppers and a bioavailability enhancer. *Indian J. Exp. Biol., 36*(1), 46-50.

Kholkute, S.D., Kekare, M.B. and Munshi, S.R. (1979). Antifertility effects of the fruits of *Piper longum* in female rats. *Indian J. Expt. Biol., 17*, 289-290.

Klaassen, C.D. and Doull, J. (1980). Evaluation of Safety: Toxicologic Evaluation. *Toxicology* (2nd ed.), pp.11-28.

Kuby, J. (1997). Chapter I: Overview of the immune system. *Immunology*. USA: Freeman W.H. & company, Sumanas Inc.

Kuenzi, F.M. and Dale, N. (1996). Effect of capsaicin and analogues on potassium and calcium currents and vanilloid receptors in Xenopus embryo spinal neurons. *Br. J. Pharmacol., 119*(1), 81-90.

Kuttan, G. and Kuttan, R. (1992). Immunomodulatory activity of a peptide isolated from *Viscum album* extract. *Immunological Investigations, 21*, 285-296.

Laszlo, F., Pavo, I., Penke, B. and Balint, G. A. (1989). Protective effect of an orally administered, highly potent somatostatin analog (RC-121) against absolute ethanol induced hemorrhagic erosions of rat gastric mucosa. *Life Sci., 44*, 1573-1578.

Lawless, H.T. (1984). Oral chemical irritation: psychophysical properties. *Chem. Senses, 9*, 143–155.

Lee, E.B., Shin, K.H. and Woo, W.S. (1984). Pharmacologogical studies on piperine. *Arch. Pahrmacol. Res., 7*, 127-132.

Liu, G.Q. and Garattini, S. (1986). Stimulation of serotonin synthesis in rat brain after administration of antiepilepsirine, an antiepileptic piperine derivative. *Chin. Med. J. (Engl.), 99*(5), 411-416.

Liu, L. and Simon, S.A. (1996). Similarities and differences in the currents activated by capsaicin, piperine and zingerone in rat trigeminal ganglion cells. *J. Neurophysiol., 76*(3), 1858-1869.

Lu, F.C. (1996). Toxicology of the immune system. *Basic toxicology: fundamental, target organs and risk assessment* (3rd ed.). UK: Taylor & Francis Inc.

Luetting, B., Pape, L., Bode, U., Bell, E.B., Sparshott, S.M., Wagner, S. and Westermann, J. (1999). Naïve and memory T lymphocyte migrate in comparable numbers through normal rat liver: activated T cells accumulate in the periportal field. *J. Immunol., 163*, 4300-4307.

Majeed, M., Badmaev, V. and Rajendran, R. (1996, 1998). Use of piperine to increase the bioavailability of nutritional compounds. United States Patent, No.5, 536, 506 and No.5, 744, 161.

Malini, T., Manimaran, R.R., Arunakaran, J. and Aruldhas, M.M. (1999). Effects of piperine on testis of albino rats. J. *Ethnopharmacol., 64*, 219-225.

Mathew, S. and Kuttan, G. (1999). Immunomodulatory and antitumour activities of *Tinospora cordifolia*. *Fitoterapia, 70*, 35–43.

Matsuura, S. and Suzuki, K. (1997). Immunohistochemical analysis of DNA synthesis during chronic stimulation with isoproterenol in mouse submandibular gland. *J. Histochem. & Cytochem., 45*(8), 1137- 1145.

Maxwell, M.W. (1967). *Clinical hematology* (6th ed.). Philadelphia: LEA & FEBIGER press.

Mcginley, J.N., Knott, K.K. and Thompson, H.J. (2000). Effect of fixation and epitope retrieval on BrdU indices in mammary carcinomas. *J. Histochem. & Cytochem., 48*(3), 355-362.

MERCK. (2000). General information on piperine Merck index, Germany, 1030.

Miller, W.L. (1988). Molecular biology of steroid hormone synthesis. *Endocr. Rev., 9*, 295–318.

Mooradian, A.D., Morley, J.E. and Korenman, S.G. (1987). Biological actions of androgens. *Endocr. Rev., 8*, 1-28.

Mori, A., Kabuto, H. and Pei, Y.Q. (1985). Effects of piperine on convulsions and on brain serotonin and catecholamine levels in E1 mice. *Neurochem. Res., 10*(9), 1269-1275.

Muffly, K.E., Nazian, S.J. and Cameron, D.F. (1993). Junction-related sertoli cell cytoskeleton in testosterone-treated hypophysectomized rats. *Biol. Repro., 49*, 122-132.

Mujumdar, A.M., Dhuley, J.N., Deshmukh, V.K., Raman, P.H. and Naik, S.R. (1990). Anti-inflammatory activity of piperine. *Jpn. J. Med. Sci. Biol., 43*(3), 95-100.

Nygard, U.C. and Lovik, M. (2002). Blood and spleen lymphocytes as targets for immunotoxic effects in the rat a comparison. *Toxicology, 174*, 153-161.

O'Donell, L., McLachlan, R., Wreford, N., de Kretser N.D.M. and Robertson D.M. (1996). Testosterone withdrawal promotes stage specific detachment of round spermatids from the rat seminiferous epithelium. *Biol. Reprod., 55*, 895–901.

Ononiwu I.M., Ibeneme C.E. and Ebong O.O.. (2002). Effects of piperine on gastric acid secretion in albino rats. Afr J Med Med Sci., 31(4), 293-295.

Orgebin-Crist, M.C., Eller, B.C. and Danzo, B.J. (1983). The effects of estradiol, tamoxifen and testosterone on the weights and histology of the epididymis and accessory sex organs of sexually immature rabbits. *Endocrinol., 113*, 1703–1715.

Parmar, V.S., Jain, S.C., Bisht, K.S., Jain, R., Taneja, P., Jha, A., Tyagi, O.D., Prasad, A.K., Wengel, J., Olsen, C.E. and Boll, P.M. (1997). Phytochemistry of genus *Piper*. *Phytochem., 46*, 597–673.

Pei, Y.Q. (1983). A review of pharmacology and clinical use of piperine and its derivatives. *Epilepsia, 24*(2), 177-182.

Pei, Y.Q., Yue, W., Cui, J.R. and Yao, H.Y. (1980). A study of the central pharmacological action of piperine and its derivatives. *Yao Xue Xue Bao, 15*(4), 98-205.

Piyachaturawat, P. and Pholpramool, C. (1997). Enhancement of fertilization by piperine in hamsters. *Cell Biol. Int., 21*(7), 405-409.

Piyachaturawat, P., Glinsukon, T. and Toskulkao, C. (1983). Acute and subacute toxicity of piperine in mice, rats and hamsters. *Toxicol. Lett., 16* (3-4), 351-359.

Piyachaturawat, P., Sriwattana, W., Damrongphol, P. and Pholpramool, C. (1991). Effects of piperine on hamster sperm capacitation and fertilization *in vitro*. *Int. J. Androl., 14*(4), 283-290.

Platel, K. and Srinivasan, K. (1996). Influence of dietary spices or their active principles on digestive enzymes of small intestinal mucosa in rats. *Int. J. Food Sci. Nutr., 47*(1), 55-59.

Platel, K. and Srinivasan, K. (2000). Stimulatory influence of select spices on bile secretion in rats. *Nutr. Res., 20*, 1493-1503.

Platel, K. and Srinivasan, K. (2001). Influence of dietary spices and their active principles on pancreatic digestive enzymes in albino rats. *Nahrung, 44*(1), 42-46.

Race, G. and Alami, S.Y. (1969). *Laboratory Medicine*. New York: Haper & Row Inc.

Rakowski, T. (2002). CCMS recommended analgesic and anesthetic dosages. *The Critical Care Medicine Section*. Los Angeles, CCMS .

Reanmongkol, W., Janthasoot, W., Wattanatorn, W., Dhumma-Upakorn, P. and Chudapongse, P. (1988). Effects of piperine on bioenergetic functions of isolated rat liver mitochondria. *Biochem. Pharmacol., 37*(4), 753-757.

Reddy, A.C. and Lokesh, B.R. (1992). Studies on spice principles as antioxidants in the inhibition of lipid peroxidation of rat liver microsomes. *Mol. Cell Biochem., 111*(1-2), 117-124.

Reen, R.K. and Singh, J. (1991). *In vitro* and *in vivo* inhibition of pulmonary cytochrome P450 activities by piperine, a major ingredient of piper species. *Indian J. Exp. Biol., 29*(6), 568-573.

Reen, R.K., Jamwal, D.S., Taneja, S.C., Koul, J.L., Dubey, R.K., Wiebel, F.J. and Singh, J. (1993). Impairment of UDP-glucose dehydrogenase and glucuronidation activities in liver and small intestine of rat and guinea pig *in vitro* by piperine. *Biochem. Pharmacol., 46*(2), 229-238.

Reen, R.K., Wiebel, F.J. and Singh, J. (1996). Piperine impairs cytochrome P4501A1 activity by direct interaction with the enzyme and not by down regulation of CYP1A1 gene expression in the rat hepatoma 5L cell line. *Biochem. Biophys. Res. Commun., 17*, 218(2), 562-569.

Roberts, K.P., Awoniyi, C.A., Santulli, R. and Zirkin, B.R. (1991). Regulation of sertoli cell transferrin and sulfated glycoprotein-2 messenger ribonucleic acid

levels during the restoration of spermatogenesis in the adult hypophysectomized rat. *Endocrinol., 129*, 3417-3423.

Sambaiah, K.L. and Srinivasan, K. (1991). Secretion and composition of bile in rats fed diets containing spices. *J. Food Sci. Technol., 28*, 35-38.

Saunders, E.J., Varedi, M. and French, A.S. (1993). Cell proliferation in the gastrulating chick embryo: a study using BrdU incorporation and PCNA localization. *Development, 118*(2), 389-399.

Sharatchandra, J.N., Platel, K. and Srinivasan, K. (1995). Digestive enzymes of rat pancreas and small intestine in response to orally administered mint leaf, and garlic pearl. *Indian J. Pharmacol., 27*, 156-160.

Shi, S.R., Cote, R.J. and Taylor, C.R. (1997). Antigen retrieval imunohistochemistry: past, present, and future. *J. Histochem. & Cytochem., 45*(3), 327-343.

Shoba, G., Joy, D., Joseph, T., Majeed, M., Rajendran, R. and Srinivas, P.S. (1998). Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Med., 64*(4), 353-356.

Silveira, M.G.D., Golovina, E.A., Hoekstra, F.A., Rombouts, F.M. and Abee, T. (2003). Membrane fluidity adjustments in ethanol stressed O*enococcus oeni* cells. *Appl. Environ. Microbiol., 69*(10), 5826–5832.

Singh, J., Dubey, R.K. and Atal, C.K. (1986). Piperine-mediated inhibition of glucuronidation activity in isolated epithelial cells of the guinea-pig small intestine: evidence that piperine lowers the endogeneous UDP-glucuronic acid content. *J. Pharmacol. Exp. Ther., 236* (2), 488-493.

Singh, J., Reen, R.K. and Wiebel, F.J. (1994). Piperine, a major ingredient of black and long peppers protects against AFB_1-induced cytotoxicity and micronuclei formation in H4IIEC3 rat hepatoma cells. *Cancer Lett., 86*(2), 195-200.

Singh, N. (1973). Studies on the analeptic activity of some *Piper longum* alkaloids. *J. Res. Ind. Med., 8* (1), 1-9.

Stohr, J.R. and Xiao, P.G. (2001). Constituents of Chinese *Piper* species and their inhibitory activity on prostaglandin and leukotriene biosynthesis *in vitro*. *J. Ethnopharmacol., 75*, 133-139.

Takaki, M., Jin, J.G., Lu, Y.F. and Nakayama, S. (1990). Effects of piperine on the motility of the isolated guinea-pig ileum: comparison with capsaicin. *Eur. J. Pharmacol., 186*, 71-77.

Tarnawski, A.S., Stachura, J., Hollander, D., Sarfeh, I.J. and Bogdal, J. (1988). Cellular aspects of alcohol-induced injury and prostaglandin protection of the human gastric mucosa. Focus on the mucosal microvessels. *Clin. Gastroenterol., 10*(1), 35-45.

Ternes, W. and Krause, E.L. (2002). Characterization and determination of piperine and piperine isomers in eggs. *Anal. Bioanal. Chem., 374*, 155-160.

Tulinska, J., Sovcikova, A.A., Liskova, A., Kubova, J. and Horakova, K. (2000). Immunotoxicity of ethy1-4-isthiocyanatobutanoate in male Wistar rats. *Toxicology, 145*, 217-225.

Turner, T.T. (1991). Spermatozoa are exposed to a complex microenvironment as they transverse the epididymis. *Ann. N.Y. Acad. Sci., 637*, 364-383.

UBC. (1996). Blood collection. Animal Care Guidelines. SOP-002. UBC Committee on Animal Care web site: http://www.acc.ubc.ca/SOP/SOP002.html.

Unchern, S., Saito, H. and Nishiyama, N. (1998). Death of cerebella granule neurons induced by piperine is distinct from that induced by low potassium medium. *Neurochem. Res., 23*(1), 97-102.

Vane, J.R. (1971). Inhibition of prostaglandin synthesis as a mechanism of action for aspirin like drugs. *Nat. New Biol., 231*, 232-235.

Vos, J.G. and Loceren, H.V. (1995). Markers for immunotoxic effects in rodents and humans. *Toxicol. Lett., 82*, 358-394.

Wakabayashi, K., Nagao, N. and Sugimura, T. (1989). Mutagens and carcinogens produced by reaction of environmental aromatic compounds with nitrite. *Cancer Surv., 8*(2), 385-399.

Walker, M. (1997). Piperine in black pepper, one of a newly recognized class of thermonutrients. *Health Foods Business*, 11-14.

WHO. (2001). Guidelines for the preparation of working papers on intake of food additives for the Joint FAO/WHO. *Expert Committee on Food Additives*, 1-21.

Winton, A.L. and Winto, K.B. (1993). *Structure and composition of foods* (pp. 336-339). New York: John Wiley and Sons Inc.

Wrba, H., el-Moffy, M.M., Schwaireb, H. and Dutter, A. (1992). Carcinogenicity testing of some constituents of black pepper (*Piper nigrum*). *Exp. Toxicol. Pathol., 44*(2), 61-65.

Zirkin, B.R., Santulli, R., Awoniyi, C.A. and Ewing, L.L. (1989). Maintenance of advanced spermatogenic cells in the adult rat testis: quantitative relationship to testosterone concentration within the testis. *Endocrinol., 124*, 3043–3049.

Zirkin, B.R. and Robaire, B. (1991). The male germ cell: spermatogonium to fertilization. Proceedings of the 6th North American testis workshop. Montreal, Quebec, *Ann. NY. Acad. Sci., 637*, 1-16.

Zutshi, R.K., Singh, R., Zutshi, U., Johri, R.K. and Atal, C.K. (1985). Influence of piperine on rifampicin blood levels in patients of pulmonary tuberculosis. *J. Assoc. Physic. India, 33*(3), 223-224.

APPENDIX

APPENDIX: I

CHEMICALS AND REAGENTS

Table 26: CHEMICALS AND REAGENTS.

Chemicals / agents / reagents	Purchased from
1) 3 – aminopropyltriethoxysilane (Silane)	Sigma Chemical Company, St. Louis, Missouri, USA.
2) 5'-Bromo-2'-deoxyuridine	
3) ABC Kit Vector stain	Vector laboratory Inc., CA., USA.
4) Absolute ethanol	Merck, Darnstatt, Germany
5) Agarose	Merck, Darnstatt, Germany
6) Anti 5 - Bromo - 2'- deoxyuridine	Dako, CA.,USA.
7) Biotin-mouse immunoglobulin	
8) Bovine serum albumin (BSA	Sigma Chemical Company, St. Louis, Missouri, USA.
9) Complete/incomplete freund adjuvant	Sigma Chemical Company, St. Louis, Missouri, USA.
10) Dipotassium - hydrogen phosphate	Merck, Darnstatt, Germany
11) Drabkin's solution	Bio-Medical Laboratory, Bangkok, Thailand
12) Glacial acetic acid	Merck, Darnstatt, Germany
13) Peroxidase substrate kit (DAB)	
14) Piperine	Aldrich Chemical. Co., USA.
15) Polyxyethelene (20) sorbitan monolaurate (Tweens 20)	Sigma Chemical Compayny, St. Louis, Missouri, USA.
16) Potassium dihydrogen phosphate	Merck, Darnstatt, Germany
17) Sodium hydroxide	Bio-Medical Laboratory, Bangkok, Thailand

APPENDIX: II

Complete Blood Count (CBC)

I) Hemoglobin.

Test: Cyamethohaemoglobin method.

This procedure was used to determine the concentration of hemoglobin (Hb) in whole blood sample. Hemoglobin is the major component of red blood cells which act as oxygen transporter and buffering carbon dioxide formed during metabolism. The Hb level is used for evaluation of anemia and polycythemia, by mean of corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC).

Specimen : Whole blood in EDTA or heparin.

Limitation of the test

For hyperlipidemia or white blood cell counts greater than 50,000 cell/mm^3, the hemoglobin may falsely elevate resulting in the increase of MCH, and MCHC. However, a method for correcting lipidemia has been suggested.

Principle

Blood sample was diluted in a solution containing potassium cyanide and potassium ferriccyanide. Hb and carboxyhemoglobin (CoHb) are converted to hemiglobincyanide (HiCN). The mixtured solution is measured by a spectrophotometer at the wavelength of 540 nm.

Materials

Potassium ferriccyanide	200	mg
Potassium cyanide	50	mg
Potassium dihydrogen phosphate	140	mg
Non ionic detergent	1	ml
Distilled water	1	L

Finally, make up total volume to 1,000 ml by add distilled water and adjust pH to 7.0 – 7.4.

Method

	Tested-sample	**Control**
Hb Reagent (ml)	2.5	2.5
Blood (ml)	0.01	-
Distilled water (ml)	-	0.01

1. The mixtures were mixed thoroughly.
2. Leave for 3-5 minutes; incubate the samples at 37^{o}C for 15 minutes before measuring.
3. Read at 540 nm by spectrophotometer.

II) Hematocrit (Hct).

Micro method test.

Hematocrit micro method (Hct) is to determine the percentage of red blood cells in whole blood and calculation of the MCV and MCHC.

Specimen: Whole blood with EDTA or heparin is recommended except clotted or hemolysed specimen

Materials: 75 mm. capillary tubes with heparin coating.

Method:

1) Allow blood sample to enter the tube by capillary force, leaving at least 15 mm. unfilled.
2) Seal the tube with paraffin.
3) Place capillary tube on hematocrit tray. Tightly fixed and closed the lid.
4) Centrifuge at 2,000 rpm for 5 minutes.
5) Measure the Hct using a reading device.

III) White blood cell count.

Specimen:

Using white blood cells with anticoagulant (EDTA/Heparin), occlude clotted blood specimen and hemolysed specimen.

Materials:

1. Counting chamber, white cell pipette, diluent fluid (2% glacial-acetic acid)
2. Light microscope.
3. Diluent fluid.

Acetic acid	5	ml
Distilled Water	500	ml

Method

Prepare a 1:20 dilution of blood with diluent fluid. Pipette blood up to 0.5 µl. and then add diluent fluid to 11 µl. Mix testing blood by a mechanical mixer or hand for 5 minutes. Pour the mixture immediately onto a clean and dry counting chamber with the cover-glass ready in position. Care should be taken for counting chamber at the right position and no fluid spills at surrounding moat. Leave the chamber undisturbed about 2 minutes, allowing the cells to settle before counting by using a 10x objective lens.

IV) Differential leukocyte count of peripheral blood.

This is the part of complete blood count, determined as relative and/or absolute number of circulating leukocytes.

Specimen: whole blood with anticoagulant (EDTA/Heparin).

Materials

-Light microscope
-Wright's stain
-Distilled water
-Tab water
-Glass slides
-Spreader

Method

1) Prepare blood smear.
2) Stain the smear with Wright's stain for 5 minutes.
3) Wash the slide with tab water.
4) Leave it to air dry at room temperature.
5) Differentiate WBC under a light microscope at 100x objective lens.

V) Red blood cell count.

Specimen:

Whole blood with anticoagulant (EDTA/Heparin), hemolysed blood is not recommended.

Diluent solution: Gower's Solution

Sodium sulfate	12.5	g
Glacial acetic acid	33.3	ml
Distilled water	200.0	ml

Method

Make up a 1:200 dilution of blood with Gower's solution. Fill the white blood cell pipette with blood to 0.5 µl. then add diluting reagent to 101 µl. Mix the testing sample with a mechanical mixer or by hand for 5 minutes by tilting the pipette through an angle of 120 degrees and rotating which allowing the air bubble to mix the suspension. Pour the mixture immediately on a clean counting chamber with the cover-glass ready in position. Care should be taken to the counting chamber at the right position and no fluid spills at surrounding moat. Leave the chamber undisturbed about 2 minutes. Then count the cells by using a 40x objective lens.

VI) Serum collection.

The clotted blood samples were centrifuged at 2,500 rpm, 5 minutes using Kokusan centrifuge, collected the supernate and kept in a freezer until use for IEP test.

APPENDIX: III

IMMUNOELECTROPHORESIS (IEP)

Specimen:

Serum samples were used to determine antibody reactivity against injected antigens.

Materials:

1. Veronal buffer pH 8.6:

Sodium babital	35.92	g
Sodium azide	2.0	g
Distilled Water	4.0	L

Adjust pH to 8.6 with concentrated HCl drop by drop, the buffer will be stable for 2 months when kept at 2-8 ^{o} C.

2. Preparation 1.5 % Agar

Noble Agar	6	g
Veronal buffer	200	ml
Distilled water	200	ml

Mixed whole ingredient in an Erlenmeyer flask and heated in boiling water, do not allow direct flame. Stir until agar dissolved and then stored in aliquots at 2-8 ^{o} C.

3. Washing buffer:

Sodium chloride	20	g
1% Sodium azide	2	g

Mixed the ingredients in a volumetric flask. Make up to 2 liters volume.

4. Destainer:

Acetic acid	60	ml
Alcohol	300	ml
Distilled water	640	ml

5. Staining:

Amido Black	10	g
Destainer	1000	ml

Put both of them in a volumetric flask (1 L). Shake well and filter before kept in a cleaned bottle at room temperature.

6. Antisera:

Anti-human γ-globulins-antiserum was used as reference –antiserum.

Equipments:

1. Slides
2. Electrophoresis chamber
3. Power supply
4. Slide holders
5. Slide cutter
6. Filter paper

Procedure:

1. Dissolve the agar under boiling water bath and warming the dissolved agar to reach 65 - 70 ^{o}C.

2. Set slides with slide holders and pour about 3 cc. agar for each slides.

3. Allow agar to solidify by 30 minutes.

4. Make two holes and a trough on each gel with needle and gel cutter.

5. Place antigen samples used as immunogens into each holes and electroseparated in the electrophoresis chamber filling with 2000 ml Veneral Buffer and run under 300 volts D.C. for 60 – 90 minutes.

6. After electrophoresis, the tested serum samples were put into each troughs.

7. Then place in a moisture chamber and allow diffusion to take place for 8 to 24 hours.

8. The reaction bands were observed before and after staining as following method.

Staining procedure

A. Place the gel in 1% NSS for 2 days and washed with DW 3 hr.

B. Oven at 56 ^{o}C until agar is dried.

C. Amido Black stain was put for 5 minutes.

D. Wash with acetic acid for 2 hours and read the result.

APPENDIX: IV
HISTOPATHOLOGICAL TECHNIQUE

I: Tissue processing

Placed the tissue samples into 70% ethanol, 2 hours (2X), then into 95% ethanol, 2 hours (2X), followed by 100% ethanol, 2 hours (2X), xylene, 2 hours (2X), Paraffin I, 2 hours, and filling in Paraffin II, 30 minutes. The formalin fixed paraffinized tissues were then cut into 3μm. in thickness and put on poly-L-lysine-L-lysine (PLL) coated slide.

II: PLL coated slides.

Glass slides were used after cleaning with detergent and dried at room temperature followed by 95% ethanol for 12-24 hours. The slides were dipped in 0.1% w/v PLL solution (Sigma-Aldrich, P8920) in ultra pure water for 5 minutes, then washed in distilled water (DW), and dried in an oven at 50^{o}C before use.

III: Deparaffinization and rehydration

The tissue sections were serially rehydrated with xylene for 15 minutes(2x), 100% ethanol, 15 minutes (2X), 95% ethanol, 15 minutes (2X), 85% ethanol, 15minutes (2X), 95% ethanol, 15minutes (2X), 70% ethanol, 15minutes (2X) and distilled water, 5minutes (1X).

III: Mounting process

After staining, the tissue sections were dehydrated and accordingly in 70% ethanol for 15 minutes (1X), 85 % ethanol for 15 minutes (1X), 95 % ethanol for 15 minutes (2X), 100 % ethanol for 15 minutes (2X), xylene for 15 minutes (2X), then mounted with permount solution and put a cover glass onto the slide and air dried at room temperature.

APPENDIX: V

IMMUNOHISTOCHEMISTRY

Immunostaining

After tissues dehydration in serial ethanol solutions (70%, 85% and 100%), the tissues were immunostained as following step**.** The tissue sections were retrieved in 250 ml of 20 mM of 0.01 M. sodium citrate buffer, pH 6.0 under a microwave oven for 3 minutes at medium power, cooled for 30 min at room temperature before staining.

The sections following procedure was carried out by immersing in 2 N HCL for 25 minutes to unfold the DNA. The sections were rinsed several changes of DW and blocked endogenous peroxidase activity with 3% H_2O_2 for 15 minutes followed by several changes of distilled water and PBS, pH 7.4, 3 times for 5 minutes. Moreover, non-specific background was blocked with 3% bovine serum albumin for 30 minutes. All steps were performed in a humidity chamber. Primary antibody of mouse anti-BrdU (Sigma, USA) prepared as 1:75 dilution was applied on the sections and incubated for 1 hr at room temperature and 4^{o}C overnight. Negative control samples using PBS instead of primary antibody was included. After 3 times PBS washing each for 5 minutes, the secondary antibody, biotinylated rabbit anti mouse IgG (DAKO, CA) at 1:400 dilution was added and incubated for 30 minutes. After PBS washing, the 3, 3'-diaminobenzidine (DAB) was applied and incubated for 5 minutes before stop with several changes of distilled water. The sections were counterstained with hematoxylin for 2 minutes, followed by distilled water washing and dehydrated with serially graded ethanol and xylene as previously mentioned.

APPENDIX: VI

DATA ANALYSIS

Data analysis of the percent of the body weight gain and food consumption averaging are according to the formula as presented below:

$$\% \text{ of control} = \frac{\text{Final day (s)} - \text{day 0 (treatment group)}}{\text{Final day(s)} - \text{day 0 (control group)}} \times 100$$

FOOD CONVERSION EFFICIENCY

$$\text{Food conversion efficiency (\%)} = \frac{\text{Body weight gain (g)} \times 100}{\text{Total food consumption (g)}}$$

RELATIVE ORGANS WEIGHT

$$\text{The relative organs weight} = \frac{\text{Weight of each organ} \times 100}{\text{Body weight}}$$

BIOGRAPHY

NAME	Miss Jiraporn Suwana
DATE OF BIRTH	29 December 1974
PLACE OF BIRTH	Ubonratchathani, Thailand
INSTITUTIONS ATTENDED	Huachiewchalerprakiet University, 1996-1999
	Bachelor of Science (Medical technology)
	Mahidol University, 2000 – 2004
	Master of Science (Pathobiology)
HOME ADDRESS	35/2 Kanasai Road., Mueng District,
	Patthalung Province, Thailand 80000.