องค์ประกอบของพิษผึ้งมิ้ม *Apis florea* Fabricius, 1787 และผลของพิษ

ที่มีต่อหนูแรทเพศผู้

นางสายชล ไชยวงศ์วัฒนกุล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาสัตววิทยา ภาควิชาชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

COMPONENTS OF RED DWARF HONEYBEE *Apis florea* Fabricius, 1787 VENOM AND ITS EFFECT ON MALE RATS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
Department of Biology
Faculty of Science
Chulalongkorn University
Academic Year 2008

Thesis Title	COMPONENTS OF RED DWARF HONEYBEE *Apis florea* Fabricius, 1787 VENOM AND ITS EFFECT ON MALE RATS
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Field of Study	Zoology
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สายชล ไขยวงศ์วัฒนกุล : **องค์ประกอบของพิษผึ้งมิ้ม *Apis florea* Fabricius, 1787 และผลของพิษที่มีต่อหนูแรทเพศผู้.** (COMPONENTS OF RED DWARF HONEYBEE *Apis florea* Fabricius, 1787 VENOM AND ITS EFFECT ON MALE RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : **ผศ. ดร. สุรีรัตน์ เดี่ยววาณิชย์**, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : **ผศ. ดร. วรินทร ชวศิริ,** 117 หน้า.

พิษของผึ้งมิ้มซึ่งถูกเก็บจากถุงเก็บพิษ มีค่าเฉลี่ยของพิษผึ้งแห้ง 0.016 $\pm$ 0.0004 มิลลิกรัมต่อตัว องค์ประกอบของพิษผึ้งมิ้มถูกนำมาวิเคราะห์ด้วยวิธีรีเวิสเฟส ไฮเปอร์ฟอร์มแมนท์ ลิควิด โครมาโทกราฟฟี (RP-HPLC) และหาลำดับกรดอะมิโนด้วยมาลดิทอฟแมสสเปกโทรเมทรี (MALDI-TOF Mass spectrometry) พบว่ามี อะปามิน (apamine) ฟอสโฟไลเปสเอ สอง (PLA_2) และเมลลิติน (melittin) เป็นองค์ประกอบเช่นเดียวกับในผึ้งพันธุ์, *A. mellifera* ในขณะที่อีก 7 แฟรคชั่นที่ศึกษายังไม่พบลำดับกรดอะมิโน ที่ตรงกับในฐานข้อมูลของแมสคอต (Mascot search) นอกจากนี้ได้ทำการศึกษาผลของพิษผึ้งมิ้มที่มีต่อเนื้อเยื่อตับ ไตและเลือดของหนูแรทเพศผู้โดยฉีดพิษที่ใต้ผิวหนัง (subcuteneous) ด้วยความเข้มข้น 0.25 และ 0.5 มล.ต่อกก. ของน้ำหนักหนู โดยละลายพิษใน PBS 0.1 มล. สำหรับกลุ่มควบคุมฉีดด้วย PBS 0.1 มล. จากนั้นเก็บผลที่ 2 8 และ 24 ชั่วโมงหลังฉีดพิษ พบว่าเนื้อเยื่อตับของกลุ่มที่ถูกฉีดด้วยพิษผึ้งทั้งสองกลุ่ม มีเม็ดเลือดแดงจำนวนมากแทรกตัวอยู่ในช่องแอ่งเลือด (sinusoid) และมีการเพิ่มจำนวนคัฟเฟอร์เซลล์ (Kupffer cell) ซึ่งต่างจากในกลุ่มควบคุมอย่างมีนัยสำคัญ ส่วนลักษณะของหยดไขมันในเซลล์ตับพบได้ในทุกกลุ่มโดยไม่มีความแตกต่างจากกลุ่มควบคุม ในเนื้อเยื่อไตพบเม็ดเลือดแดงจำนวนมากแทรกตัวอยู่ทั้งในชั้นไตส่วนนอก (cortex) ไตส่วนใน (medulla) ในบริเวณท่อและลักษณะพิคโนติค นิวเคลียส (pycnotic nucleus) นอกจากนี้ยังพบการเพิ่มจำนวนนิวโตรฟิว (nutrophil) และการลดจำนวนของลิมโฟไซต์ (lymphocyte) ต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญอีกด้วย

ลักษณะการเพิ่มจำนวนของคัฟเฟอร์เซลล์และเม็ดเลือดแดงจำนวนมากในช่องแอ่งเลือดของเนื้อเยื่อตับในชั้นไตส่วนนอก ส่วนใน บริเวณในท่อและลักษณะพิคโนติคนิวเคลียสในเนื้อเยื่อไต รวมถึงการเปลี่ยนแปลงจำนวนเม็ดเลือดขาวนั้น แสดงว่าพิษของผึ้งมิ้มน่าจะเป็นสาเหตุทำให้เกิดความเสียหายได้ในเนื้อเยื่อตับ ไตและส่งผลกระทบต่อระบบภูมิคุ้มกันของร่างกาย

ภาควิชา	ชีววิทยา	ลายมือชื่อนิสิต	
สาขาวิชา	สัตววิทยา	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก	
ปีการศึกษา	2551	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม	

4972521423 : MAJOR ZOOLOGY
KEYWORDS : *Apis florea* / honeybee / venom / rat

SAICHON CHAIWONGWATTANAKUL : COMPONENTS OF RED DWARF HONEYBEE *Apis florea* Fabricius, 1787 VENOM AND ITS EFFECT ON MALE RATS. ADVISOR : ASST. PROF. SUREERAT DEOWANISH, CO-ADVISOR : ASST. PROF. WARINTHORN CHAWASIRI, 117 pp.

Venom of red dwarf honeybee was collected from venom sac of the bees. Average dried weight of venom was 0.016 $\pm$ 0.0004 mg per bee (n=500). The components of venom were analyzed by RP-HPLC. Determinated the peptide mass fingerprint by MALDI-TOF Mass spectrometry. Three major components such as apamine, pla_2 and melittin were found as in *A. mellifera*. Other seven subfractions were collected and they were not matched with amino acid sequence in database of protein venom from Mascot search. The effects of *A. florea* venom on liver, kidney and blood tissues of male rats were also investigated. The venom was injected into subcutaneous layer of rats at concentration of 0.5 and 0.25 mg/kg (in PBS 0.1 ml) for the treatment groups and PBS 0.1 ml was used for a control group. Liver and kidney tissues of rats were collected for histological study at the time point of 2, 8 and 24 hours after treated with venom. The liver tissue of both treatment groups showed significant different of many red blood cells in the sinusoid capillaries and increasing of Kupffer cells number from a control group. A few number of lipid droplets was found in hepatocyte and there was not difference from a control group. The kidney tissue of treated groups showed significant different of many red blood cells in cortex, medulla, tubule and pycnotic nucleus. Increasing of neutrophils and decreasing of lymphocytes were showed significant different from control group.

The characters of increasing of Kupffer cells, many red blood cells infiltration in sinusoid of liver tissues, cortex, medulla, tubule and pycnotic nucleus in kidney tissues and the changing number of white blood cells suggested that venom of *A. florea* might be a cause of liver, kidney tissues damage and have the effects to immune system.

Department : Biology Student's Signature : .....

Field of Study : Zoology Advisor's Signature : .....

Academic Year : 2008 Co-Advisor's Signature : .....

Acknowledgements

I wish to express my deepest gratitude to my advisor, Assistant Professor Dr. Sureerat Deowanish for her encouragement, valuable suggestions and guidances throughout my study. I am truly greatful to my thesis co-advisor, Assistant Professor Dr. Warinthorn Chavasiri for his valuable guidance and suggestions of my thesis.

I wish to express my greatful thank to Associate Professor Dr. Kumthorn Thirakhupt, chairman of thesis committee and Associate Professor Dr. Chanpen Chanchao for serving on my thesis committee.

I am truly thank to Assistant Professor Dr. Guntima Suwannapong and Dr. Jirarach Srijunngam for their kind suggestions and encouragements.

My thanks are also extended to Mr. Anantanart Ladaphan for his love and valuable helping in many ways.

I would like to thank the Graduate School of Chulalongkorn university for financial supports and also thank to many helping hand throughout my study from member of Department of Biology, Faculty of Science, Chulalongkorn University and Center of excellence in Entomology: Bee Biology, Biodiversity of Insects and Mites, Department of Biology, Faculty of Science, Chulalongkorn University.

Finally, I would like to express my deepest appreciation to my parents and everyone in my family for their love and understanding throughout my study.

CONTENTS

Pages

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure | Page

LIST OF ABBREVIATIONS

A. andreniformis	*Apis andreniformis*
A. cerana	*Apis cerana*
A. dorsata	*Apis dorsata*
A. florea	*Apis florea*
A. mellifera	*Apis mellifera*
ACN	acetronitrile
°C	degree Celcius
cm	centimeter
g	gram
GPC	gel permeation chromatography
KDa	kilodalton
LD_{50}	median lethal dose
M	molar (concentration)
MALDI	matrix-assisted laser desorption/ionization
MALDI-TOF	matrix-assisted laser desorption/ionization/time of flight analyzer
MS	mass spectrometry
MCDP	mast cell degranulating peptide
MeOH	methanol
PLA_2	phopholipase A2
RP-HPLC	reverse phase high performance liquid chromatography
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid
µm	micrometer
µg	microgram
mg/kg	milligram/kilogram

Liver tissues

B. C.	blood congestion
C. V.	central vein
F	fatty droplet

H	hepatocyte
K	Kupffer cell

Kidney tissues

C. D.	collecting duct
H. M.	haemorhage
M. D.	medullary ray
P. C.	pycnotic nucleus
RBCs	red blood cells

CHAPTER I

INTRODUCTION

There are many miscellaneous assortments of sting, biting and venomous arthropods that may cause discomfort or affect human's health. Sting insects, comprising of the bees, wasps, hornets and ants, are in the order Hymenoptera. Also, honeybees known as pollinator insects help to increase many products of agriculture. Honeybee products such as honey, bee pollen, royal jelly, bee wax and bee venom, have important properties and restore human health. Not only it is used as a component in many healthy products but also use for treatment of many diseases such as arthritis, rheumatism, back pain, cancerous tumors and skin disease, etc. (Hider, 1988). From the reason of stinging, they annoy frequenly to man but normally they usually sting only as a defensive reaction. Defensive behaviour of honeybee workers is induced by visual stimuli such as moving of a dark object, and inflated by alarm pheromone secreted from the dufour glands of attacked nest mates (Boch *et al*., 1962). The building up to stage of the defensive behaviour is the "stinging response", which involves the penetration of the shaft of the sting and release venom from venom sac (Collins *et al*., 1980).

The sting apparatus is a modified ovipositor of sting insects. Wasps and ants have unbarbed stings which able to withdraw their apparatus quickly after stinging and escape. On the other hand, the sting of honeybees have barbs. Mostly, after stinging the sting will be leaved on victim's skin or it takes some time to pull out. The victim usually brushes them aside which result in the stinging apparatus being torn from bee and remaining in the body (Service, 1980). The whole stinging apparatus,

which is enfolded within the sting chamber, includes two set of parts. One is a long tapering shaft, which consists of an unpaired dorsal component (stylet) and paired ventral elements (lancets). The other is the large basal part, which is the principal motor apparatus and three cuticular plates (the quadrate plate, oblong plate and triangular plate) by which the sting is attached within the sting chamber of the abdomen (Ogawa, Kawakami and Yamaguchi, 1995). Upon delivering the stinger, the bee will subsequently die from losing part of its insides, since the portion where the stinger bulb was removed draws out of its insides. Venom still spread into victim body by actively pumping of muscle function which without requiring the presence of bee. The best safety way for victim is to remove the stinger quickly by scrape along the skin underneath the stinger and flick it out. A stinging bee also releases “alarm pheromone” which can alert other bees to sting at the same site (Crane, 1999; Ghent and Gary, 1962). The sting of honey bees genus *Apis* are different in morphology when studied by scanning electron microscope. It’s able to used identification this genus included the other characters of external morphology (Jayasvati and Limbipichai, 1988).

The high mortality rate from bee, hornet and wasp stings is attributed to anaphylactic shock resulting from hypersensitivity of venom polypeptides (Anthony, 1984). Different kinds of enzyme and protein cause allergy in each honeybee species, were found by gel electrophoresis method (Schmidt, 1982).

Southeast Asia is the center of honeybees diversity. There are eight endemic species of genus *Apis* in this area. *Apis mellifera* is the most studied and economic benefit species. This species called “exotic species” because it was recently imported to Asia. The other seven species, *A. cerana*, *A. dorsata*, *A. florea*, *A. andreniformis*, *A. koschevnikovi*, *A.*

nigrocincta and *A. nuluensis*, with only partially discovered patterns of distribution, are southeast Asian natives. There are five species in Thailand, *A. cerana*, *A. dorsata*, *A. florea*, *A. andreniformis* and *A. mellifera*. This rich of species diversity can create both basic and applied research on honeybee biology (Connor *et al*., 1993). The study of *A. florea* in Thailand has been done on behaviour, ecology, distribution and morphology (Wongsiri *et al*., 1997). The red dwarf honey bee, *A. florea* is distributed throughout Thailand and present in both natural and disturbed areas (Oldroyd and Wongsiri, 2006). In general, the nesting site of *A. florea* is the plain up to 500 m from sea level. But in seasonal migration can high up to 1,500 m from sea level and higher in the north of Thailand and in the south of China (Free, 1981; Wongsiri *et al*., 1997). The migrations may be an adaptation response to seasonal change and lack of food (Ruttner, 1986). The nest of red dwarf honey bee is single – comb nest in daylight like a nest of *A. dorsata* (giant honey bee) but very much smaller size, attached under the branch or a small tree (Butler, 1979). Cavity-nesting honeybees *A. cerana* and *A. mellifera* can be kept and managed in hives for commercial beekeeping (Ruttner, 1988). Meanwhile the wild species such as *A. florea* and *A. dorsata* can not manage so they are hunted.

A. florea is the species which wildly distributed in Thailand. Villagers usually hunt and collect its comb for consuming and selling. So, many people have got stung by this bee. It is interesting in the changing of the tissues when the body recieves toxic from bee venom. The important organs of metabolism, detoxification, storage and excretion of toxicant are liver and kidney (BrzÓska *et al*., 2003). The amount of bee venom when we got stung is also important for medical treatment. The most previous researches on bee venom showed only the quantity,

compound and properties of venom from *A. mellifera* but it is still little known in *A. florea*. In this study reverse phase high performance liquid chromatography technique (RP-HPLC) and MALDI-TOF mass spectrometry were used to analyze and identify the molecular weight include N-terminal 8 amino acid sequence of venom. The study of its effect to liver and kidney tissues of male rats by histological technique was also carried out.

The objectives of this research were

1. To measure the amount of *A. florea* venom per bee.
2. To study the components of *A. florea* venom.
3. To study the effect of *A. florea* venom on liver, kidney and blood tissues of male rats *Rattus norvegicus*.

This research is not only to increase basic knowledge of native honeybees in Thailand but also provide the beneficial of medicinal properties of bee venom for treatment of some disease in the future.

CHAPTER II

LITERATURE REVIEW

2.1. Biology of *Apis florea*, Fabricius 1787

Taxonomy of *A. florea* has been recognized as follows (Wongsiri, 1989)

Kingdom Animalia
Phylum Arthropoda
Class Insecta
Order Hymenoptera
Super-family Apoidea
Family Apidae
Genus *Apis*
Species *Apis florea*

Honeybees in the genus *Apis* that are found in Thailand composed of 5 species: *A. dorsata* Fabricius 1793; *A. cerana* Fabricius 1793; *A. mellifera* Linnaeus, 1758; *A. florea* Fabricius 1787 and *A. andreniformis* Smith, 1858 (Wongsiri *et al*., 1991). Two dwarf honeybees are *A. florea* and *A. andreniformis*. *A. florea* is reddish at the first abdominal segment and known as red dwarf honeybee, while *A. andreniformis* is black at the first abdomenal segment and known as black dwarf honeybee. Both are about 10 mm. long. They are small honeybees of southern and southeastern Asia. They are very distinct evolutionarily and are probably the result of allopatric speciation. *A. florea* has more widely distribution,

beside *A. andreniformis* shows more aggressive than *A. florea* (Arias and Sheppard, 2005).

Figure 2-1. Nest of *A. florea.* (Photoed by S.Wongvilas)

Figure 2-2. Nest of *A. andreniformis*. (Photoed by S. Wongvilas)

Figure 2-3. Workers of *A. florea.* (Photoed by S.Wongvilas)

Figure 2-4. Workers of *A. andreniformis*. (Photoed by S. Wongvilas)

A. florea is a native wild honeybee and the most common species in Thailand. The distribution areas of *A. florea* cover from north to south and east to west of Thailand.

2.2. Biology of bee venom and toxic effects

2.2.1 Bee sting

The sting is a modified ovipositor of bees so only females (queen and workers) can sting. Honeybees can sting only one time cause from the characteristic barbs of sting. They defend their nests and themself with sting, then possess a stinger into the victim's skin. The stinger and venom sac are pulled out of the bee's abdomen and the bee dies in soon (Reisman, 1994). The stinging process of many bees composes three steps. First, sliced sting continues to move and embed itself deeply into the victim's skin. Second, venom was pumped into skin. Third, the sting releases alarm pheromones (isopentyl acetate) which is evaporate from surface of the sting. This pheromone is a communicative chemical which called alarm pheromone. It can alert other worker bees in a colony and attract them to proven vulnerable area (Vetter and Visscher, 1998).

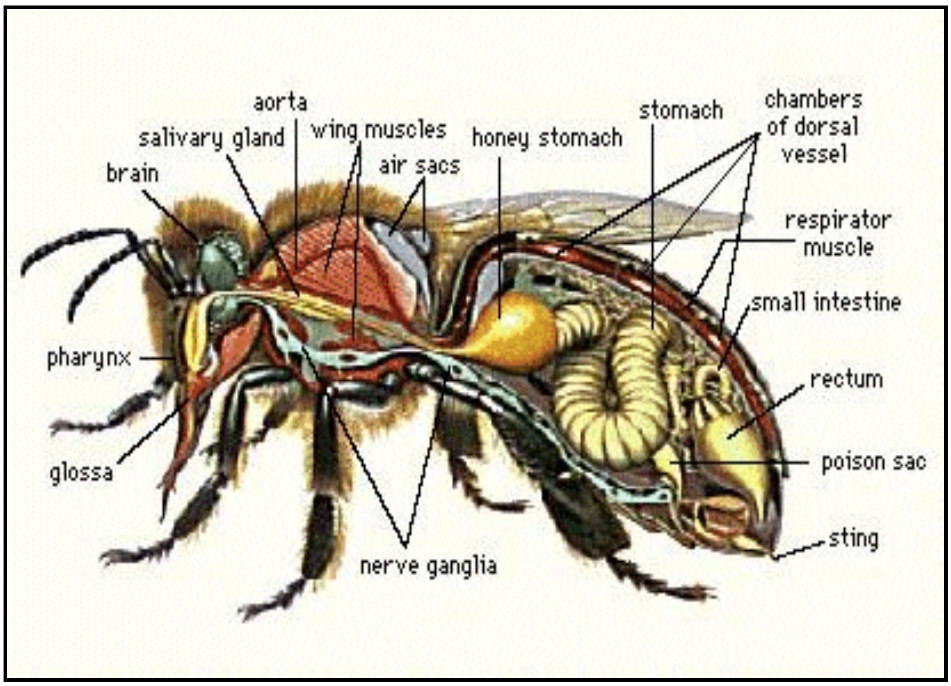

Figure 2-5. Anatomy of honeybee (www. beemaster.com).

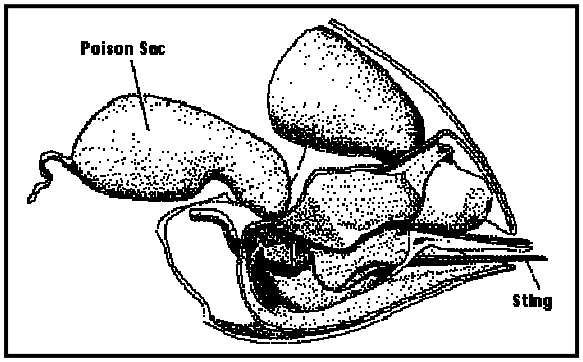

Figure 2-6. Venom gland of honeybee (www.bees.ucr.edu).

Figure 2-7. *A. florea* venom gland (X 100).

The sting composes of two barb lancets and one stylet which can slice back and forth along its length. Lancets and stylet are moved by muscles in the base of the sting. Attached to the lancets have two membranous pump diaphragms which pump venom down through a channel to sting shaft (Vetter and Visscher, 1998).

2.2.2 Component of bee venom

Venom of honeybee is stored in a poison sac. The venom is produced since newly emerged until reach to 18 days old of guard bee, after that bee venom is not produced. Moreover, Lauter and Vrla (1939) found that the sugar diet has not related with the production of bee venom. The same components of proteins in venom were found from different times and area of country indicated that pollen source independent with bee venom production (Benton, 1965). Bee venom is a clear liquid, bitter test, an aromatic odor and a specific gravity of 1.1313. It dried quickly at room temperature, to 30-40 % of the original liquid weight. Components of bee venom may have potential in medical use. Crude bee venom can be used in two ways

1. Treatment of rheumatoid arthritis which is known as “bee venom therapy” or “apitherapy”. This idea came from the observation of bee keepers are seldom afflicted.

2. Desensitization of hypersensitive individuals (Witherell, 1979).

However, injection of whole insect extract will contain many proteins that unrelated treatment proteins in pure venom. Used an extract of the whole bee body showed lower toxin content than the material injected by the sting. Injection by sting may be contain a specific bee-protein allergen, so some allergists choosed an extract of the whole honeybee body for treatment (Ordman, 1968). Mild electric shock was used to excite stinging of bee in order to collect the venom. Worker bees in a hive were stimulated by electric shock. They will sting and venom deposited on a glass. Then the venom was removed by razor blade. By this method, honeybees can survive (Witherell, 1979).

The active components of honeybee venom contain several biochemically or pharmacologically active substances, including enzymes (PLA_2 and hyaluronidase), other smaller proteins peptides (melittin, apamine, mast cell degranulating peptide (MCDP) and minimine) and amines (histamine and dopamine) (Witherell, 1979).

Melittin is the main component which can be found about 50% of venom dry weight. It is a peptide containing 26 amino acid residues and is the main lethal component in honeybee venom, direct haemolytic agent, cause the lysis of erythrocytes and release of histamine from mast cells (Schmidt, 1995; Habermann and Kowallek, 1970). Two forms of melittin are found in bee venom. The amino acid sequence of melittin I is showed in Figure 2-8. (Habermann and Jentsch, 1967; Jentsch, 1969).

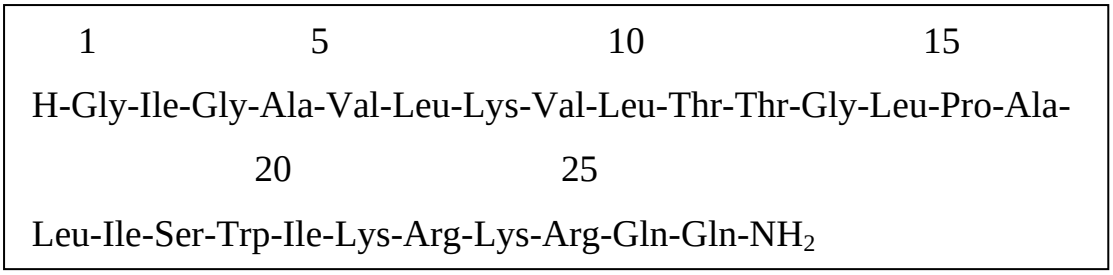

Figure 2-8. An amino acid sequence of melittin I.

Melittin II has 27 amino acid residues and shows a little difference in sequence after residue 21 (Figure 2-9) (Schröder *et al*., 1971).

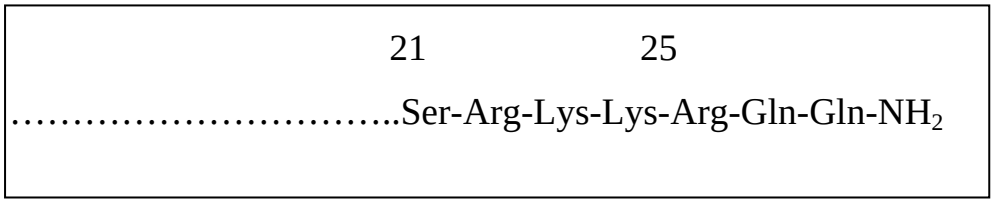

Figure 2-9. An amino acid sequence of melittin II.

The sequence of amino acids in melittin indicated species specific in *A. dorsata*, *A. florea*, and *A. mellifera* venom. Melittin that isolated from *A. cerena* and *A. mellifera* are alike, indicated that two species are closely related biologically. Positions of amino acid residues were reported as in Figure 2-10.

	5	10	15	22	25	26
A. mellifera						
A. cerana	Val	Thr	Ala	Arg	Gln	Gln
A. dorsata	Ile	Ser	Ala	Arg	Gln	Glu
A. florea	Ile	Ala	Thr	Asn	Lys	Gln

Figure 2-10. Positions of amino acid residues (Kreil, 1975).

Apamine is a small peptide and neurotoxic properties. It was first isolated by Habermann and Reiz (1965) and contained less than 2% in venom dry weight. The local effects such as burning or stinging pain, swelling and redness. This peptide had specific effects on synaptic functions and acting directly on the central nervous system. Moreover, it have effects to Na^{+} and Ca^{++} channel in several cell and does not present lytic properties (Schmidt, 1982). The isolation can be done by electrophoresis or by chromatography (Figure 2-11). (Hartter and Weber, 1975; Räder *et al*., 1987)

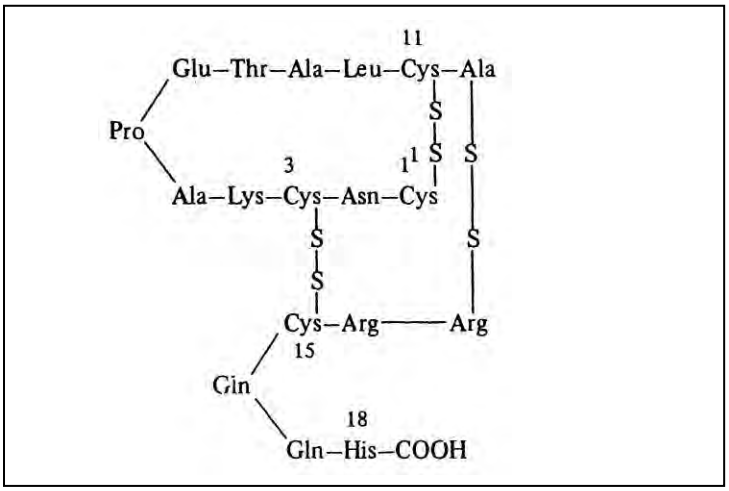

Figure 2-11. Amino acid sequence of apamin (Haux *et al*., 1967).

Mast cell degranulating peptide (MCD-peptide, peptide 401) is highly effective in degranulating mast cells (Fredholm, 1966). The sequence and position of disulfide bond were studied by Billingham *et al*. (1973) (Figure 2-12).

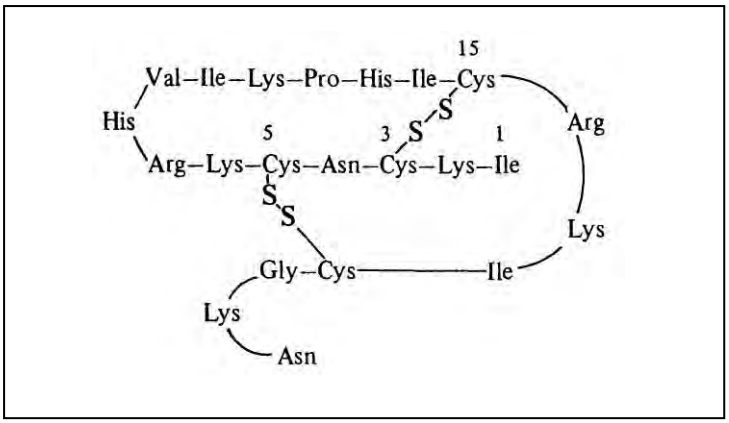

Figure 2-12. Amino acid sequence of MCD-peptide.

Other peptides such as secapin, tertiapin and melittin F were found in *A. mellifera* venom. The percentages of peptide present in whole venom are 45% melittin, 3% apamin, 2-3% MCD-peptide, 1% secapin, <1% tertiapin and <1% melittin F. Procamine is also found in *A. mellifera* venom. It is a polypeptide which contain a histamine residues at the C-terminal. Peck and O'Connor (1974) had been studied and isolated this peptide. The amino acid sequence of procamine is as following (Figure 2-13).

Ala-Gly-Pro-Gln-His

Ala-Gly-Gln-Gly-His

Figure 2-13. Amino acid sequence of procamine (Peck and O'Connor 1974).

Hyaluronidase is an enzyme isolated from bee venom compose of arginine as the N-terminal group. It is known as spreading factor of venom, which helps the diffusion and infiltration of venom among tissues. PLA_2 is also found about 12% of dry weight venom (Han *et al*., 2000). Amino acid sequence of PLA_2 was isolated from bee venom as in Figure 2-14. It is show powerful hemolytic activity which will cause dropping of blood pressure.

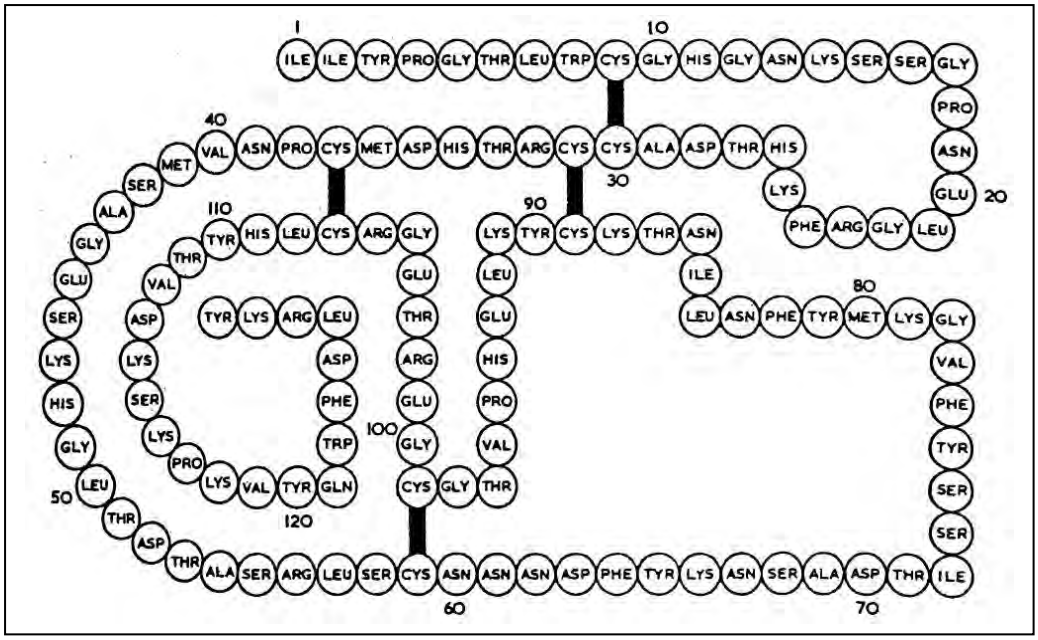

Figure 2-14. Structure of Phospholipase A_2 (Shipolini *et al*., 1974).

Moreover, *A. mellifera* venom was reported to contain enzymes such as esterase and alkaline phosphatase (Benton, 1967). In bee venom, there is protease inhibitor, which protects venom components such as hyaluronidase, PLA_2 and toxic peptides from hydrolysis by proteolytic enzymes of victim (Shkenderov, 1973).

Non peptide components can be found in bee venom such as histamine, free amino acid, carbohydrate, lipid and other biogenic amines. The concentrations of histamine and histidine were related to age of honeybee. These could not be found in newly emerged worker bee. Appearance of these components starts in one-week bees, highly at 3-4 weeks and then decline at 6 weeks old bees (Owen *et al*., 1974).

Amino acids such as alanine, arginine, cystine, glutamic acid, histidine, proline, carbohydrate (glucose and fructose), and phospholipids (about 5% dry weight) were also found in bee venom (O' Conner *et al*., 1967). Dopamine, noradrenaline and acetylcholine were present in bee venom (Owen, 1971)

Natzir *et al*. (1999) discovered a novel venom protein molecular size 50 Kda (p50) in Asian bee (*A. cerana*, *A. nigrocincta* and *A. dorsata*) which had never been reported in the venom of the Western honeybee, *A. mellifera*. Moreover its effect was very similar to PLA_2 of *A. mellifera.* The sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed the main allergic components of *A. mellifera* venom composed of PLA_2**,** hyaluronidase and acid phosphatase.

Africanized bees, *A. mellifera scutellata* contained less venom but have more PLA_2 than European bees, *A. mellifera mellifera*. European bees contained greater than 300 μg of venom and PLA_2 concentration varied between 1.8% and 27.4% (w/w). Concentration of PLA_2, melittin and dry weight of venom from different colonies were also differed (Schumacher *et al*., 1992).

Schmidt *et al*. (1997) found the (Z)-11-eicosen-1-ol is a major component of *A. cerana* venom. Gas chromatography- mass spectrometry (GC-MS) revealed that there are 81.2% eicosenol in venom. Averaging over 250 μg per bee, which is absent or present in small quantities in other parts of the sting apparatus. Meanwhile this component can not be found in venom of *A. mellifera* and *A. dorsata*. Expected function of eicosenol in venom of *A. cerana* may use to mark the enemies or floral resources. So the components of bee venom can be different either same species or different species.

2.2.3 Toxic effect of bee venom

A chemical absorbed into the bloodstream through barriers (skin, lungs and alimentary canal) is distributed, throughout the body, including the site where it produces damage. Toxicant may have one or several target organs and several toxicants may have the same target organ.

Subcutaneously administer toxicants are usually slower absorbed but enter directly into the general circulation (Figure 2-15). The rate of absorbtion by these route can be altered by changing the blood flow to the injection site. Liver and kidney have a high capacity for binding a multitude of chemicals. These two organs probably concentrate more toxicants than other organ obtained. Kidney is a very efficient organ for the elimination of toxicants from body. A toxicant filtered at glomeruli may remain in the tubular lumen and be excreted with urine. Depending on the physicochemical properties of a compound, it may be reabsorbed across the tubular cells of the nephron back into the bloodstream. So toxicants with a high liquid/water partition coefficient are reabsorbed efficiently, whereas polar compound and ion are excreted with urine.

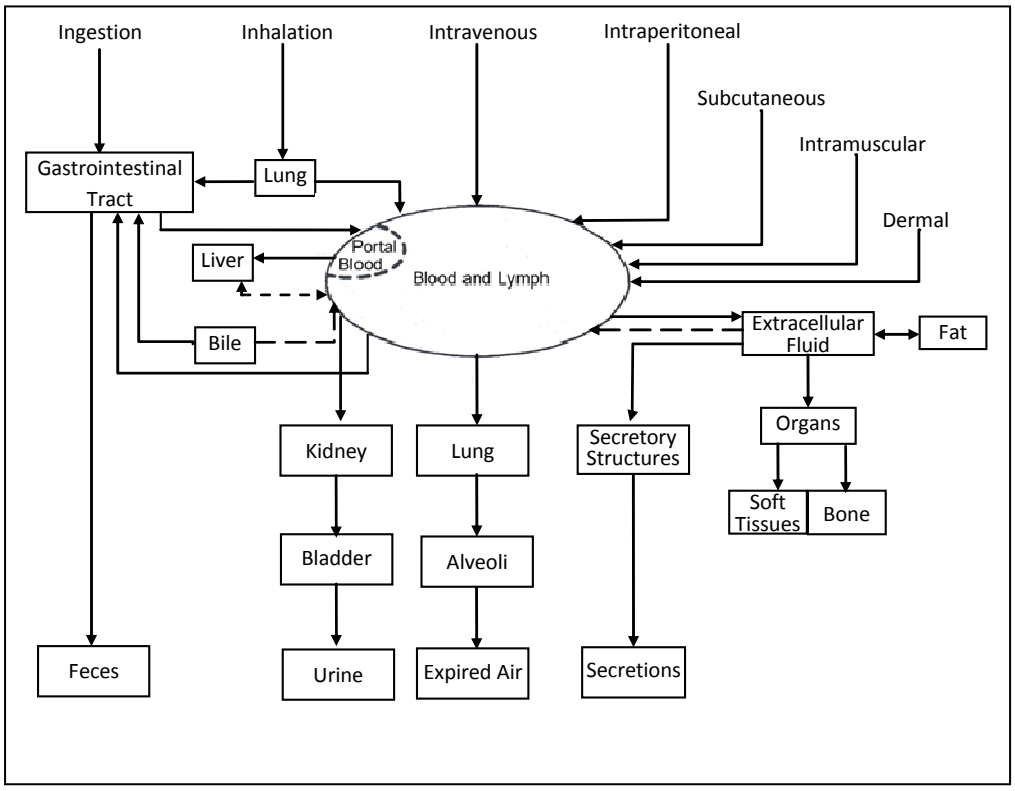

Figure 2-15. Routes of absorbtion, distribution, and excretion of toxicants in the body (Rozman and Klaassem, 2001).

The renal proximal tubules reabsorb small plasma proteins that are filtered at the glomerulus. Thus, if a toxicant binds to these small proteins, it can be carried into the proximal tubule cells and excreted with urine, whereas molecules between 350-700 kDa are excreted in bile. The liver is an organ for removing toxic agents from blood after absorbtion from gastrointestinal (GI) tract, because blood from GI tract passes through the liver before reaching the general circulation. Thus, the liver can extract compounds from blood and prevent their distribution to other part of body (Rozman and Klaassem, 2001).

One or few stings can induce allergic reactions. Meanwhile hundred to thousand stings can cause severe systemic injury on different organ. This cause from mast cell degranulating peptide may induce more endogenous histamine release (Schumacher and Egen, 1995).

Used venom for stinging have different objectives between queen and worker bees. Newly emerged queen used their venom only for stinging other queens. Beside that in 1-2 years queen, found only half lethal to mice when compared with worker bees. Meanwhile the worker bees use venom only to defend their nest and themself (Schmidt, 1995).

Vogt *et al*. (1970); Humblet *et al*. (1982) and de Roodt *et al*. (2005) found that melittin and PLA_2 have a synergism to lyses erythrocytes membranes and muscle cell. Moreover, melittin and PLA_2 can cause haemolysis, kidney tubular degeneration, necrosis and can cause necrosis of skeletal muscle cells within 30 minutes after i.m. injection at 4 μg/g (Grisotto *et al*., 2006). In contrast to previous reports, bee venom components showed independent lethal activities of melittin and PLA_2. Melittin can damage the vascular endothelium and vasoconstriction. Moreover, it can insert itself into phospholipid bilayer of cell membranes which cause hydrolysis and cell disruption.

Meanwhile, PLA_2, may induce renal blood flow impairment by release of vasoconstrictor eicosanoids and catecholamine (Schumacher and Egen, 1995; Koyama *et al*., 2002).

Table 2-1. Toxicitys of *A. mellifera* venom and its components after treated in mice (Anthony, 1984)

Sample	LD_{50} (µ/g)	Route of injection	References
venom	3.5	-	Vick *et al.*, 1974
apamine	15.0	-	Vick *et al.*, 1974
	4.0	i.v.	Habermann and Cheng-Raude, 1975
	5.0	-	Hartter and Weber, 1975
pla_2	0.37	i.p.	Slotta *et al.*, 1971

i.p. is intraperitoneal; i.v. is intravenous; i.c. intracerebral

Radiotracer is the method for studies of the bioavailability process, and kinetics of any molecule which labeled with an isotope. Analysis of the data that obtained from *A. mellifera* venom demonstrated that the kidney and liver showed higher radiotracer concentration from observed time, while spleen, heart, and brain showed lowest quantities. The quantities found in the liver suggested that a hepatic metabolism may be happened. The venom radioactivity profile detected in the kidneys over 24 hours suggested that it may excrete the toxin or some of its metabolites. It was expected that the kidney would be the main elimination route. This fact may be caused by the venom nephrotoxicity. Therefore, any venom compound could have hepatic and renal metabolism (Yonamine *et al*., 2005)

When the immune system response to insect's venom, an allergic reaction will occurs. Immunoglobulin E (IgE, antibody) was produced from leucocytes and attaches to protein in venom. The release of

histamine and other chemicals were occured and showed allergic symptoms. Histamine can induce vasodilation of arterioles and constriction of bronchioles in the lung, also known as bronchospasm.

There are many reports about components toxicity of *A. mellifera* in mice, which revealed in LD_{50} (Table 2-1). The composition and lethality of venom are similar in European and Africanized bees (Schmidt, 1995; Schumacher *et al*., 1990). Azevedo-Marques *et al*. (1991) reported that Africanized bee venom can cause damage to skeletal muscle of Wistar rats by intramuscular (i.m.) injection. In rats treated with bee venom by i.m. injection revealed acute inflammatory reaction which followed the report in experimental treated with snake venom (Gutierrez and Lomonte, 1989). Meanwhile in rats treated with intravenous (i.v.) injected bee venom showed damaging in skeletal muscle at a distance from the injection site (lateral tail vein), which not found in i.m. injection route. However, in i.v. injected route the rats can longest survival from 70 seconds to 13 minutes. On the other hand, in rats i.m. injected route can survive until sacrifice at 4, 7 and 24 hours. Total creatine kinase (CK) was measured in both groups. It showed highly CK level at the first period of both i.m. and i.v. route before slightly at 24 hours. Increasing of CK level and acute renal tubular necrosis were found in victim of multiple Africanized bee stings (Mejia *et al*., 1986; Patrick *et al*., 1987). Franca *et al*. (1994) found that creatinine level increase as soon as 2 hours in patients after attacked with bee venom.

Oliveira *et al*. (2007) reported the kidney of dog treated with Africanized bee venom showed degeneration tubular necrosis, severe liver congestion and adrenal haemorrhage.

2.3. HPLC technique for purification of bee venom

Purification of proteins

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), reverse-phase high pressure liquid chromatography (RP-HPLC), ion-exchange chromatography, and Gel permeation chromatography (GPC) are the several methods for purification of proteins. Individual protein components from these methods will be identify by Mass spectrometry technique. The success of HPLC separation depends on the appropriate choice of column dimensions, column packing, mobile phase, and gradient. The speed, simplicity, extremely high resolving power, and compatibility with mass spectrometry detection of RP-HPLC cause it was preferred technique for the fractionation of protein digests (Dass, 2001).

The different columns for chromatographic separation conditions were used in research about chemical composition of various venom in HPLC separation or in capillary electrophoresis and at obtaining a high-degree of purity of the separated components (Ameratunga *et al*., 1995; Dotimas and Hider, 1987). The best separation of the bee venom protein fraction was obtained on 180 and 300 A° pore size columns at a temperature of 25C°. Bee venom was analyzed for major protein fractions. Average of major proteins were measured as melittin 64.40%, PLA_2 13.00% and apamine 3.10%. It is interesting that the average of melittin was found significant differences in year-to-year (Rybak-Chmielewska and Szczęsna, 2004). The Averaged melittin was differ from the report of Szokan *et al*. (1994) which found 50-60%.

Dotimas *et al*. (1987) found that gel permeation chromatography on Sephadex G-50 and reverse-phase HPLC possible to isolate melittin (<1 in 10^7) and phospholipase (<1 in 10^5) in high yield. Molecular weight of primary proteins (melittin and apamine) in wasp *Pimpla turionellae*

venom components were estimated by SDS-PAGE and RP-HPLC (Uckan *et al*., 2004).

Chen *et al*. (2006) had been isolated and purified five subfractions from whole bee venom by gel chromatography (GC) and RP-HPLC. After separation both matrix-assisted laser desorbtion/ionization time-of-flight mass spectrometry (MALDI-TOF) and amino acid sequence analysis were used to identified the active polypeptide in the fractions. This result indicated that whole bee venom composed of four active peptide components, which characterized as apamin, MCDP, PLA_2-related peptide and melittin.

Information of *A. florea* venom components and its effect on a victim is still unknown in Thailand. So this research will provide basic knowledge and increasing medicinal value of natural product from the native species of honeybee.

CHAPTER III

MATERIALS AND METHODS

3.1 Preparation of bee venom

3.1.1 Collecting of *A. florea* venom

Venom collecting method was applied from Funari *et al*. (2001). Five hundreds of *A. florea* workers were anesthetized by freezing. Sting and venom sac from 100 bees were pulled out and punctured to get venom on a glass slide under stereo microscope with cool light. The venom on slide was kept dried about 2 days at room temperature. The dried venom from each slide then scratched and weighted. Mean amount of venom per bee was calculated from the 5 replications. The dried venom was stored in dark at -20°C until used.

3.1.2 Components of *A. florea* venom

Analytical standards of venom compounds apamine, PLA_2 and melittin were isolated from *A. mellifera* venom; $\geq$ 95% (HPLC). These standards composed apamine 0.5 mg, melittin 250 μg and phospholipase A_2 1 mg. The concentration used in chemical analyzed was 20 mg crude bee venom dissolved in 0.01 M acetic acid solution (20mg/ml). Analytical standards of venom were also dissolved in 0.01 M acetic acid solution. Centrifuged at 10,000 rpm for 5 min at 4°C and kept supernatant in microcentrifuge tube which wraped with aluminium foil. Filtered by 0.45 μm syring filter nylon before analysis of the venom components by RP-HPLC. Each RP-HPLC subfractions were kept at -20°C before identifying the molecular weight and amino acid sequence by MALDI-TOF Mass spectrometry.

3.1.3 Effects of *A. florea* venom on rat

The venom was dissolved in PBS (Phosphate buffer saline). Concentration of bee venom at 0.5 and 0.25 mg/kg body weight in 0.1 ml PBS were used. The solution was filtered through a 0.45 μm syringe filter nylon before used.

3.2 Chemical analysis

The purification of fractions was performed by C18 RP-HPLC column (4.6 x 250 mm, Apollo C18 5u) equipped on a HPLC system (autosampler 410). Analytical standard compound of bee venom; apamine, melittin and phospholipase A_2 were supplied by Sigma Chemicals Co. (St Luis, MO, USA). Acetonitrile was obtained from Sigma, trifluoroacetic acid was obtained from Aldrich Chemical Co. (Milwaukee WI USA) and membrane filters 0.45 μm were manufactured by Pall Gelman Laboratory (USA). The eluents employed were 0.1 % trifluoroacetic acid (TFA) as solvent A and acetonitrile with 0.05% TFA as solvent B. Elution was performed with linear gradients of solvent B in solvent A at flow-rate of 0.7 ml/min. Detector was UV spectrophotometry (UV-VIS detector 335) at 220 nm.

The molecular weight and amino acid sequence of each RP-HPLC subfractions were identified by MALDI-TOF Mass spectrometry (PRF-S04, ultraflex TOF/TOF). Sample fraction about 0.5–1.0 ml was used for each sequence run. Mascot search result was used for identification of peptide mass fingerprint.

3.3 Animals

This experiment was done under the protocol review no. 0823002. The male albino wistar rats which obtained from Nation Laboratory

Animal Centre, Mahidol University were used in this study. They were kept in Laboratory Animal's house, Department Biology, Faculty of Science Chulalongkorn University. Body weights of rat were about 230-250 g. Five rats were housed in a stainless steel cage with solid bottom and open top under strictly hygienic conventional as, temperature 25±1 °C, relative humidity 50 ± 10 % and standard fluorescent 12:12 hr light-dark cycle. Cage size was 24x45x14 cm. (W x L x H). Drinking fluid was tap water. Standard diet was C.P. mice feed on ad libitum and changed every days. Bedding was sawdust that changed every 3 days.

The rats were randomly divided into three groups. Each group contained 15 male rats. In control group, rats were injected with s. c injection of PBS 0.1 ml. Two treatment groups, the rats were injected with s.c. injection of 0.25 and 0.5 mg bee venom/kg body weight in 0.1 ml PBS respectively. Injection area was back neck subcutaneous layer. Liver, kidney and blood tissues were collected at 2 (n=5), 8 (n=5) and 24 (n=5) hrs. after treating with bee venom for histological technique.

3.4 Histological study

The rats were anesthetized by using 99.7 % diethyl ether until heart rate and movement could not detect. Liver and kidney tissues of each groups were collected at 2 (n=5), 8 (n=5) and 24 (n=5) hrs. after treated with bee venom. Slices of the left liver lobe and left kidney from five rats of each group were fixed in 10% formalin buffer for 24 hrs. before preserved in 70% ethanol. Then routine paraffin technique was used and tissues were embedded in paraffin at 6 μm sections. The slices were routinely stained with haematoxylin and eosin (H&E) and observed under a light microscope. All alterations from the normal structure were observed. The following criterias were used for scoring liver and kidney

histology. The characteristic: -, not found any change in rats of a group; +, mild to moderate change in rats of a group; ++, severe changes in rats of a group.

3.5 Blood collecting

Animals were get rid off any food except water about 8 hours before collecting the blood. Syringe size 3-10 ml and Needle 21 G were used to collect the blood.

3.5.1 Haematology

The rats were anaesthesied by 99.7 % diethyl ether until deep sleep. Blood was collected from cardiac puncture about 1 ml in microcentrifuge tube size 1.5-2.0 ml and mixed with K_3-EDTA 5 μl/1 ml and stored at 2-8 **°C** until used. A part of blood was smeared as thin film on the glass slide and dried at room temperature. Blood smear on slide was fixed by methanol about 4 minutes and then stained with Giemsa stain. Finally, blood characters and complete blood count (CBC) were investigated including amount of red blood cell (per μl), hemoglobin (g/dl), % hematocrit, platelets (per μl), blood morphology, white blood cell (per μl) and % differential count (neutrophils, eosinophils, lyhmphocytes and monocytes) which were analyzed by Faculty of Veterinary Science, Chulalongkorn University.

3.6 Statistical analysis

Statistical analysis was performed using the One way ANOVA. Analysis of variance venom effects on number of Kupffer cells around the central vein of liver tissue the central vein of liver tissue and hematology analysis data were done. In order to discern the different between certain parameters, ANOVA post hoc analysis (Duncan or

Brown-Forsythe test) and multiple comparison (LSD) were used. P value < 0.05 was considered to be statistically significant. All statistical calculations were done using SPSS 16.0 computer program.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Amount of *A. florea* venom

Average dried weight of *A. florea* venom from this experiment was 0.016 ± 0.0004 mg per bee as shown in Table 4-1.

Table 4-1. Dried weight of *A. florea* venom (mg)

Time	Dried weight *A. florea* venom (mg)	
	100 bees	average/ bee
	mg	mg
1	1.60	0.0160
2	1.50	0.0150
3	1.70	0.0170
4	1.70	0.0170
5	1.50	0.0150
Mean	1.60	0.0160
± S.E.	0.04	0.0004

Previously, venom collection by electrostimulation to learn more about action of toxin and transport mechanism was reported (Yonamine *et al*., 2005) However, the electrostimulation apparatus was not suitable to collect venom of *A. florea* in this study, because of the different habitat of honeybees. The amount of dried venom of *A. florea* in this study was lesser than the same species that collected in Thellua, Sri Lanka (9.5 mg dried weight, n=56) (Funari *et al*., 2001). However, the amount of bee venom was related to the size of honeybees (Schmidt, 1995). *A. florea* has lesser venom quantity when compare with Africanized honeybee (*A. mellifera scutellata* 0.117 ± 0.015 mg), European hybrid (*A. mellifera ligustica* 0.139 ± 0.0020 mg) and *A. mellifera carnica* (0.147 ± 0.023 mg) by the same method of venom quantity in venom sac (Funari *et al*., 2001).

Although Africanized honeybee produces the less venom than European honeybee, but they liberated larger venom in the extraction apparatus (Schumacher *et al*., 1992 and Funari *et al*., 2001). The amount of venom when bee stinging in each time is not depend on the volume of venom in venom sac.

4.2 Components of *A. florea* venom

Dried venom of *A. florea* was analyzed by RP-HPLC (C18, RP-HPLC column (4.6 x 250 mm, Apollo C18 5u), solvent A: 0.1 % trifluoroacetic acid (TFA), solvent B: acetonitrile 0.05% TFA and flow-rate 0.7 ml/min). Elution was performed with linear gradients. Standard component of bee venom in this study composed of apamin, pla_2 and melittin from *A. mellifera* venom. The separated fraction of major components were eluted at retention time 18.02 minutes (apamin), 31.04 minutes (phospholipase A_2) and 39.39 minutes (melittin) (Figure 4-1).

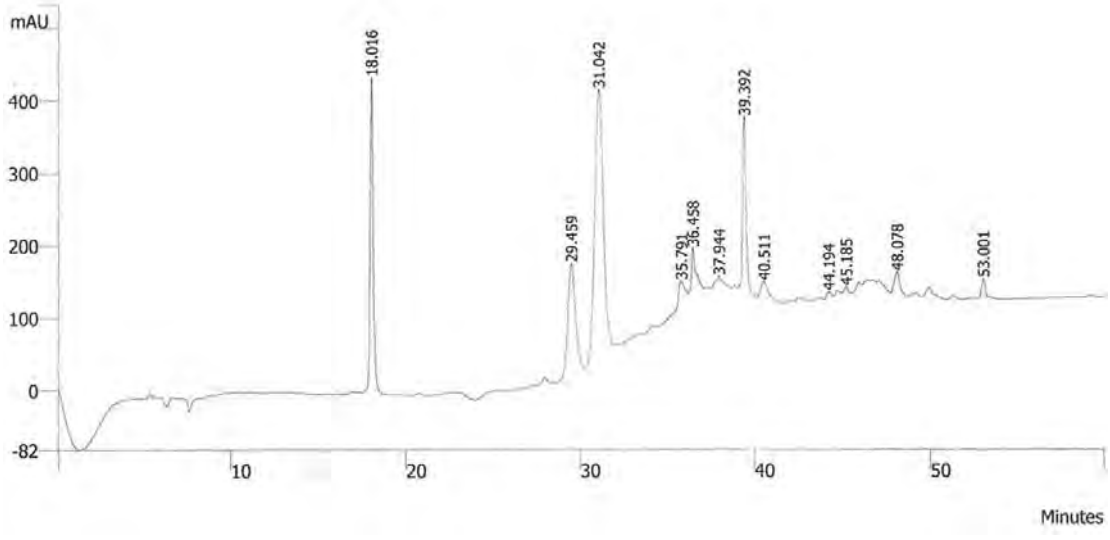

Figure 4-1. Major fraction of bee venom, *A. mellifera* component by RP-HPLC.

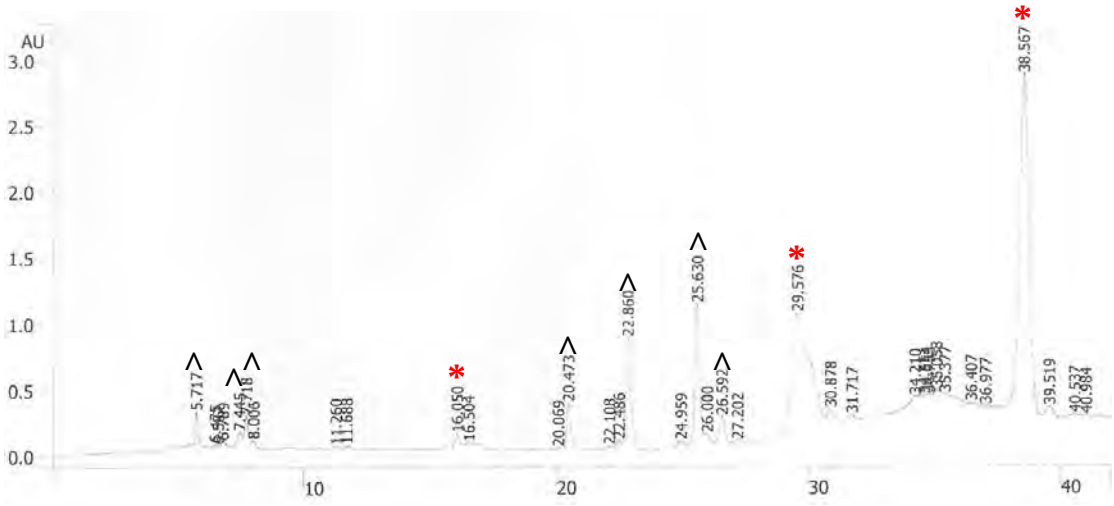

Figure 4-2. Separated fraction of bee venom, *A. florea* component by RP-HPLC.

Rybak-Chmielewska and Szczesna (2004) found that the average retention time of apamine, pla_2 and melittin were 8.62 $\pm$ 0.13 min, 20.47 $\pm$ 0.37 min and 28.08 $\pm$ 0.34 min, which using the DISCOVERY® C18, 180 A° column for linear gradient elution 5% B-80%B for 40 min, flow rate of mobile phase 2.0 ml/min and separation temperature 25C°. Chromatographic separation was performed using the following eluents: A, 0.1% TFA in water and B, 0.1% TFA in the solution acetonitrile: water (80: 20). UV detector at 220 nm wave length was used for the best separation of bee venom protein fraction. The different of retention time between major components in *A. mellifera* venom of previous studied and in this study were about 10.3 min.

Three fractions of *A. florea* venom in this study showed relative retention time with standard at 16.05, 29.58 and 38.57 min (Figs 4-2 with * sign). This study had used the same condition to analyse in *A. florea* and standard of *A. mellifera* venom. From the relation of the retention time it revealed that the frist fraction was apamine, the second fraction was PLA_2 and the third was melittin. Different of retention time between *A. florea* and *A. mellifera* in the same condition was about 1-2 minutes.

Meanwhile the retention time of *A. mellifera* major component in previous study was shorter than *A. florea* about 8.6 min. The differences in retention time of major components in *A. mellifera* venom from previous study and in this study were probably the different column and used condition.

In each protein major peak from *A. florea* could calculated area as 0.07 % for apamine, 8.88 % for pla_2 and 11.95 % for melittin. *A. florea* venom showed that melittin is the highest and then is PLA_2 and apamine, respectively (Table 4-2). This suggested that melittin is the main component in *A. florea* likes as in *A. mellifera* which Schmidt (1995) had been described is the main component in honeybees. The highest seven subfractions, which excepted for three major components, were collected at retention time 5.71, 7.45, 7.72, 20.47, 22.86, 25.63 and 26.59 min respectively (Figs 4-2 with ^ sign).

Table 4-2. Bee venom components compared between *A. mellifera* (Rybak-Chmielewska and Szczesna 2004) *and A. florea* from this study.

Bee venom components	***A. mellifera* (%)**	***A. florea* (%)**
apamin	**3.10**	**0.07**
PLA_2	**13.00**	**8.88**
melittin	**64.40**	**11.95**

Then seven subfractions from RP-HPLC were identified for the molecular weight and amino acid sequence determination by MALDI-TOF Mass spectrometry. Type of search for amino acid sequence determination was peptide mass fingerprint. Trypsin was used to be the digest enzyme. Seven subfractions were identified and showed as chromatogram in figures 4-3 - 4-9 (A)

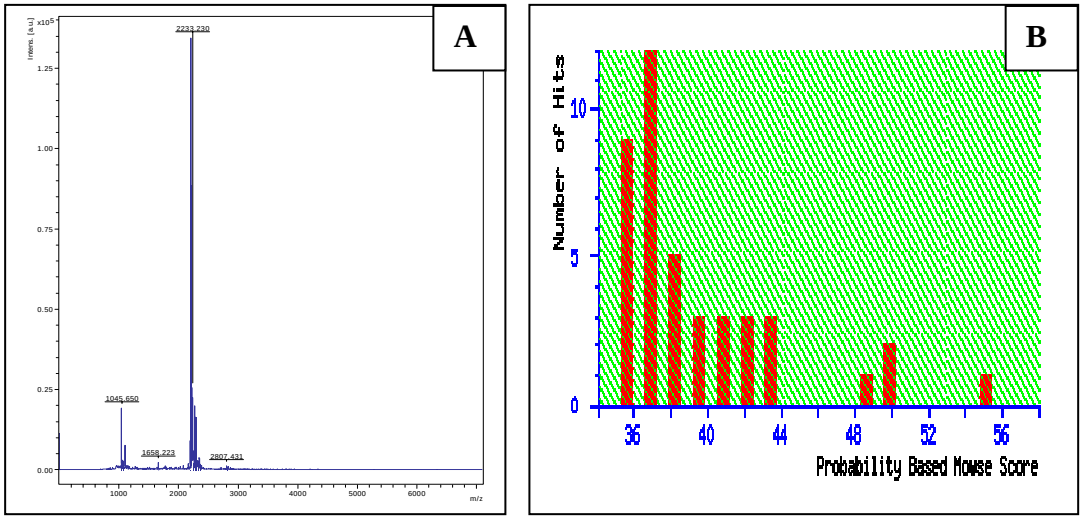

Figure 4-3. Chromatogram of fraction 1 (A) and Mascot search results of fraction 1 (B)

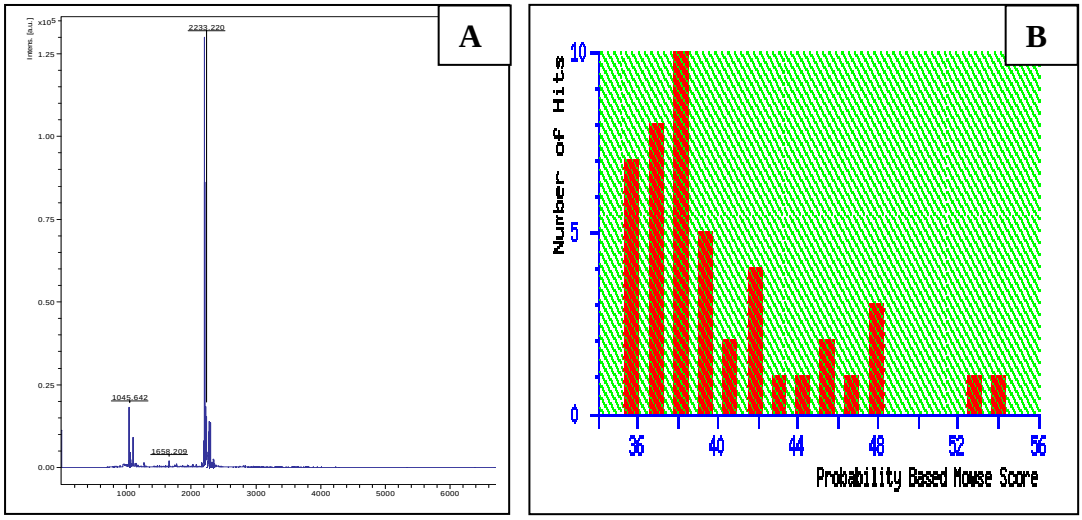

Figure 4-4. Chromatogram of fraction 2 (A) and Mascot search results of fraction 2 (B)

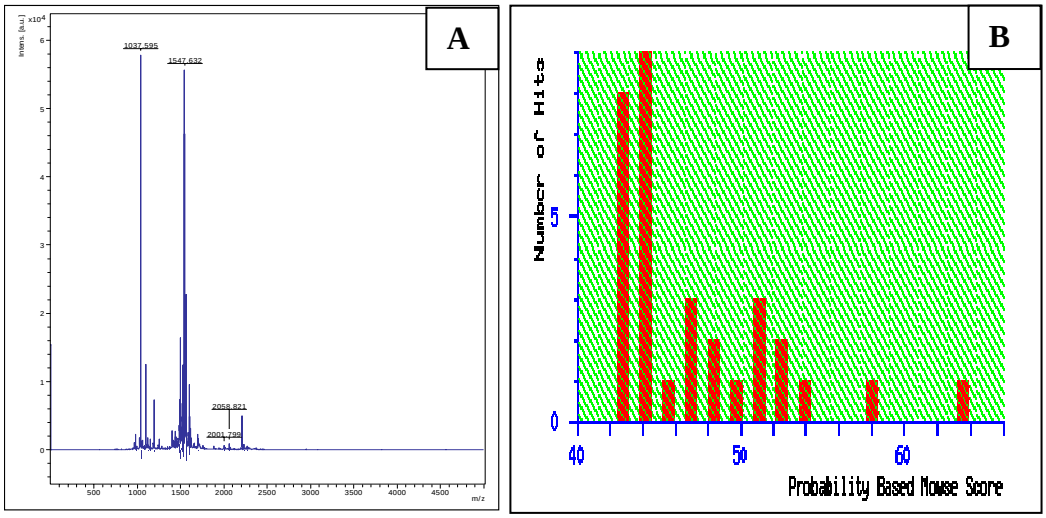

Figure 4-5. Chromatogram of fraction 3 (A) and Mascot search results of fraction 3 (B)

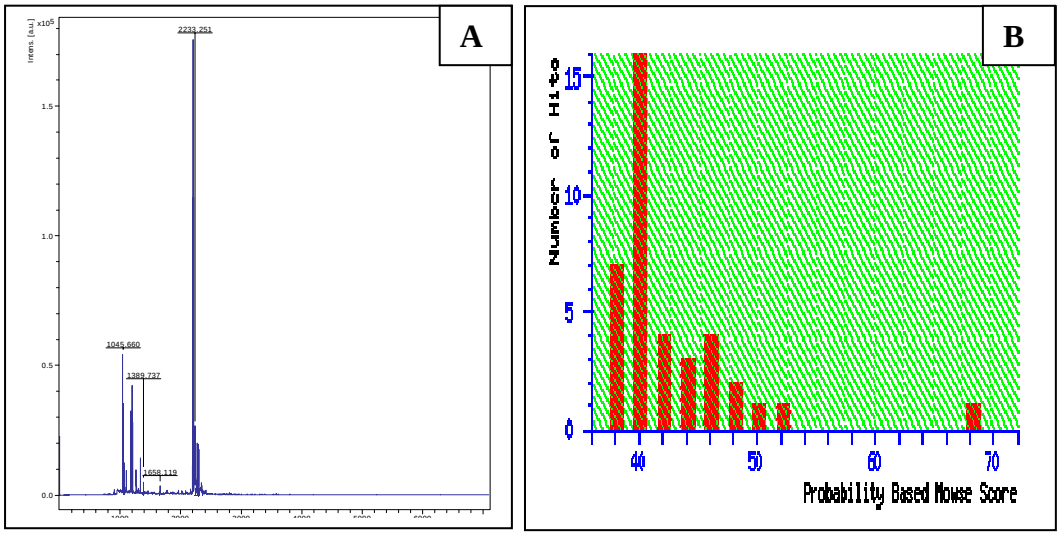

Figure 4-6. Chromatogram of fraction 4 (A) and Mascot search results of fraction 4 (B)

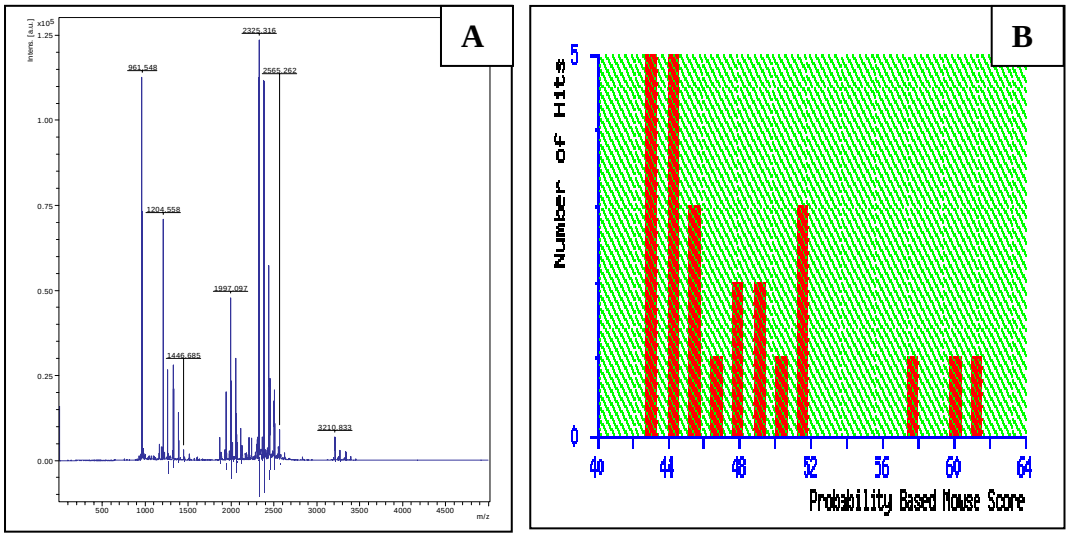

Figure 4-7. Chromatogram of fraction 5 (A) and Mascot search results of fraction 5 (B)

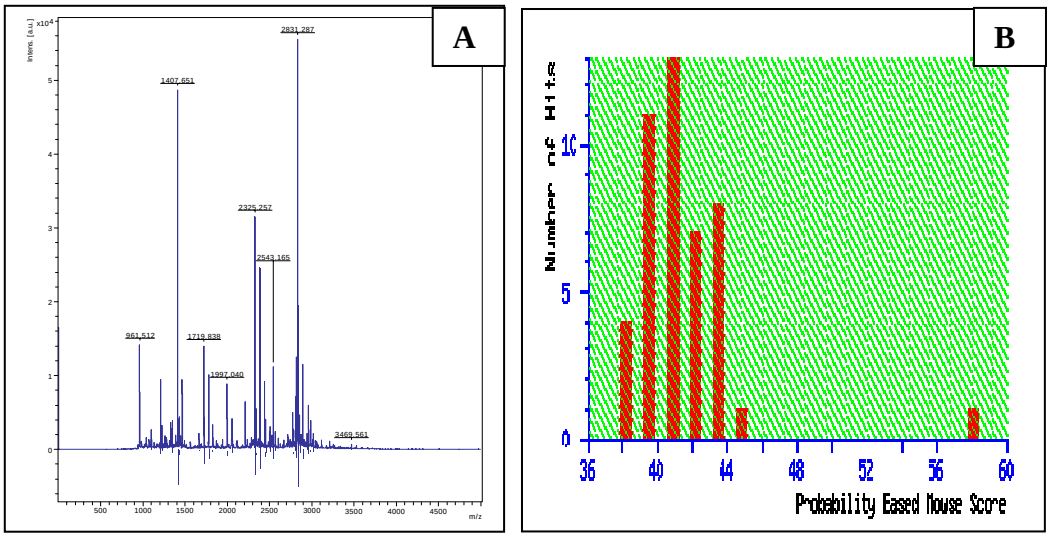

Figure 4-8. Chromatogram of fraction 6 (A) and Mascot search results of fraction 6 (B)

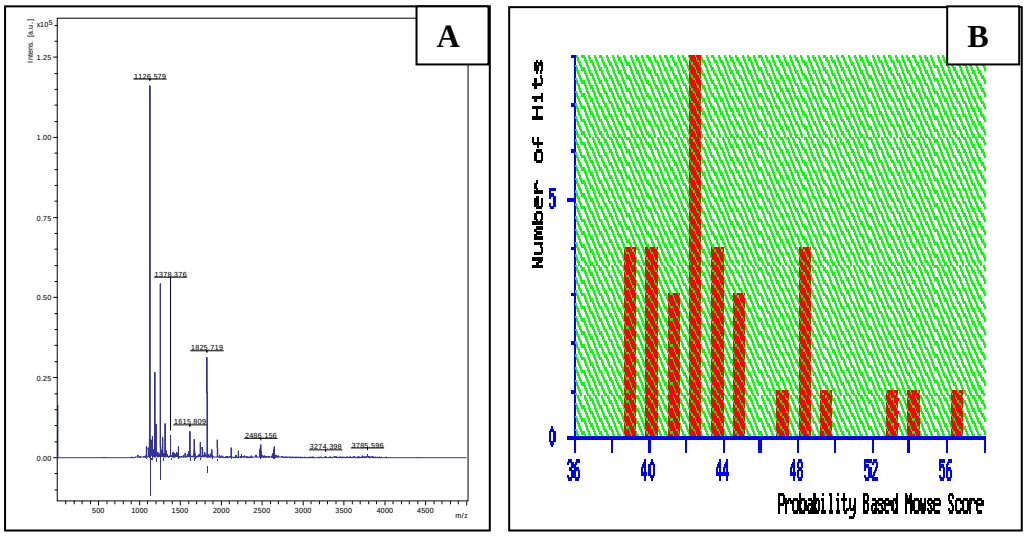

Figure 4-9. Chromatogram of fraction 7 (A) and Mascot search results of fraction 7 (B)

Mascot search results from the matrix science showed the probability based mowse score of each subfraction in figures 4-3 to 4-9 (B). Protein score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 75 are significant ($p<0.05$). However, all of subfractions were not matched in group of database protein venom.

4.3 Effects of *A. florea* venom on rats

4.3.1 Effects of *A. florea* venom on liver tissues

The liver of control rats showed normal structure (Figs 4-11 to 4-13 : A). The tissues compose of hexagonal lobules with central vein and separated by sinusoid containing a few Kupffer cells. There was clearly different characters between control and treatment groups. The groups treated with 0.25 and 0.5 mg/kg bee venom in 0.1 ml PBS showed almost sinusoids overfilled with erythrocytes and there were numerous of Kupffer cells at the walls of most sinusoids (Figs 4-11 to 4-13 : B and C).

The different time points at 2, 8 and 24 hrs. after treated with bee venom were noted. Both treatment groups showed clearly different of

severe blood congestion and numerous number of Kupffer cells at time point of 2, 8 and 24 hrs. compared with the control (Table 4-3).

Table 4-3. Effects of *A. florea* venom to liver tissue of rats.

Groups	Time points	Fatty droplets	Blood congestion
Control	**2 (n=5)**	**-**	**+**
0.1 ml PBS	**8 (n=5)**	**-**	**-**
	24 (n=5)	**++**	**+**
Treatment 1	**2 (n=5)**	**-**	**++**
0.25 mg/kg bee venom	**8 (n=5)**	**-**	**++**
	24 (n=5)	**-**	**++**
Treatment 2	**2 (n=5)**	**+**	**++**
0.5 mg/kg bee venom	**8 (n=5)**	**-**	**++**
	24 (n=5)	**-**	**++**

The characteristics: ++, severe changes in rats of a group; +, mild to moderate change in rats of a group; -, not found any change in rats of a group.

Blood congestion or sinusoidal congestion indicated the severe inflammation occured in liver tissues which cause from toxin (El-Arab *et al*., 2006). This character was also found in a control group but less than in the both treatment groups. All the time points also showed in the same way between treated groups (Figs 4-21: A, B and C).

Fatty droplets were a little found in hepatocyte of some samples but there was not difference between control and treatment groups (Table 4-2 and Figs 4-11: C). A few fatty droplets were found but not different among the group. Severe fatty droplets character was show in little rats number of control group. Meanwhile the normal structure were showed in many rats of both treatment groups (Figs 4-20: A, B and C). This result may be related with the role of the fat storing cell in rat liver. Fatty droplets in the cytoplasm were significantly increased in number at two weeks after treated with fat emulsion intravenously (Hirohiko and Kyuichi, 1972). Although, fatty droplets were found in some groups, it was not difference and need more information to explain.

Amount of Kupffer cells in the group treated with 0.25mg/kg were higher than control at 2 hrs. The highest was found at 8 hrs and then declined at 24 hrs. Meanwhile the group treated with 0.5 mg/kg showed high number at 2 hrs, lightly declined at 8 hrs and then increased again at 24 hrs (Figs 4-10 and Table 4-4).

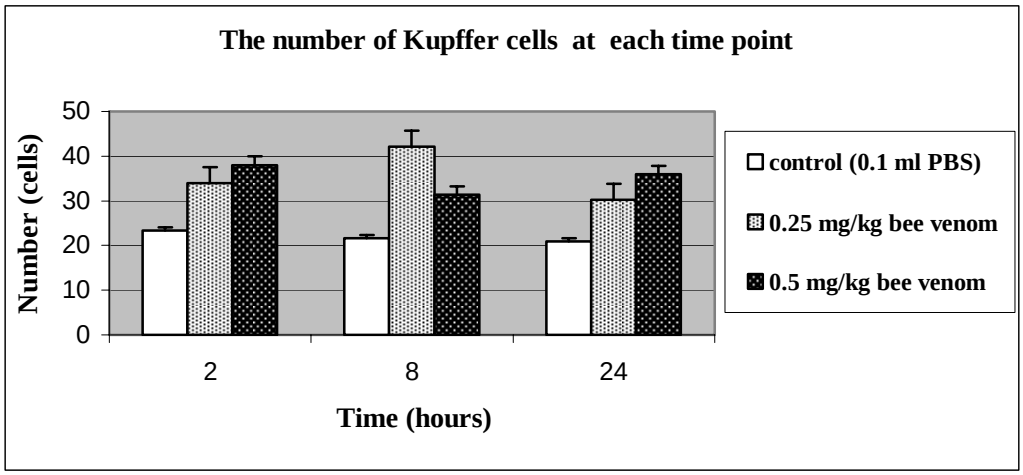

Figure 4-10. The number of Kupffer cells at 2, 8 and 24 hrs. after treated with 0.1 ml PBS (control), 0.25 mg/kg and 0.5 mg/kg bee venom.

Increasing of Kupffer cells were found in 2 hrs. of both treatment groups. Kupffer cells have been reported to contribute to injury during ischemia (Tsung *et al*., 2005; Ito *et al*., 2003; Laskin *et al*., 1986). Inhibition of Kupffer cells function or depletion of Kupffer cells appears against liver injury from the immunostimulants concanavalin A and Pseudomonas exotoxin (Schumann *et al*., 2000). On the other hand, depletion of Kupffer cells brought about the apoptosis of sinusoidal cells which increases liver injury from partial hepatectomy (Prins *et al*., 2004; Toshinori *et al*., 2001). Rapid necrosis occurred with Kupffer cells as reported by Manickan *et al*. (2006). Kupffer cells in mice, which adenovirus injected intravenously, were decreased by 4 hrs. after injection cause from rapid necrosis. The decreasing number of Kupffer cells at 8 hrs. after treated with the 0.5 mg/kg venom may be a cause of

rapid necrosis. Increasing of Kupffer cells at 24 hrs. may showed the activity of Kupffer cells to phagocyte the rest venom for recovery from the liver tissue injury.

Table 4-4. The number of Kupffer cells in control and treatment groups.

Groups	Time points	Kupffer cells (cell)
Control 0.1 ml PBS	**2 hours**	**23.16 ± 0.81**
	8 hours	**21.58 + 0.41**
	24 hours	**21.34 ± 0.69**
Treatment 1 0.25 mg/kg bee venom	**2 hours**	**33.96 ± 0.92***
	8 hours	**42.16 ± 0.89$^{*, a}$**
	24 hours	**30.24 ± 1.33$^{*, a, b}$**
Treatment 2 0.5 mg/kg bee venom	**2 hours**	**37.50 ± 0.68$^{*, **}$**
	8 hours	**31.32 + 0.76$^{*, **, a}$**
	24 hours	**35.92 ± 0.69$^{*, **, b}$**

mean ± S.E.

the mean difference is significant at the 0.05 level : ^{a} compared to 2 hrs., ^{b} compared to 8 hrs., * compared to control, ** compared to treatment 1,

4.3.2 Effects of *A. florea* venom on kidney tissues

Normal structure of the cortex, medulla and tubular epithelium of kidney were observed in the control rats. It was showed normal structure of the tube with the relatively low simple cuboidal epithelium contained clearly spheroidal nucleus of tubule. There was not found haemorrhage in the cortex (Figs 4-14 to 4-16: A) and medulla of kidney (Figs 4-17 to 4-19: A and B).

In both treatment groups, haemorrhage in cortex and medulla, red blood cells in tubule and pycnotic nucleus were observed (Table 4-5). After treated with 0.25 and 0.5 mg/kg bee venom, the capillaries filled with erythrocytes in the both cortical and medullary parts of the kidney (Figs 4-14 to 4-16, cortical: B and C, Figs 4-17 to 4-19, medulla: C, D, E and F).

All the time point of both treatments also showed severe haemorhage between tissue and ducts. It followed to the report of El-Arab *et al*. (2006) which found toxin may cause many red blood cells in vascular channels of kidney tissues. In treated groups many of rats showed severe haemorrhage in both cortex and medulla. Meanwhile, mild to moderate of haemorhage were found in a few rats among the groups (Figs 4-22 and 23: A, B and C).

Beside that in medullary part showed small number of red blood cells in some tubules included collecting ducts. All time points showed a few red blood cells in some tubules. Both treatment groups also found mild to moderate of this characters in many rats (Figs 4-17 to 4-19 : D and F and Figs 4-24: A, B and C). Decrease of renal function closely correlated with the presence of red blood cells casts in tubular lumina and the occurance of tubular necrosis (Praga *et al*., 1985; Yasuaki *et al*., 2001).

Table 4-5. Effects of *A. florea* venom to kidney tissue of rats.

Groups	Time points	Cortex	Medulla		
		Haemorrhage	Haemorrhage	RBCs in tubule	Pycnotic nucleus
Control 0.1 ml PBS	2 (n=5)	-	-	-	-
	8 (n=5)	-	-	-	-
	24 (n=5)	-	+	-	-
Treatment 1 0.25 mg/kg bee venom	2 (n=5)	++	++	+	++
	8 (n=5)	++	++	+	++
	24 (n=5)	++	++	+	++
Treatment 2 0.5 mg/kg bee venom	2 (n=5)	++	++	+	++
	8 (n=5)	++	++	+	++
	24 (n=5)	++	++	+	++

The characteristics: ++, severe changes in rats of a group; +, mild to moderate change in rats of a group; -, not found any change in rats of a group.

Meanwhile pycnotic nucleus of tubule cell had showed severe character which observed in almost rats of both treatment groups and all the time points (Figs 4-17 to 4-19: C, D, E and F). Although, number of rats , mild to moderate of pycnotic nucleus were found in all group but severe character was not found in control group (Figs 4-25: A, B and C). Histological changes in cortex and medulla of treatment groups were more serious than those observed in a control group.

The previous studied on mice treated with *A. mellifera* venom showed decreasing of adenosine deaminase (ADA), collagen, histamine, IgE, myoglobin and nerve growth factor (NGF) level (Lipps, 2002). Especially, ADA deficiency resulted in combined immunodeficiency by a severe T, B, NK cell lymphopenia, kidney, adrenal and chondroosseous tissue alterations (Ratech *et al*., 1985). ADA deficiency was also associated with bony and renal abnormalities, hepatocellular damage, neurological disorders and pulmonary insufficiency (Bollinger *et al*., 1996). Inflammatory cell of hepatic and renal histopathology showed pycnotic cell, numerous of Kupffer cells and some blood sinusoid appeared to be filled with erythrocytes (Ebaid *et al*., 2007). Injection of

bee venom into rats can increase circulating levels of corticosterone which cause to anti-inflammatory (Couch and Benton, 1972). Lorenzetti *et al*. (1972) found that subcutaneous injection 4 mg/kg of bee venom is beneficial in adjuvant-induced arthritis in rats.

4.3.3 Effects of *A. florea* venom on blood tissues

Normal character of red blood cells were found in both treatment groups. The results of complete blood count were showed in Table 4-6.

A. florea venom also was not showed effect to number of red blood cell and white blood cells in treated groups (Figs 4-26 and 4-29). Increasing of hemoglobin and hematocrit were found at 8 hrs. after treated with bee venom. The concentration of 0.5 mg/kg bee venom showed highly increase when compared to 0.25 mg/kg bee venom and control group. They were not different from group treated with 0.25 mg/kg bee venom and control at 24 hrs. (Figs 4-27 and 4-28). This increasing was related with the component of bee venom which caused vaso-dilation and increasing vascular permeability. This allows plasma to leak into the interstitial spaces (out side of the vessels). This fluid loss further decreases the intravascular volume available for the heart to pump. The combined effects of the vaso-dilation and the vascular permeability results in an acute state of shock (Jackie, 2008). Bee venom has effect on blood and blood vessels. It is not only increase blood circulation but also cause blood cells transmigrate into the tissues after bee venom injection. The venom opens capillary wall, thus allowing the body a better elimination of waste matter as increased metabolism, which enriches the supply of oxygen within body. Better circulation and intensified oxidation also help to destroy bacterial growth.

Table 4-6. Mean of complete blood count in each group.

Time	group	R.B.C. per µl	Hemoglobin g/dl	Hematocrit %	Platelets per µl	W.B.C. per µl	Differential count %			
							N	E	L	M
2 hrs.	Control 0.1 ml PBS	5.36e6± 1.166e5	13.40 ± 0.25	37.40 ± 0.87	5.24e5 ± 1.730e4	4780.00 ± 247.79	21.00 ± 1.58	1.40 ± 0.60	75.80 ± 1.66	1.80 ± 0.49
	Treatment 1 0.25 mg/kg bee venom	5.36e6+ 1.631e5	13.20 ± 0.37	37.60 ± 1.03	4.53e5 ± 6120.457*	4960.00 ± 587.02	37.60 ± 3.28*	0.40 ± 0.25	58.60 ± 2.98*	3.00 ± 0.71
	Treatment 2 0.5 mg/kg bee venom	5.64e6 ± 1.208e5	13.20 ± 0.49	37.60 ± 1.36	4.78e5 ± 2.188e4	4880.00 ± 333.77	39.00 ± 5.51*	1.00 ± 0.45	57.80 ± 5.96*	1.8 ± 0.37
8 hrs.	Control 0.1 ml PBS	4.90e6± 6.325e4a	12.20 ± 0.20a	34.40 ± 0.25a	5.08e5 ± 3.723e4	5120.00 ± 251.79	27.40 ± 2.36a	2.00 ± 0.71	69.40 ± 1.86a	1.20 ± 0.20
	Treatment 1 0.25 mg/kg bee venom	5.54e6+ 1.400e5*	13.20 ± 0.37*	36.80 ± 0.74*	5.30e5 ± 1.000e4	5880.00 ± 643.74	43.60 ± 3.98*	0.60 ± 0.40	53.80 ± 3.67*	2.00 ± 0.45
	Treatment 2 0.5 mg/kg bee venom	5.93e6 ± 1.184e5*	14.40 ± 0.25*,**	40.00 ± 0.55*,**	4.02e5 ± 7.241e4	6060.00 ± 712.46	57.20 ± 3.97*,**,a	0.80 ± 0.37	38.40 ± 3.36*,**,a	1.20 ± 0.49
24 hrs.	Control 0.1 ml PBS	5.09e6 ± 8.405e4	12.80 ± 0.20	35.60 ± 0.60	4.73e5 ± 2.221e4	3520.00 ± 338.23	15.20 ± 1.16a,b	0.80 ± 0.20	82.20 ± 0.86a,b	1.60 ± 0.40
	Treatment 1 0.25 mg/kg bee venom	5.45e6 ±1.404e5	13.40 ± 0.51	36.60 ± 1.98	3.79e5 ± 8.676e4	4380.00 ± 524.79a,b	20.20 ± 3.02a,b	1.20 ± 0.58	78.60 ± 3.80a,b	2.00 ± 0.45
	Treatment 2 0.5 mg/kg bee venom	5.36e6 ± 1.990e5	13.00 ± 0.63	35.60 ± 1.17b	5.23e5 ± 3929.377	4500.00 ± 242.90	37.60 ± 5.46*,**,b	0.60 ± 0.40	60.20 ± 5.01*,**,b	1.60 ± 0.40

the mean difference is significant at the 0.05 level

mean ± S.E.: a compared to 2 hrs., b compared to 8 hrs., * compared to treatment 1, ** compared to treatment 2

Sequentially, blood congestion in liver tissue and hemorrhage in kidney tissues were occured. Activation of inflammatory cascade can lead to the development of disseminated intravascular coagulation, resulting in leeding. Liver damage contributes to the bleeding because the liver's ability to manufacture clotting factors is diminished. Direct vasoactive toxic effect of the venom could lead to acute renal failure. Hemodialysis may be started to remove the low-molecular weight components of the venom (such as melittin) or acute renal failure develops (Mitchell, 2009). These characters can occur in both tissues but it is not cause abnormal or death of rats in this study. However, it need more information to explain. Injection large amounts can cause sharp drop blood pressure. In treated concentration of this study were not more enough to caused severe until the state of shock.

Presenting abnormal number of neutrophil and lymphocyte were showed clearly response between *A. florea* venom and immune system. The alterations in the neutrophil count are indicative of systemic inflammatory response related cytokine release; response was more marked in the natural venom group, showing its greater toxicity (Netto *et al*., 2004). At the first time of 2 hrs. in this study can find increasing of neutrophil. It is showed highly number of neutrophil at 8 hrs. which related to concentration of bee venom. The number of neutrophil number may be decline after 24 hrs. Concentration of 0.25 mg/kg bee venom was not showed the different number of neutrophil when compare to control at 24 hrs. (Figs 4-30). Meanwhile lymphocyte showed cytotoxicity function to destroy protein allergen of bee venom. From this study, decreasing of lymphocyte was found at the first 2 hrs. when compared with control. At 8 and 24 hrs. after treated with 0.5 mg/kg bee venom, its showed more decreasing of lymphocyte than control and group treated

with 0.25 mg/kg bee venom (Figs 4-31). The decrease in the number of lymphocyte mainly caused by the generalized pain (Nogueira *et al*., 2007).

Eosinophil and monocyte from this study were not showed the different number from control group. They were showed the same results in both of two treatment groups and all the time points (Figs 4-32 to 4-33). These may cause from the concentration of protein allergen in *A. florea* venom less than to excited the function of these white blood cells. Platelets show decreasing number only in 0.25 mg/kg bee venom at 2 hrs. (Figs 4-34). But on the other time points of both treatment groups were not showed the different from control. This may cause from incomplete in mixing between K_3 EDTA and blood of this group. Blood clotting can occurred in some part of this group which was showed in low platelets result.

However, trend of effect on blood tissue of treatment group was not different to control when the time past to 24 hrs. It may reveal that half-life of *A. florea* venom (in this study) was short. Meanwhile the effect to liver and kidney tissues may need more than 24 hrs. for recovery.

In future, *A. florea* venom may be used to treat for in some disease like to *A. mellifera* venom. The data support about *A. florea* venom effects in other tissues will be necessary.

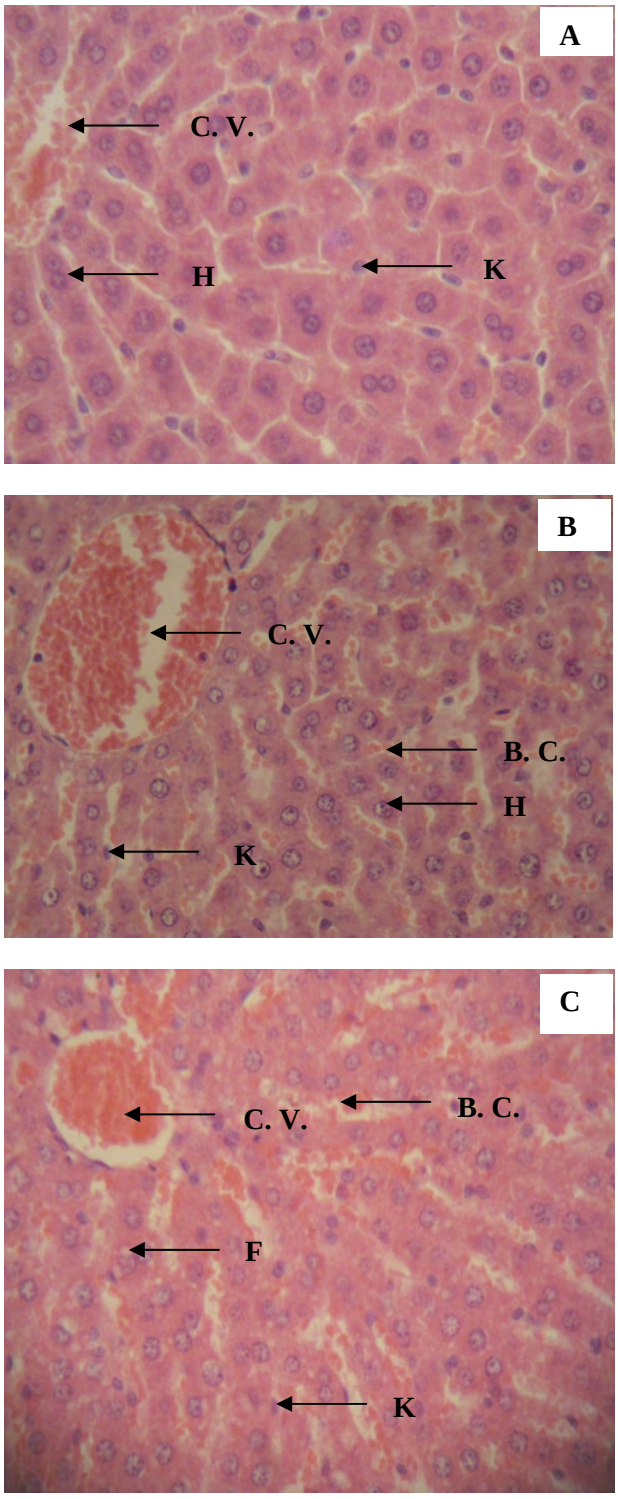

Figure 4-11. Liver tissues of a control rat (A) and treatments with 0.25 and 0.5mg/kg bee venom at 2 hrs (B, C). H&E, X 400.

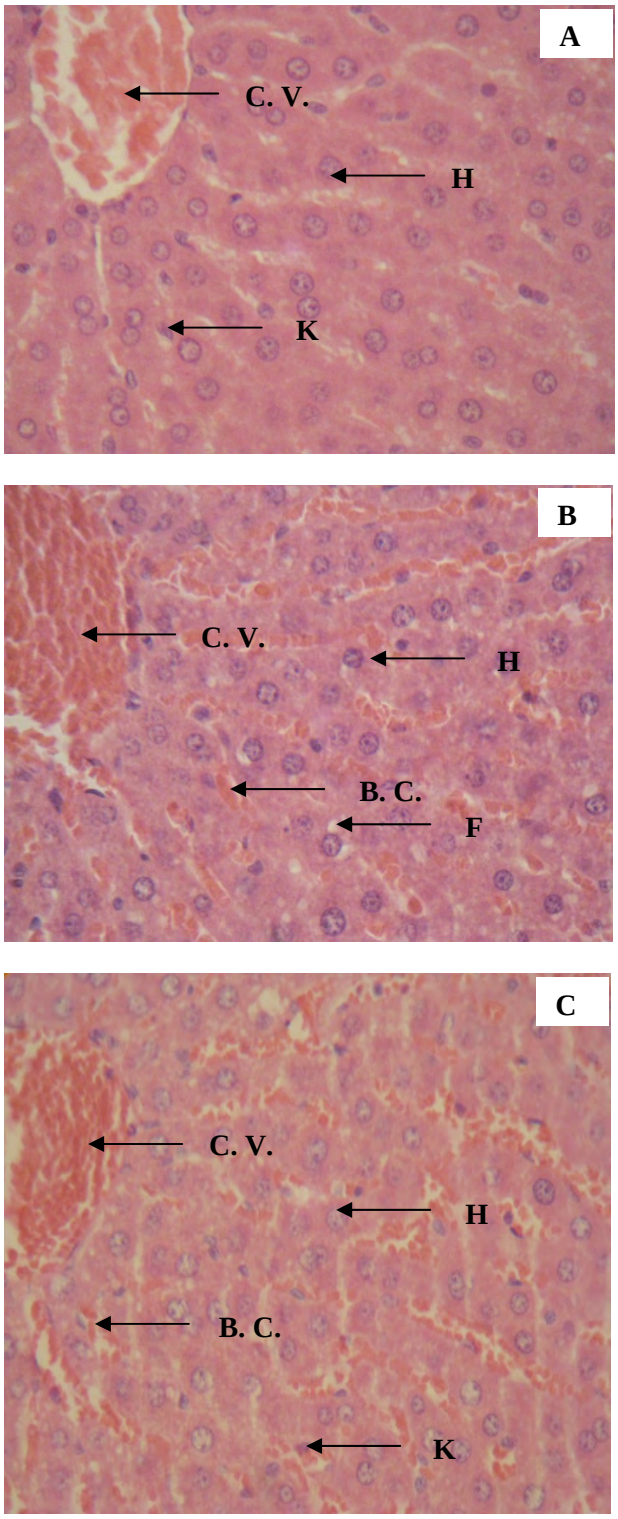

Figure 4-12. Liver tissues of a control rat (A) and treatments with 0.25 and 0.5 mg/kg bee venom at 8 hrs (B, C). H&E, X 400.

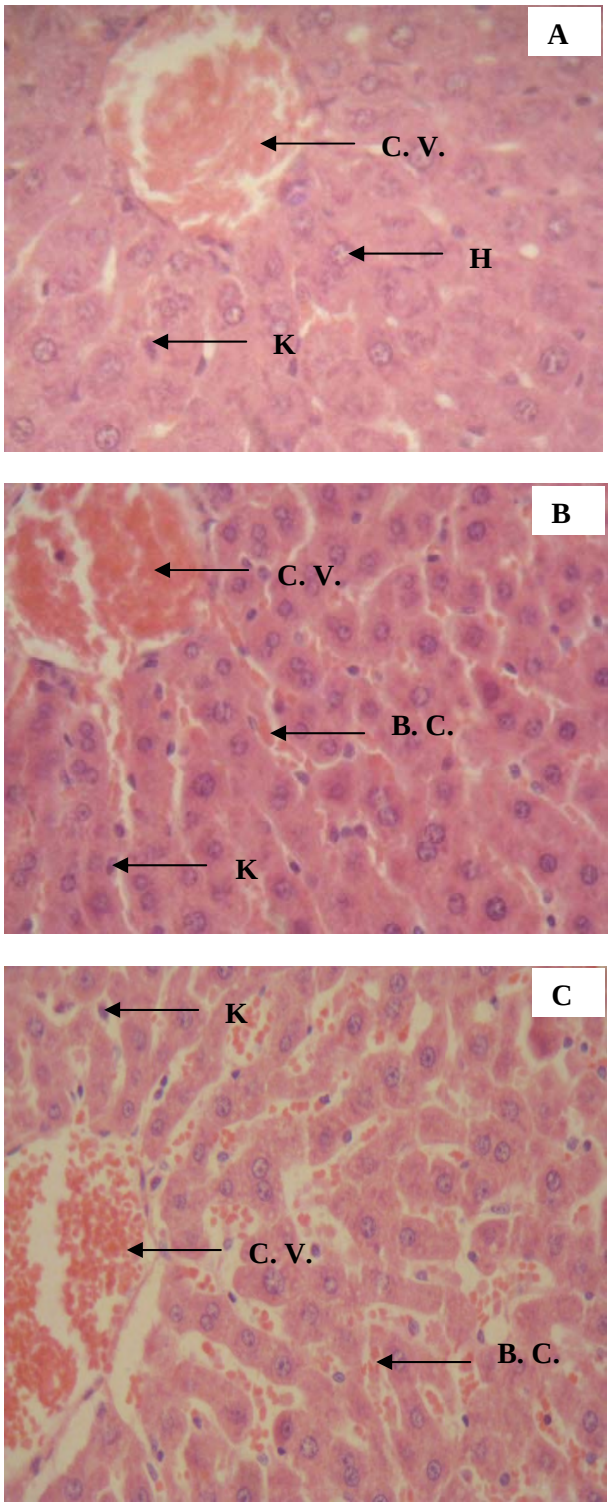

Figure 4-13. Liver tissues of a control rat (A) and treatments with 0.25 and 0.5 mg/kg bee venom at 24 hrs (B, C). H&E, X 400.

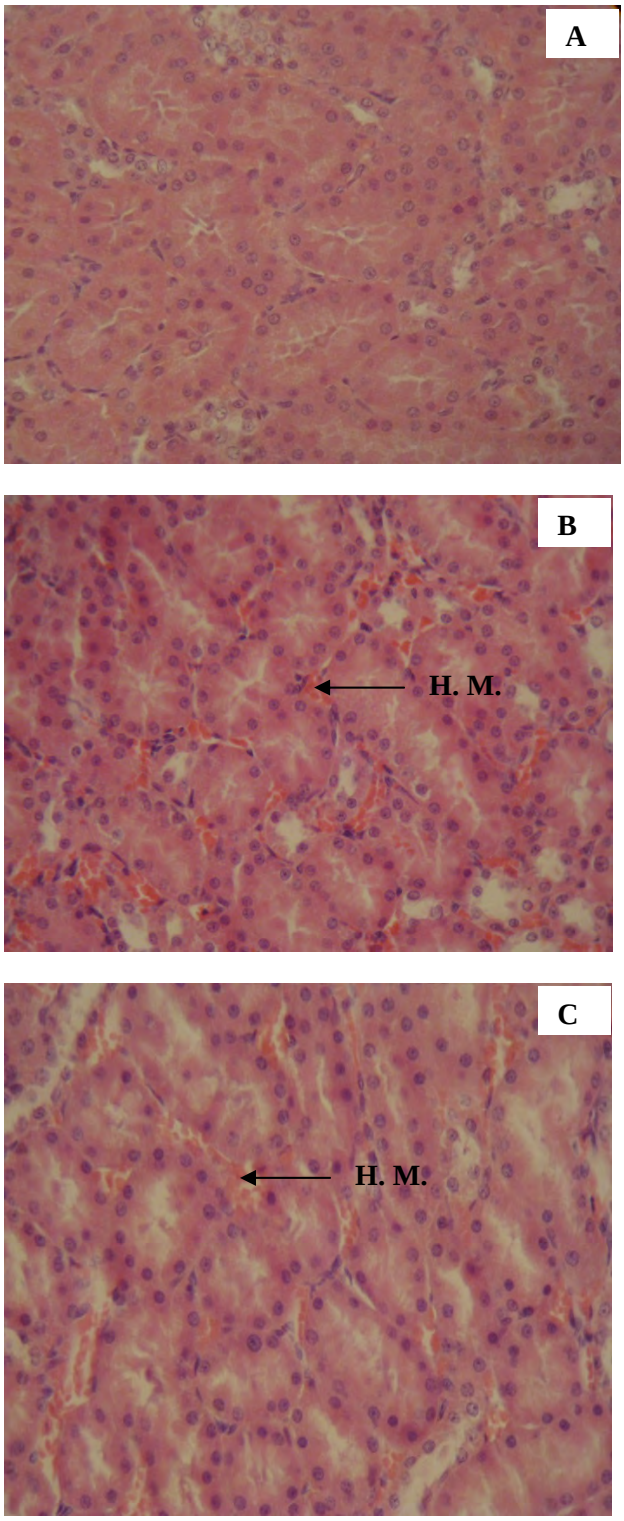

Figure 4-14. Kidney tissues (cortex) of a control rat (A) and treatments with 0.25 and 0.5 mg/kg bee venom at 2 hrs. (B, C), H&E, X 400.

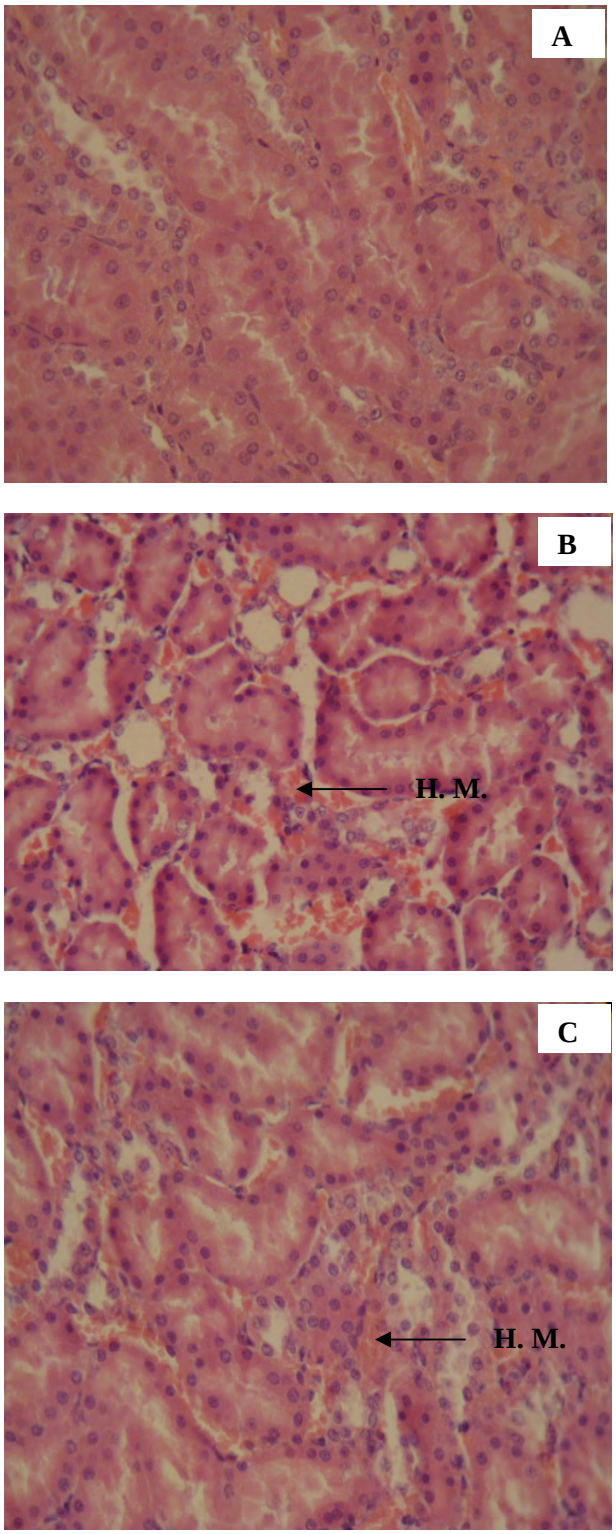

Figure 4-15. Kidney tissues (cortex) of a control rat (A) and treatments with 0.25 and 0.5 mg/kg bee venom at 8 hrs. (B, C), H&E, X 400.

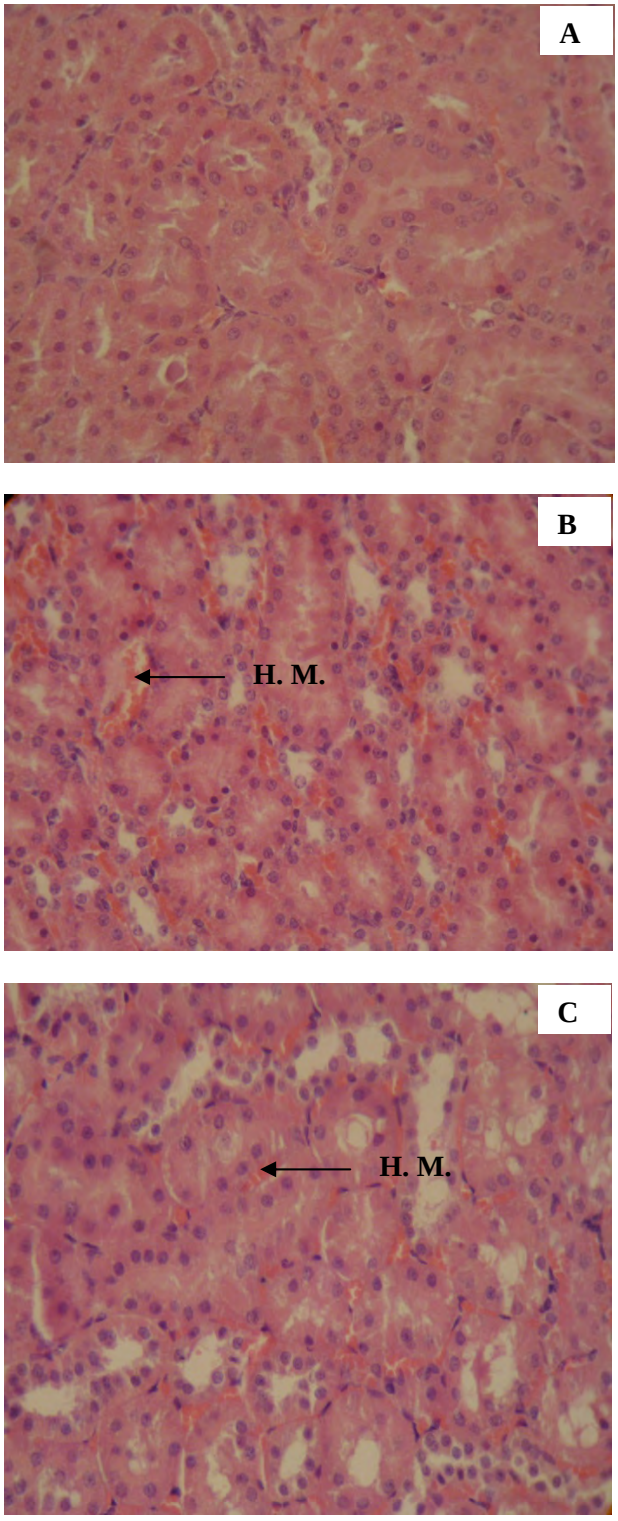

Figure 4-16. Kidney tissues (cortex) of a control rat (A) and treatments with 0.25 and 0.5 mg/kg bee venom at 24 hrs. (B, C), H&E, X 400.

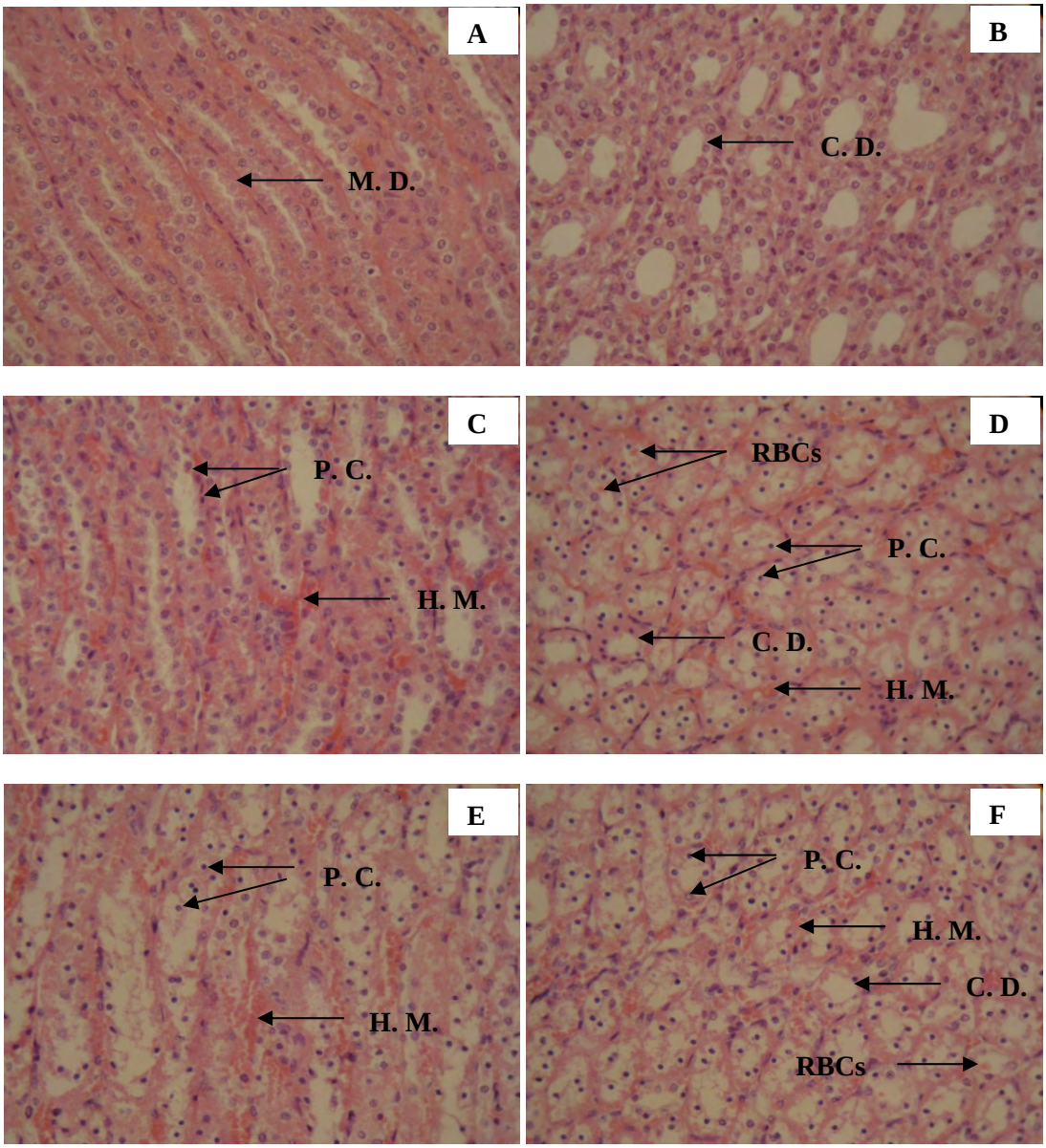

Figure 4-17. Kidney tissues (medulla) of a control rat (A, B) and treatments with 0.25 (C, D) and 0.5 mg/kg bee venom (E, F) at 2 hrs., H&E, X 400.

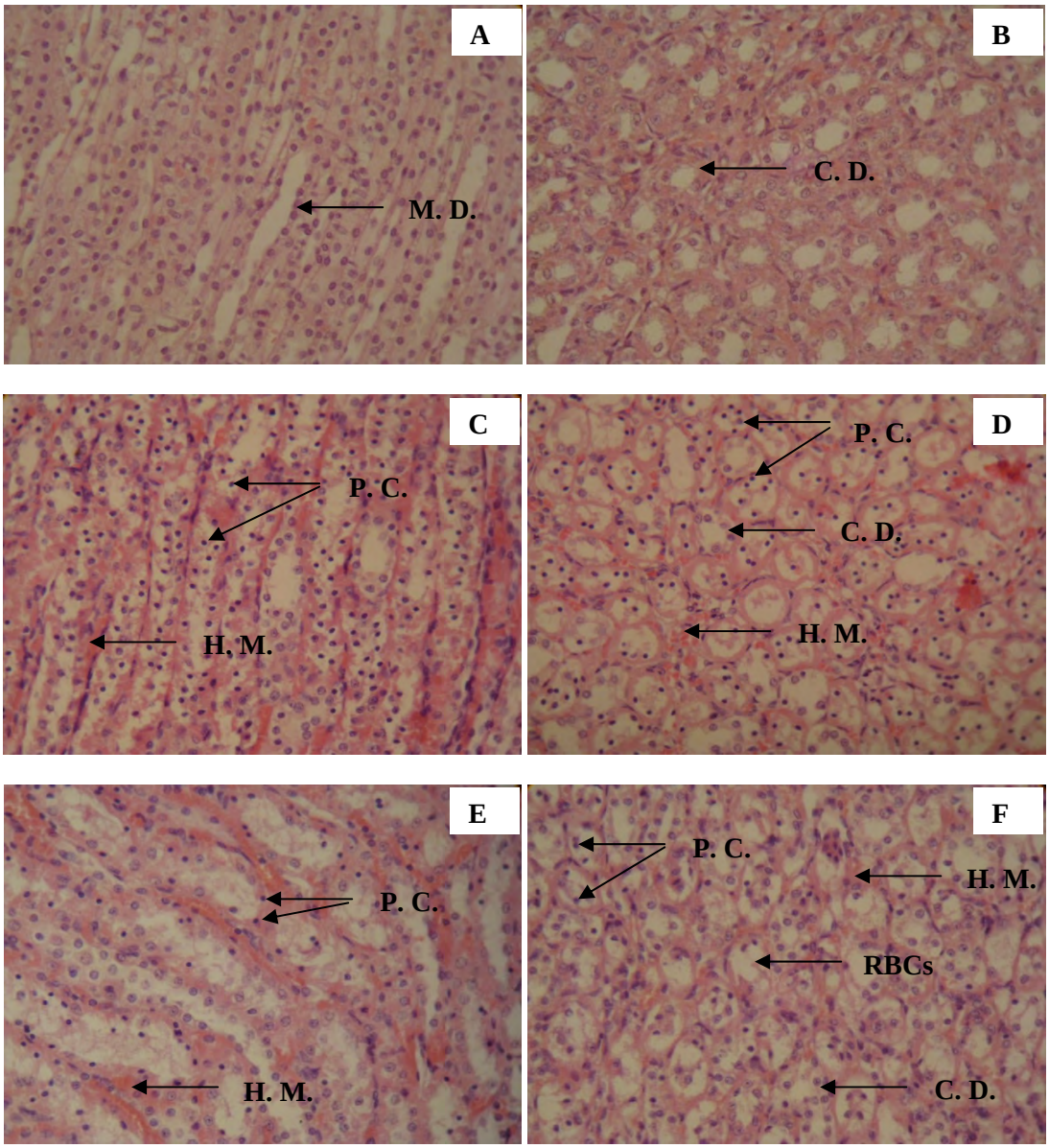

Figure 4-18. Kidney tissues (medulla) of a control rat (A, B) and treatments with 0.25 (C, D) and 0.5 mg/kg bee venom (E, F) at 8 hrs., H&E, X 400.

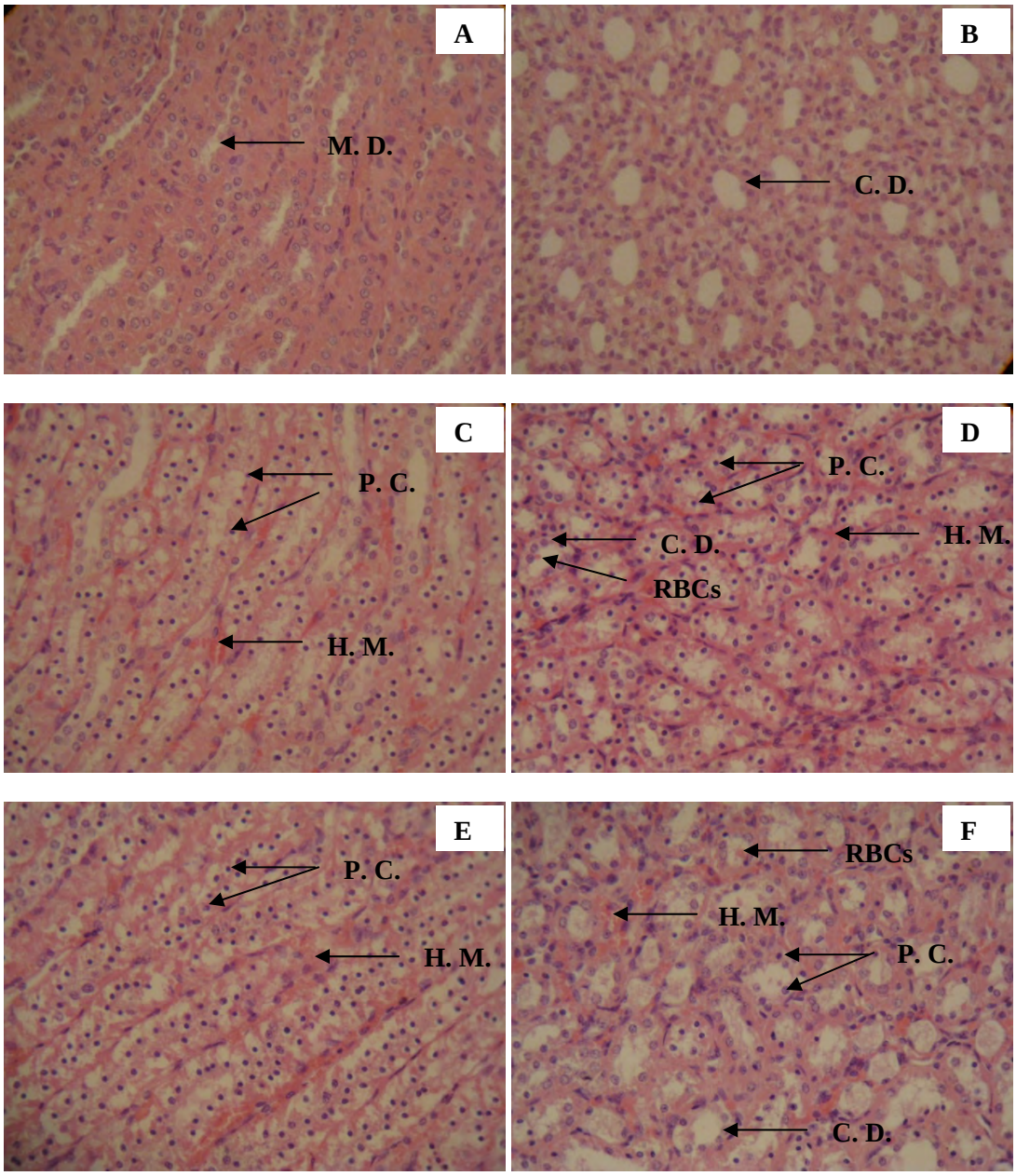

Figure 4-19. Kidney tissues (medulla) of a control rat (A, B) and treatments with 0.25 (C, D) and 0.5 mg/kg bee venom (E, F) at 24 hrs., H&E, X 400.

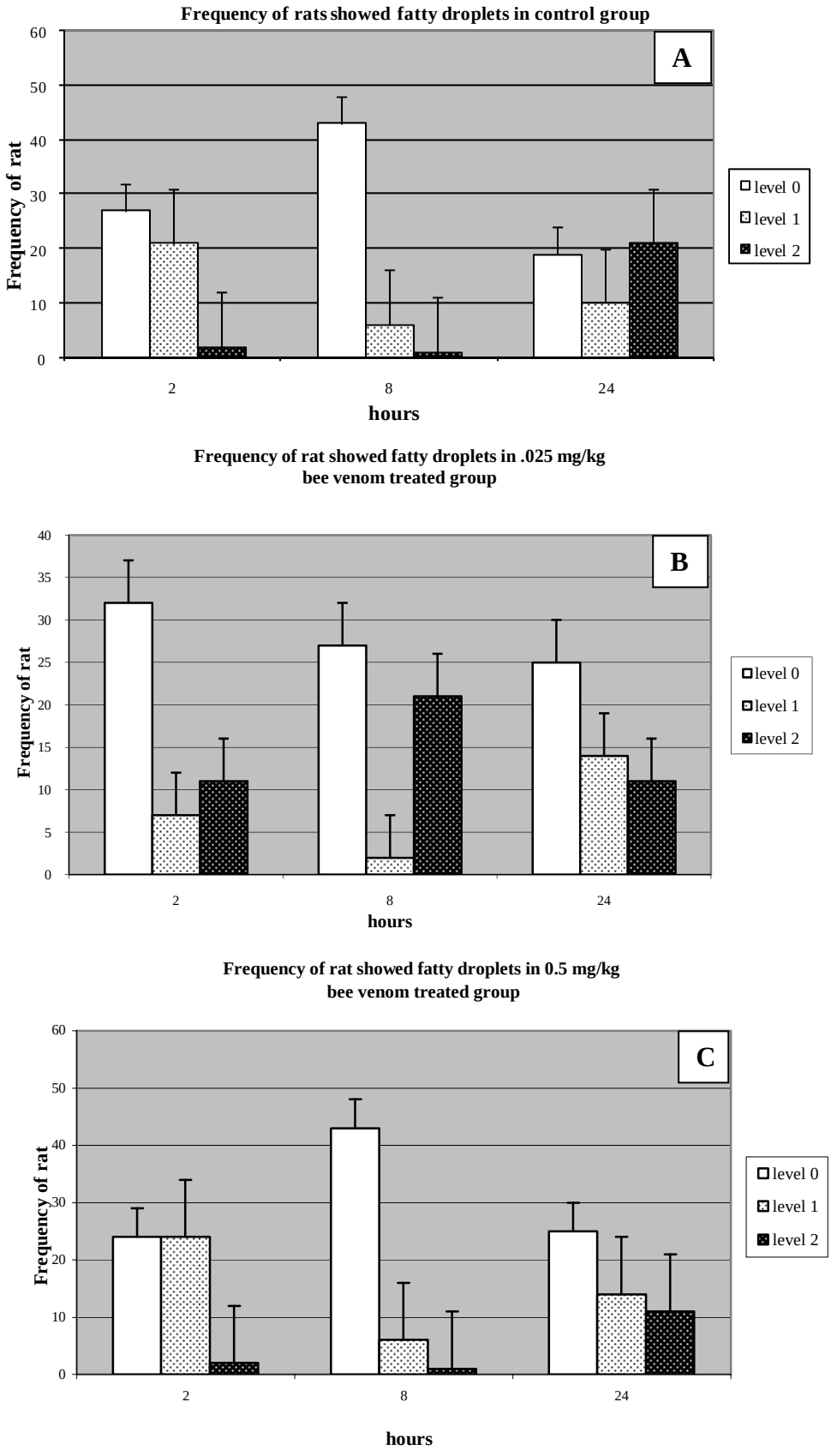

Figure 4-20. Frequency of rat showed fatty droplets in liver tissues at 2, 8 and 24 hrs. control (A), 0.25 mg/kg (B) and 0.5 mg/kg (C)
level 0: not found any change
level 1: mild to moderate change
level 2: severe change

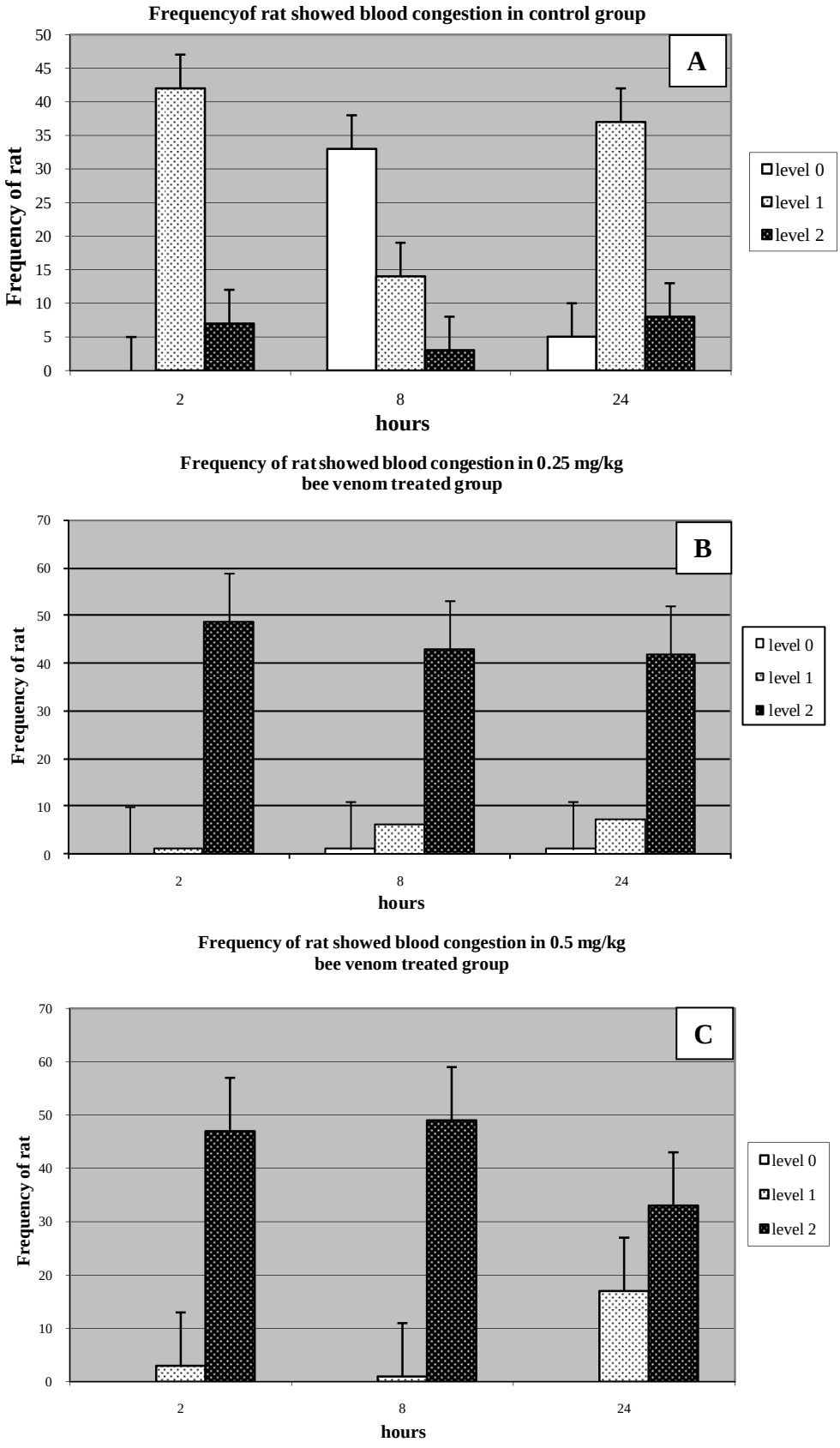

Figure 4-21. Frequency of rat showed blood congestion in liver tissues at 2, 8 and 24 hrs. control (A), 0.25 mg/kg (B) and 0.5 mg/kg (C)
level 0: not found any change
level 1: mild to moderate change
level 2: severe change

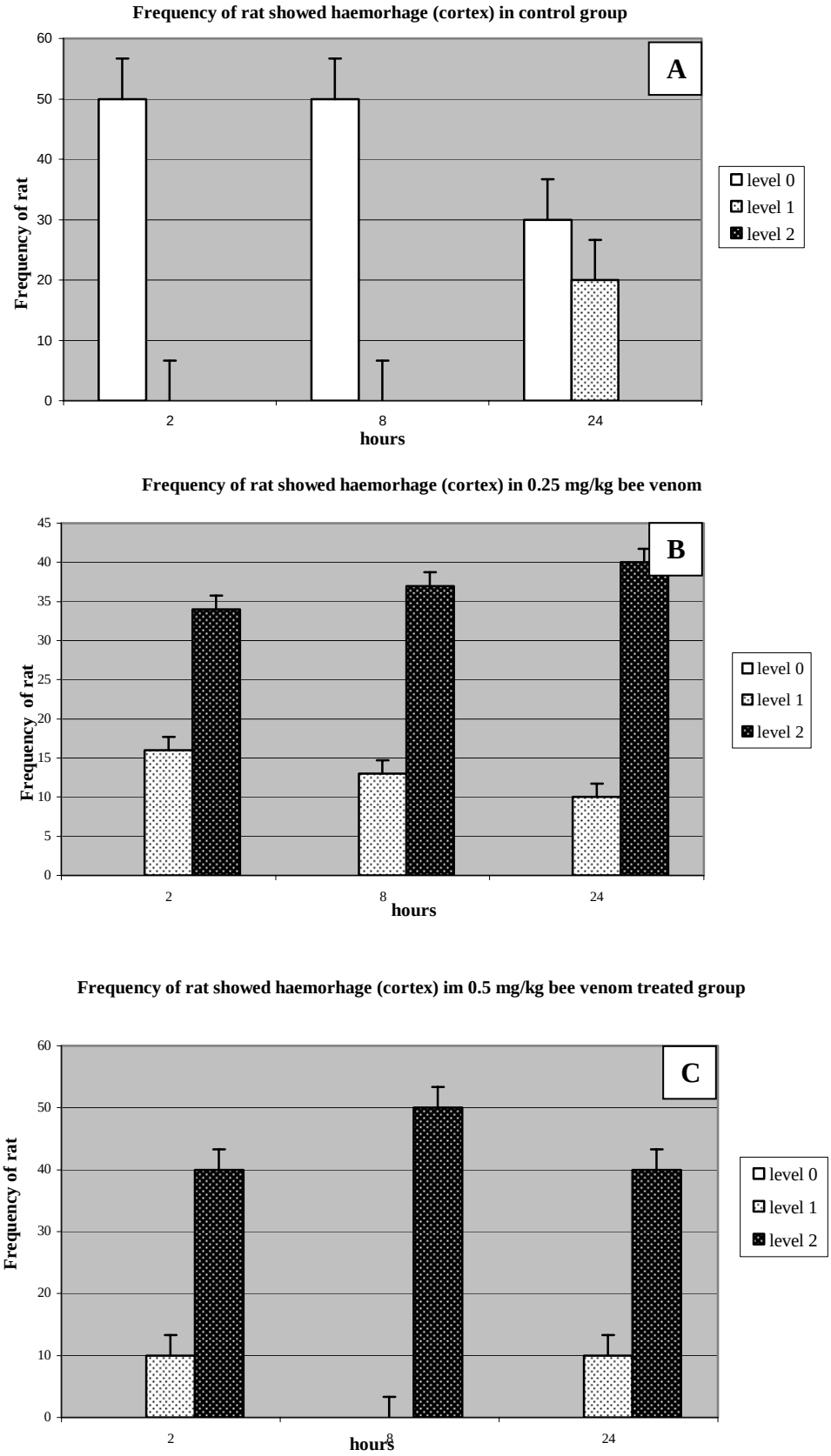

Figure 4-22. Frequency of rat showed haemorrhage in kidney tissues (cortex) at 2, 8 and 24 hrs. after treated with 0.1 ml PBS control (A), 0.25 mg/kg (B) and 0.5 mg/kg (C) bee venom.
level 0: not found any change; level 1: mild to moderate change; level 2: severe change.

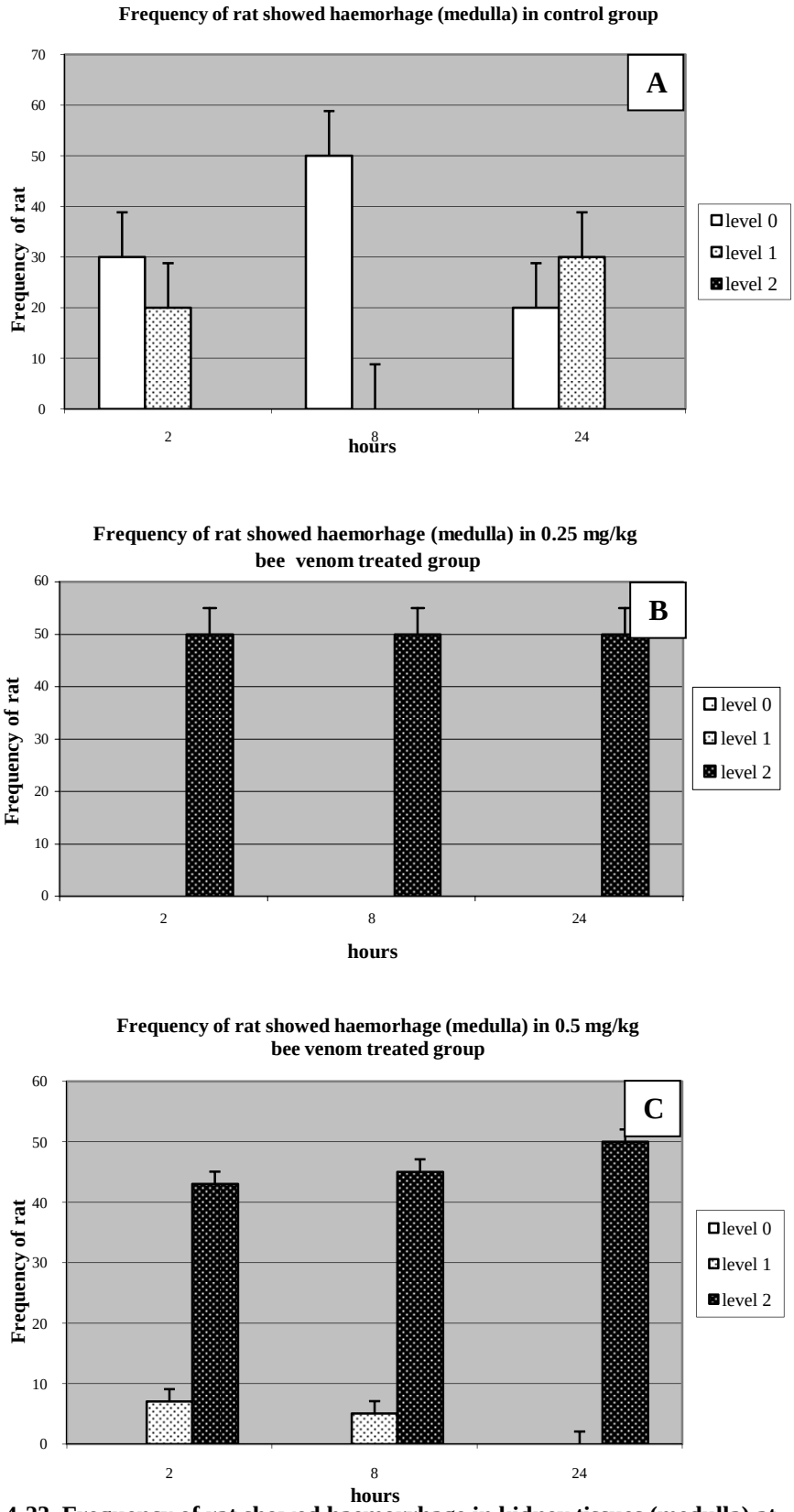

Figure 4-23. Frequency of rat showed haemorrhage in kidney tissues (medulla) at 2, 8 and 24 hrs. after treated with 0.1 ml PBS control (A), 0.25 mg/kg (B) and 0.5 mg/kg (C) bee venom.
level 0: not found any change; level 1: mild to moderate change; level 2: severe change.

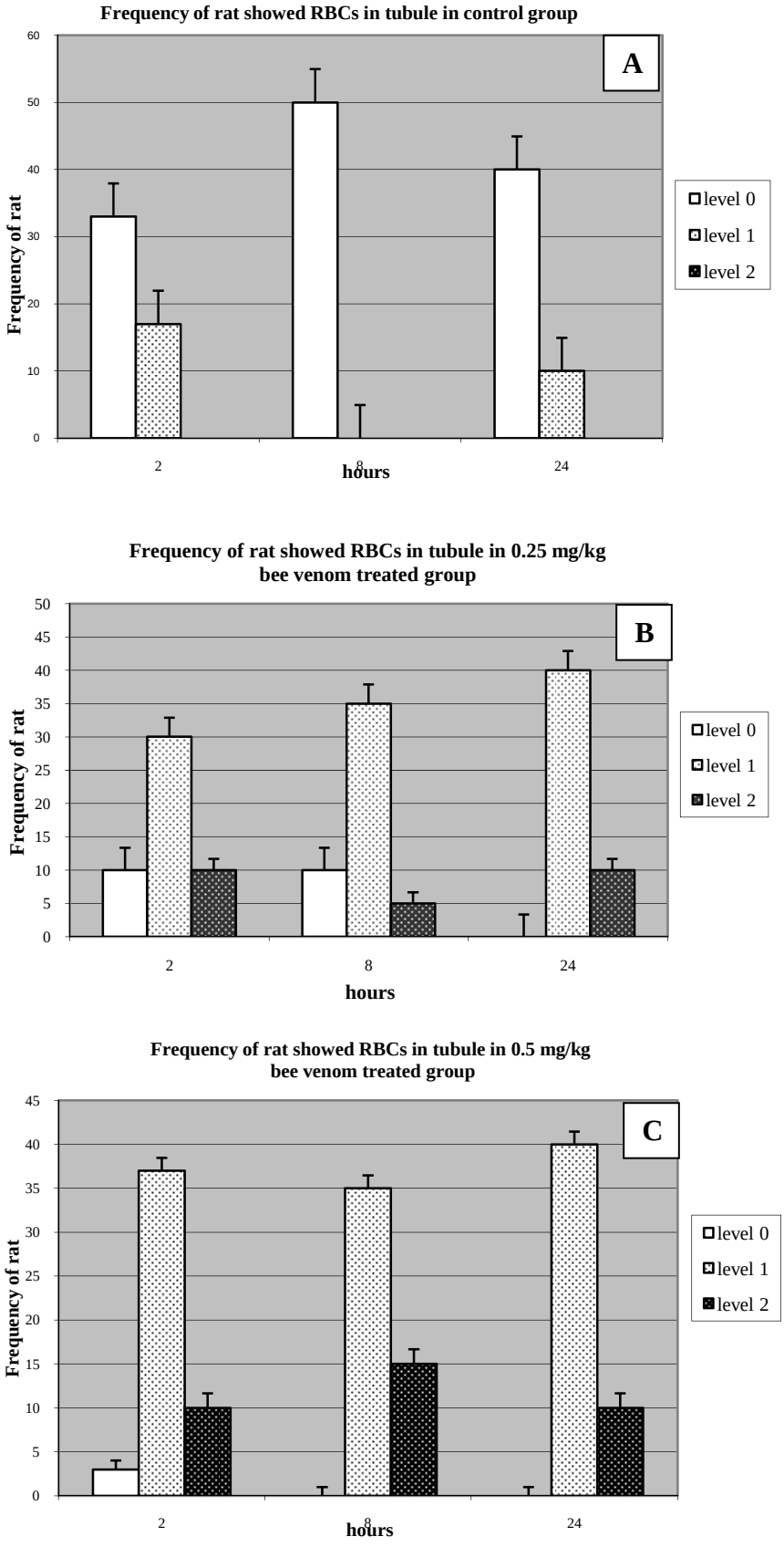

Figure 4-24. Frequency of rat showed RBCs in tubule of kidney tissues at 2, 8 and 24 hrs. after treated with 0.1 ml PBS control (A), 0.25 mg/kg (B) and 0.5 mg/kg (C) bee venom.
level 0: not found any change; level 1: mild to moderate change; level 2: severe change.

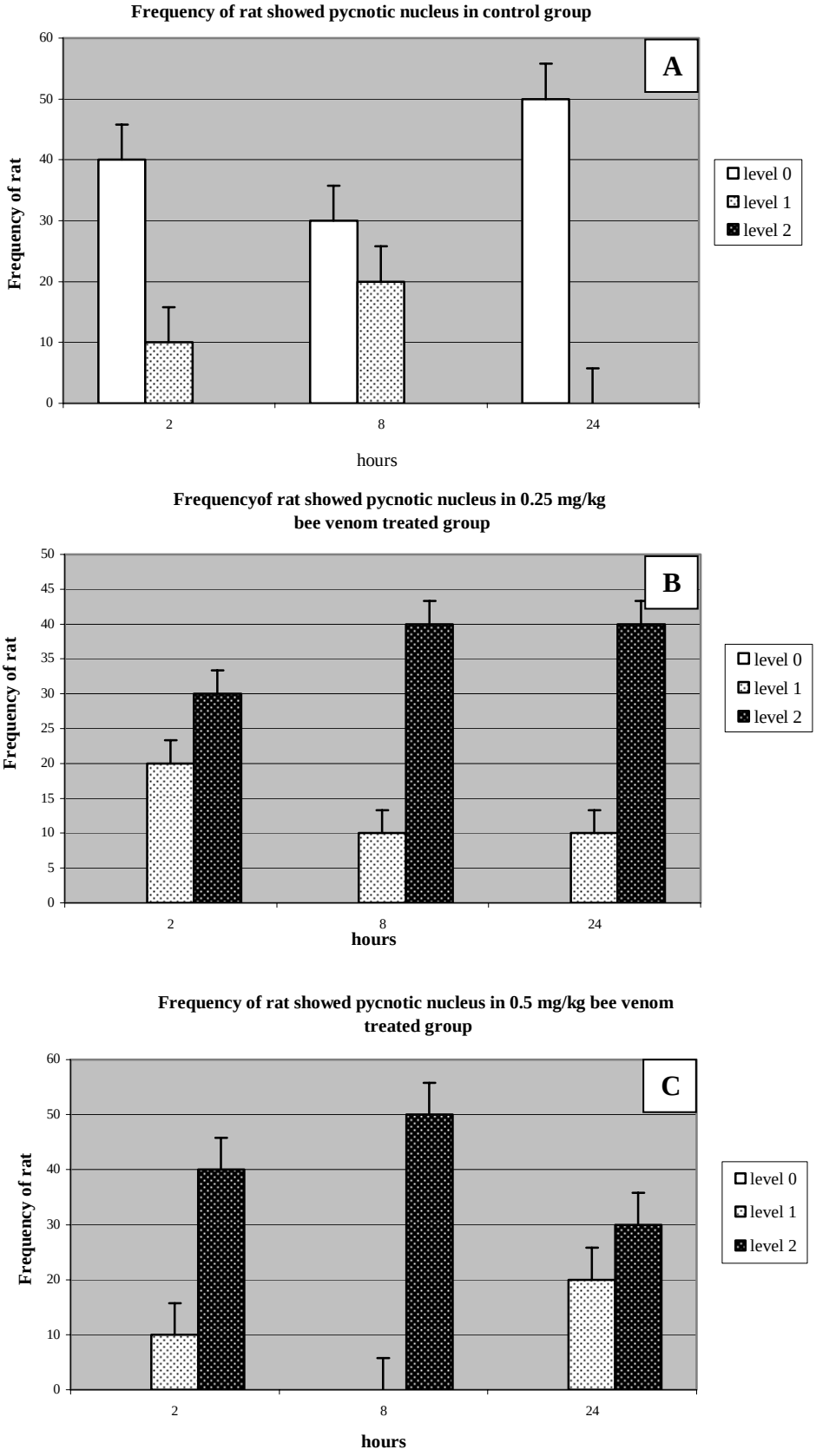

Figure 4-25. Frequency of rat showed pycnotic nucleus in kidney tissues at 2, 8 and 24 hrs. after treated with 0.1 ml PBS control (A), 0.25 mg/kg (B) and 0.5 mg/kg (C) bee venom.
level 0: not found any change; level 1: mild to moderate change; level 2: severe change.

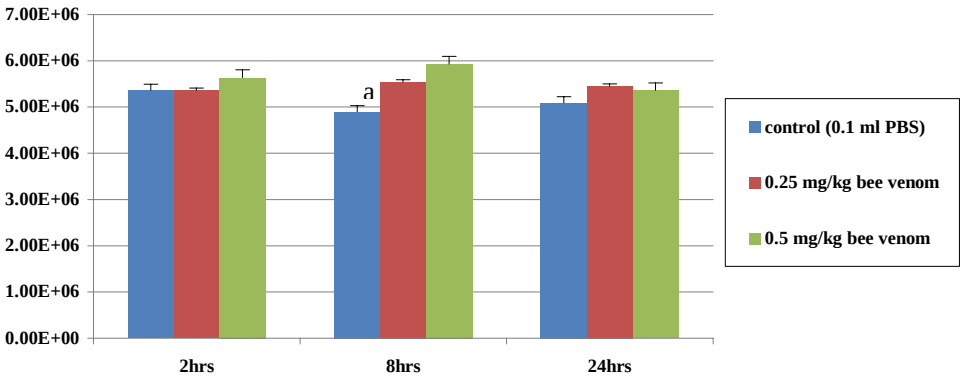

Figure 4-26. The number of red blood cell in each time point (per µl)

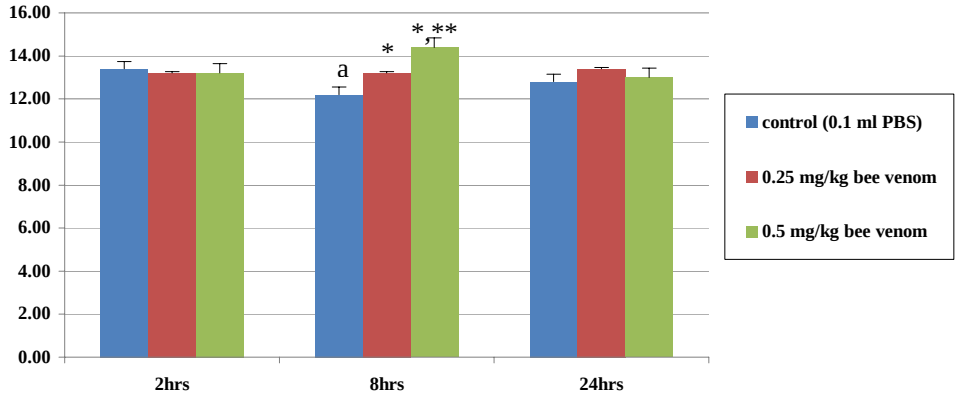

Figure 4-27. The number of hemoglobin in each time point (g/dl)

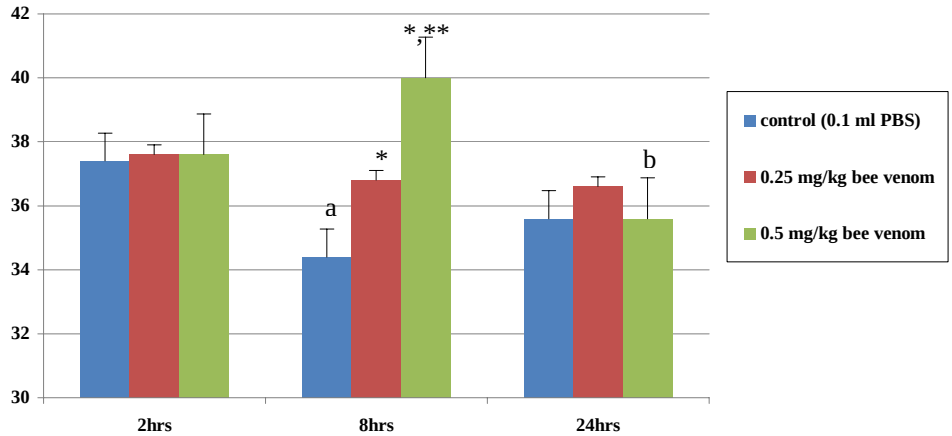

Figure 4-28. %hematocrit in each time point (g/dl)

The mean difference is significant at the 0.05 level (mean ± S.E.)

a compared to 2 hrs. **b compared to 8 hrs.**
*** compared to control** **** compared to treatment 1**

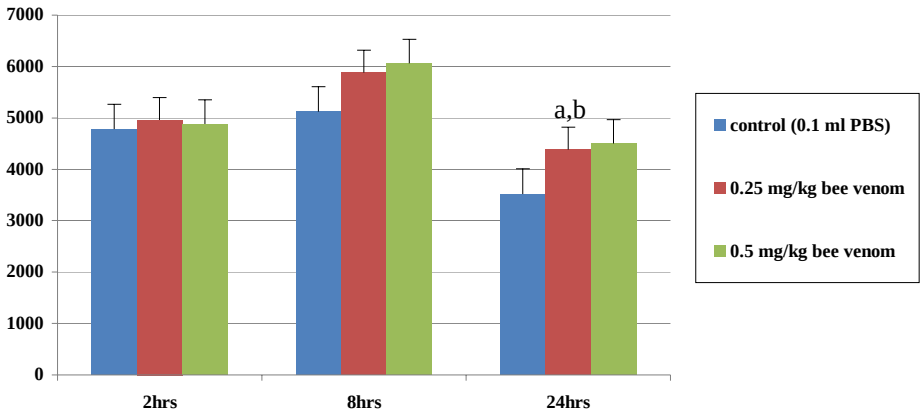

Figure 4-29. The number of white blood cells in each time point (per µl)

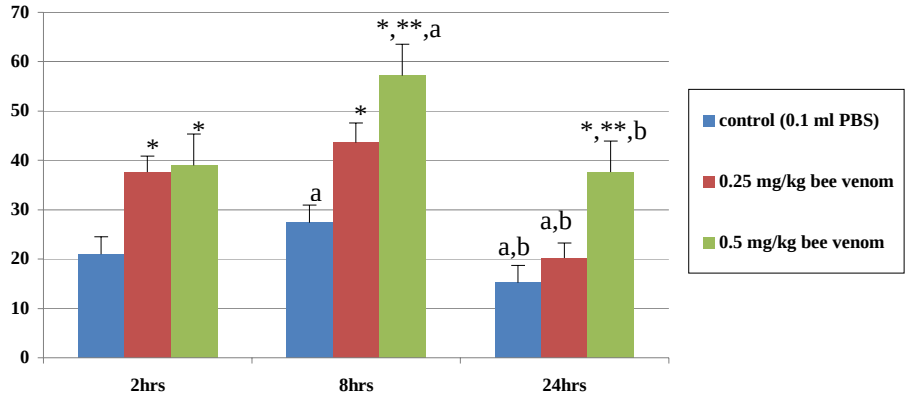

Figure 4-30. % neutrophil in each time point.

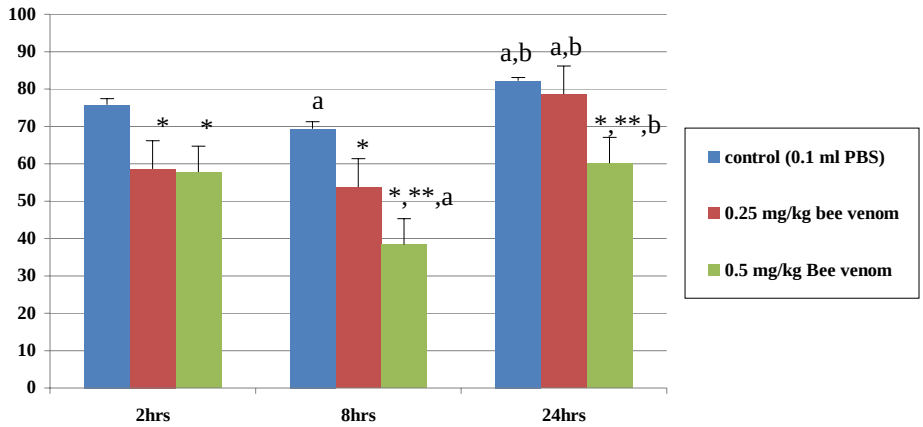

Figure 4-31. % lymphocyte in each time point.

The mean difference is significant at the 0.05 level (mean ± S.E.)

a compared to 2 hrs. **b compared to 8 hrs.**
*** compared to control** **** compared to treatment 1**

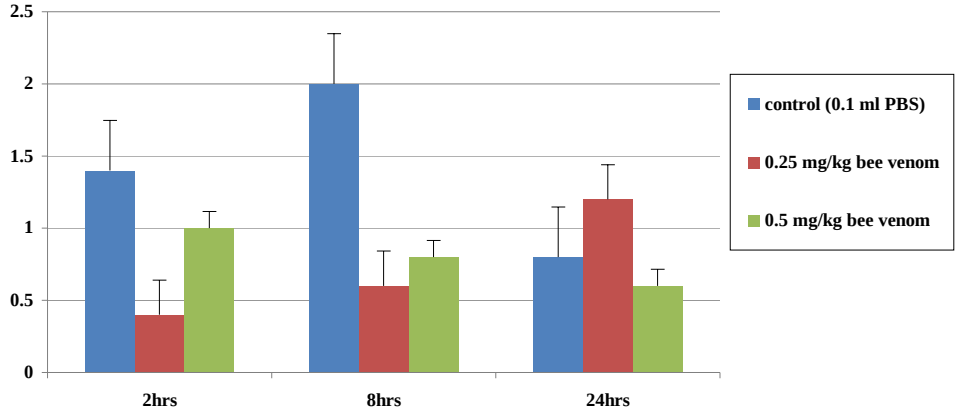

Figure 4-32. % eosinophil in each time point.

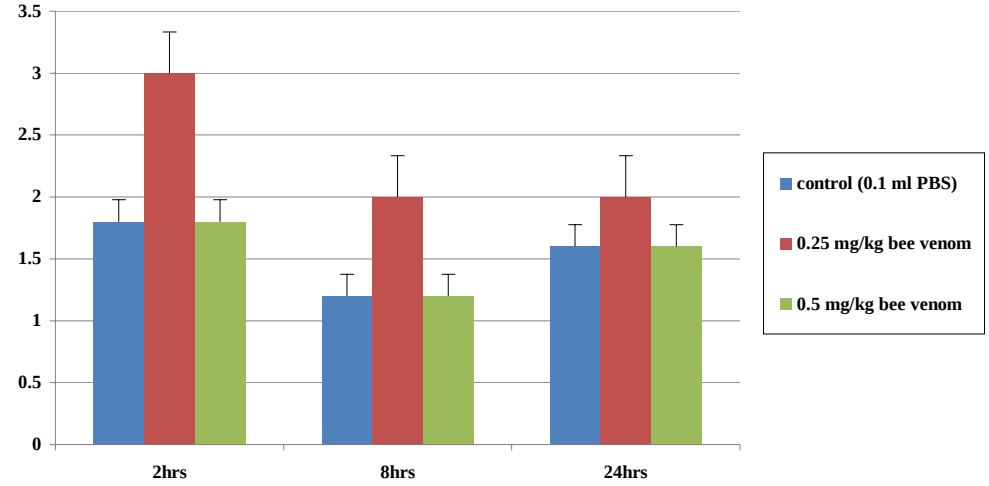

Figure 4-33. % monocyte in each time point.

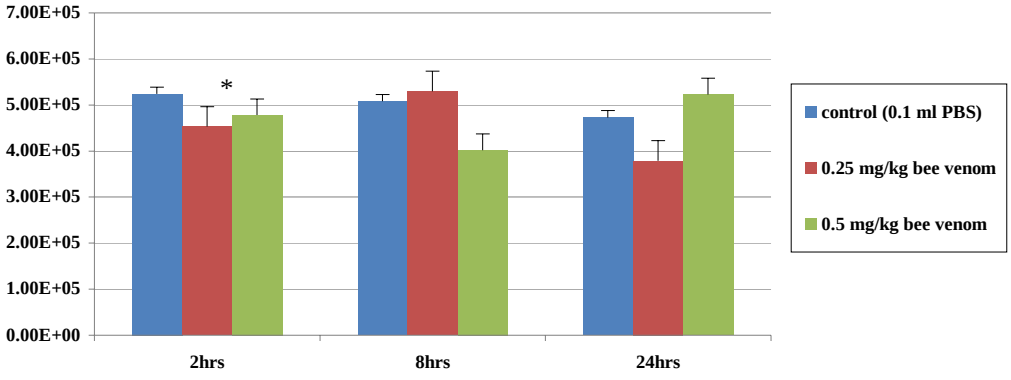

Figure 4-34. The number of platelets in each time point.

The mean difference is significant at the 0.05 level (mean ± S.E.)

a compared to 2 hrs. **b compared to 8 hrs.**
*** compared to control** **** compared to treatment 1**

CHAPTER V

CONCLUSION AND RECOMMENDATIONS

Average dried weight of *A. florea* venom was 0.016 $\pm$ 0.0004 mg per bee, which were related to the size of honeybees. *A. florea* venom contained the same major components like *A. mellifera* but different in quantity. RP-HPLC analysis was showed the different retention time which the major components eluted. Differences of retention time between *A. florea* and *A. mellifera* in the same condition were about 1-2 min. The major components of *A. florea* venom in this experiment were apamine, PLA_2 and melittin. However, seven subfractions from RP-HPLC were not matched in group of database protein venom. Fraction component of *A. florea* should be more purified with any method.

The red dwarf honeybee, *A. florea* venom had an effect to liver, kidney and blood tissues on male rats. It showed the effects both on liver and kidney tissues rats after treated with 0.25 mg/kg and 0.5 mg/kg by body weight at 2, 8 and 24 hrs. The main characters which were found in both liver and kidney tissues such as blood congestion and haemorrhage, respectively. There were many red blood cells in sinusoid of liver tissue. In addition many red blood cells were found in both cortex and medulla of kidney. Beside that many of pycnotic nucleus were found in tubular cell of kidney tissue.

Components of bee venom can cause vaso-dilation and increasing vascular permeability. But temporary increasing of hemoglobin and hematocrit were showed at 8 hrs.

Red blood cell in both treated groups showed normal character. Meanwhile the changing number of white blood cells (such as increasing

of neutrophil and decreasing of lymphocyte) were showed immune response to bee venom.

However, the amount of venom components and their effects on rat tissues in other honeybees of genus *Apis* should be explored and compared to *A. florea*.

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APPENDICES

APPENDIX A

Chemical reagents

10% Buffered formalin

Sodium acid phosphate ($NaH_2PO_4H_2O$)	4 g
Anhydrous disodium phosphate (Na_2HPO_4)	6.5 g
10% formalin	1000 ml

Eosin Y

ddH_2O	50 ml
Eosin Y	10 g
95% alcohol	940 ml

Giemsa stain

Absolute methanol	1 g
Giemsa powder	66 ml
Glycerol	66 ml

Mayer’s Hematoxylin

Ammonium alum	8 g
ddH_2O	400 ml
Glacial acetic acid (CH_3COOH)	40 ml
Glycerol	400 ml
Hematoxylin	8 g
95% alcohol	400 ml

Routine paraffin technique

70% Ethanol (several days)

⇩

90% Ethanol (60 min)

⇩

95% Ethanol (60 min)

⇩

95% Ethanol (30 min)

⇩

Absolute ethanol (60 min)

⇩

Absolute ethanol (180 min)

⇩

Absolute ethanol (180 min)

⇩

Absolute ethanol (180 min)

⇩

Xylene (30 min)

⇩

Xylene (30 min)

⇩

Xylene in paraplast 1:1 (60 min)

⇩

Paraplast (60 min)

⇩

Paraplast (60 min)

⇩

Embedded in paraplast

Routine H&E staining

Deparaffinize sections in xylene (2x; 10 min each)

⇩

Re-hydrate in absolute alcohol (2x; 5 min each)

⇩

95% (v/v) ethanol for 2 min

⇩

70% (v/v) ethanol for 2 min

⇩

Briefly wash in distilled water

⇩

Stain in Mayer's hematoxylin solution for 8 min

⇩

Wash in running tap water for 5 min

⇩

Differentiate in 1% acid alcohol for 30 second

⇩

Wash running tap water for 1 min

⇩

Dips in 95% ethanol

⇩

Stain in eosin Y solution for 40 seconds

⇩

Dehydrate through 95% ethanol

⇩

Absolute ethanol (2x; 5 min each)

Routine H&E staining (cont.)

Clear in xylene (2 times; 5 mins each)

⇩

Mounting slide

APPENDIX B

Toxicity test data

Number of Kupffer cells of group treated with PBS 0.1 ml in each time point

ber of Kupffer cells of group treated with PBS 0.1 ml in each time p

Group	time points (hrs.)		
	2	8	24
1	22	27	12
1	25	26	16
1	27	21	14
1	28	23	12
1	24	22	12
1	20	22	14
1	28	23	17
1	31	24	14
1	29	20	16
1	36	23	19
1	25	20	27
1	22	20	35
1	30	20	30
1	25	25	25
1	26	20	24
1	32	23	27
1	21	20	24
1	39	22	24
1	28	21	25
1	23	18	26
1	22	25	20
1	18	20	18
1	25	16	18
1	26	23	18
1	31	23	16
1	32	13	19
1	25	16	20
1	26	19	18
1	19	25	18
1	30	21	21
1	18	25	24
1	22	17	30
1	19	22	21
1	29	25	18
1	26	18	24
1	16	24	22
1	23	19	24
1	19	19	20
1	23	23	22
1	18	23	21
1	19	21	23
1	18	21	23
1	17	20	18
1	13	20	27
1	13	21	25
1	19	27	23
1	15	25	23
1	17	22	22

Number of Kupffer cells of group treated with 0.25 mg/kg bee venom

Group	time points (hrs.)		
	2	**8**	**24**
3	28	45	35
3	23	45	32
3	27	56	41
3	24	48	40
3	30	53	42
3	22	54	36
3	28	43	30
3	25	50	43
3	30	53	31
3	26	54	43
3	36	38	39
3	37	50	38
3	33	46	35
3	37	39	41
3	36	40	32
3	31	39	36
3	42	48	33
3	36	37	32
3	38	38	37
3	38	38	38
3	44	38	39
3	42	37	39
3	38	39	31
3	37	35	46
3	36	42	41
3	34	35	30
3	29	32	35
3	37	38	34
3	25	30	33
3	37	34	34
3	29	42	24
3	40	42	10
3	33	51	17
3	32	35	10
3	33	47	16
3	29	43	18
3	33	40	21
3	24	34	14
3	30	38	13
3	30	50	19
3	48	38	24
3	40	40	22
3	50	47	23
3	32	43	21
3	42	40	23
3	40	41	24
3	34	42	26
3	32	39	24
3	46	37	31
3	35	45	36

Number of Kupffer cells of group treated with 0.5 mg/kg bee venom

Group	time points (hrs.)		
	2	**8**	**24**
2	33	38	37
2	38	38	35
2	30	44	36
2	28	41	34
2	30	35	37
2	39	32	27
2	35	35	35
2	35	34	34
2	41	37	36
2	29	35	36
2	50	21	33
2	42	28	25
2	40	30	30
2	37	31	21
2	36	25	43
2	42	20	44
2	36	27	37
2	34	24	42
2	36	32	38
2	36	30	43
2	36	33	30
2	47	40	33
2	47	32	41
2	40	34	41
2	36	38	30
2	44	29	30
2	38	38	42
2	34	33	32
2	38	33	36
2	40	35	37
2	46	28	31
2	40	27	36
2	38	22	41
2	39	22	30
2	38	24	36
2	36	28	38
2	40	27	35
2	38	29	39
2	40	29	42
2	38	34	40
2	32	28	35
2	35	35	39
2	39	26	40
2	38	31	37
2	37	33	31
2	32	28	39
2	46	38	42
2	33	31	34
2	30	31	38
2	33	33	38

Toxicity data of liver tissues treated with 0.1 ml PBS (control).

No.	fatty droplets										blood congestion									
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
1	0	1	0	1	0	0	0	0	1	0	1	2	2	1	2	1	1	1	1	1
2	0	1	0	0	0	0	1	1	1	0	1	1	1	1	1	1	1	1	1	1
3	1	1	2	1	2	0	0	0	1	1	1	1	1	1	1	1	1	2	1	2
4	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
5	0	0	0	1	1	1	0	1	1	1	1	1	1	2	1	1	1	2	1	1
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
7	1	0	1	1	1	1	0	2	0	0	1	0	1	1	1	1	1	1	0	1
8	0	0	0	0	0	0	0	0	0	0	1	1	0	2	1	2	2	1	1	1
9	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1
12	0	0	2	2	1	1	1	1	2	0	0	1	1	1	2	1	1	1	1	1
13	0	0	0	0	1	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
14	0	1	2	1	2	2	2	2	2	2	0	1	1	2	0	1	2	1	1	2
15	0	2	0	1	0	0	1	1	0	0	0	2	2	1	1	1	1	2	2	0

Note: Level 0: not found any change in tissues/ eye view.

Level 1: > 0-50% were occured in tissues/ eye view (mild to moderate).

Level 2: > 50% were occured in tissues/ eye view (severe).

Rat no. 1-5 were data collected at 2 hrs.

Rat no. 6-10 were data collected at 8 hrs.

Rat no. 11-15 were data collected at 24 hrs.

Toxicity data of liver tissues treated with 0.25 mg/kg bee venom.

No.	fatty droplets										blood congestion									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0	0	0	2	0	0	1	1	1	1	2	2	2	2	2	2	2	2	2
2	2	0	2	2	2	1	0	0	0	0	2	2	2	2	2	2	2	2	2	2
3	0	0	0	0	0	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2
4	0	0	1	0	0	0	2	2	0	1	2	2	2	2	2	2	2	2	2	2
5	0	0	0	1	0	0	0	0	0	0	2	2	2	2	2	2	2	2	2	2
6	2	2	2	2	2	2	2	1	0	2	2	2	2	2	1	1	0	1	2	2
7	0	0	2	2	0	0	0	2	2	0	2	2	2	2	2	2	2	2	2	2
8	2	2	2	2	0	0	0	2	2	0	2	2	2	1	2	1	2	2	2	2
9	2	2	0	2	0	0	0	0	0	0	2	2	2	3	2	2	2	2	2	2
10	0	0	0	1	0	0	0	0	0	0	2	2	2	2	2	2	1	2	2	2
11	1	1	1	1	1	2	1	0	2	2	2	2	2	2	2	2	2	2	2	2
12	0	0	0	0	0	0	1	1	0	1	2	2	2	2	2	2	2	1	2	2
13	0	0	0	0	0	0	2	0	2	2	2	1	1	1	2	2	1	1	2	2
14	0	1	2	2	0	0	0	0	0	2	2	2	2	2	2	2	2	2	2	2
15	0	2	0	1	1	1	2	0	1	0	2	2	2	0	2	2	2	2	1	2

Note: Level 0: not found any change in tissues/ eye view.

Level 1: > 0-50% were occured in tissues/ eye view (mild to moderate).

Level 2: > 50% were occured in tissues/ eye view (severe).

Rat no. 1-5 were data collected at 2 hrs.

Rat no. 6-10 were data collected at 8 hrs.

Rat no. 11-15 were data collected at 24 hrs.

Toxicity data of liver tissues treated with 0.5 mg/kg bee venom.

No.	fatty droplets										blood congestion									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1	0	1	1	1	0	1	1	0	1	2	2	2	2	2	2	2	2	2	2
2	1	0	0	2	2	1	1	1	0	0	2	1	2	2	2	2	2	2	1	1
3	0	0	0	1	1	1	1	1	0	1	2	2	2	2	2	2	2	2	2	2
4	0	1	0	1	1	0	0	1	1	1	2	2	2	2	2	2	2	2	2	2
5	0	0	0	0	1	0	0	0	0	0	2	2	2	2	2	2	2	2	2	2
6	0	1	0	0	0	0	0	1	2	1	2	2	2	2	1	2	2	2	2	2
7	0	1	0	0	0	0	1	0	0	0	2	2	2	2	2	2	2	2	2	2
8	0	0	0	0	0	0	0	0	0	0	2	2	2	2	2	2	2	2	2	2
9	0	0	0	0	0	0	0	0	1	0	2	2	2	2	2	2	2	2	2	2
10	0	0	0	0	0	0	0	0	0	0	2	2	2	2	2	2	2	2	2	2
11	0	0	0	0	0	0	0	0	0	0	2	2	2	2	1	2	2	2	2	2
12	0	1	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	2	2
13	2	2	2	2	2	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2
14	0	0	1	1	1	1	1	1	1	0	1	2	1	1	1	1	1	2	2	1
15	1	2	1	2	1	0	0	1	1	0	1	2	2	2	2	2	2	2	2	2

Note: Level 0: not found any change in tissues/ eye view.

Level 1: > 0-50% were occured in tissues/ eye view (mild to moderate).

Level 2: > 50% were occured in tissues/ eye view (severe).

Rat no. 1-5 were data collected at 2 hrs.

Rat no. 6-10 were data collected at 8 hrs.

Rat no. 11-15 were data collected at 24 hrs.

Toxicity data of kidney tissues treated with 0.1 ml PBS.

	haemorhage																				RBC in tubular										pyctotic nucleus									
	cortex										medullar										medullar										medullar									
No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	1	1	1	1	1	1	1	1	1	1
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1
9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0	0	0	0	0

Note: Level 0: not found any change in tissues/ eye view.

Level 1: > 0-50% were occured in tissues/ eye view (mild to moderate).

Level 2: > 50% were occured in tissues/ eye view (severe).

Rat no. 1-5 were data collected at 2 hrs.

Rat no. 6-10 were data collected at 8 hrs.

Rat no. 11-15 were data collected at 24 hrs.

Toxicity data of kidney tissues treated with 0.25 mg/kg bee venom.

	haemorhage																				RBC in tubular										pyctotic nucleus									
	cortex										medullar										medullar										medullar									
No.	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	2	2	2	2	2	2	2	2	2	2
2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
6	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
7	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
8	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	0	0	0	0	0	0	0	0	0	0	2	2	2	2	2	2	2	2	2	2
9	2	2	2	2	2	2	2	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
10	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
11	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
12	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
13	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
14	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
15	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2

Note: Level 0: not found any change in tissues/ eye view.

Level 1: > 0-50% were occured in tissues/ eye view (mild to moderate).

Level 2: > 50% were occured in tissues/ eye view (severe).

Rat no. 1-5 were data collected at 2 hrs.

Rat no. 6-10 were data collected at 8 hrs.

Rat no. 11-15 were data collected at 24 hrs.

Toxicity data of kidney tissues treated with 0.5 mg/kg bee venom.

	haemorhage																				RBC in tubular										pyctotic nucleus									
	cortex										medullar										medullar										medullar									
No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
3	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	0	0	0	2	2	2	2	2	2	2	2	2	2
4	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
5	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1
6	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
7	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
8	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
9	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
10	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
11	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
12	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
13	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
14	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
15	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2

Note: Level 0: not found any change in tissues/ eye view.

Level 1: > 0-50% were occured in tissues/ eye view (mild to moderate).

Level 2: > 50% were occured in tissues/ eye view (severe).

Rat no. 1-5 were data collected at 2 hrs.

Rat no. 6-10 were data collected at 8 hrs.

Rat no. 11-15 were data collected at 24 hrs.

Oneway ANOVA of Kupffer cells in group treated with 0.1 ml PBS at all time points.

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
2 hour	50	23.16	5.748	.813	21.53	24.79	13	39
8 hours	50	21.58	2.893	.409	20.76	22.40	13	27
24 hours	50	21.34	4.910	.694	19.94	22.74	12	35
Total	150	22.03	4.712	.385	21.27	22.79	12	39

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	97.773	2	48.887	2.239	.110
Within Groups	3210.120	147	21.838		
Total	3307.893	149			

Robust Tests of Equality of Means

	Statistic[a]	df1	df2	Sig.
Brown-Forsythe	2.239	2	120.690	.111

a. Asymptotically F distributed.

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
10.633	2	147	.000

Oneway ANOVA of Kupffer cells in group treated with 0.25 mg/kg bee venom at all time points.

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
2 hour	50	33.96	6.518	.922	32.11	35.81	22	50
8 hours	50	42.16	6.267	.886	40.38	43.94	30	56
24 hours	50	30.24	9.395	1.329	27.57	32.91	10	46
Total	150	35.45	8.993	.734	34.00	36.90	10	56

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
6.697	2	147	.002

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3719.413	2	1859.707	32.811	.000
Within Groups	8331.760	147	56.679		
Total	12051.173	149			

Robust Tests of Equality of Means

	Statistic[a]	df1	df2	Sig.
Brown-Forsythe	32.811	2	127.180	.000

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2 hour	8 hours	-8.200*	1.506	.000	-11.18	-5.22
	24 hours	3.720*	1.506	.015	.74	6.70
8 hours	2 hour	8.200*	1.506	.000	5.22	11.18
	24 hours	11.920*	1.506	.000	8.94	14.90
24 hours	2 hour	-3.720*	1.506	.015	-6.70	-.74
	8 hours	-11.920*	1.506	.000	-14.90	-8.94

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of Kupffer cells in group treated with 0.5 mg/kg bee venom at all time points.

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
2 hour	50	37.50	4.812	.680	36.13	38.87	28	50
8 hours	50	31.32	5.355	.757	29.80	32.84	20	44
24 hours	50	35.92	4.882	.690	34.53	37.31	21	44
Total	150	34.91	5.639	.460	34.00	35.82	20	50

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
.612	2	147	.544

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1030.813	2	515.407	20.438	.000
Within Groups	3707.060	147	25.218		
Total	4737.873	149			

Robust Tests of Equality of Means

	Statistic[a]	df1	df2	Sig.
Brown-Forsythe	20.438	2	145.617	.000

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

(I) time	(J) time	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
2 hour	8 hours	6.180*	1.004	.000	4.20	8.16
	24 hours	1.580	1.004	.118	-.40	3.56
8 hours	2 hour	-6.180*	1.004	.000	-8.16	-4.20
	24 hours	-4.600*	1.004	.000	-6.58	-2.62
24 hours	2 hour	-1.580	1.004	.118	-3.56	.40
	8 hours	4.600*	1.004	.000	2.62	6.58

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of Kupffer cells at 2 hours.

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	50	23.16	5.748	.813	21.53	24.79	13	39
high dose	50	37.50	4.812	.680	36.13	38.87	28	50
low dose	50	33.96	6.518	.922	32.11	35.81	22	50
Total	150	31.54	8.361	.683	30.19	32.89	13	50

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
2.915	2	147	.057

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5580.120	2	2790.060	84.825	.000
Within Groups	4835.140	147	32.892		
Total	10415.260	149			

Robust Tests of Equality of Means

	Statistic[a]	df1	df2	Sig.
Brown-Forsythe	84.825	2	138.994	.000

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	high dose	-14.340*	1.147	.000	-16.61	-12.07
	low dose	-10.800*	1.147	.000	-13.07	-8.53
high dose	control	14.340*	1.147	.000	12.07	16.61
	low dose	3.540*	1.147	.002	1.27	5.81
low dose	control	10.800*	1.147	.000	8.53	13.07
	high dose	-3.540*	1.147	.002	-5.81	-1.27

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of Kupffer cells at 8 hours.

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	50	21.58	2.893	.409	20.76	22.40	13	27
high dose	50	31.32	5.355	.757	29.80	32.84	20	44
low dose	50	42.16	6.267	.886	40.38	43.94	30	56
Total	150	31.69	9.810	.801	30.10	33.27	13	56

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
12.409	2	147	.000

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10598.493	2	5299.247	208.298	.000
Within Groups	3739.780	147	25.441		
Total	14338.273	149			

Robust Tests of Equality of Means

	Statistic[a]	df1	df2	Sig.
Brown-Forsythe	208.298	2	117.218	.000

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	high dose	-9.740*	1.009	.000	-11.73	-7.75
	low dose	-20.580*	1.009	.000	-22.57	-18.59
high dose	control	9.740*	1.009	.000	7.75	11.73
	low dose	-10.840*	1.009	.000	-12.83	-8.85
low dose	control	20.580*	1.009	.000	18.59	22.57
	high dose	10.840*	1.009	.000	8.85	12.83

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of Kupffer cells at 24 hours.

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
control	50	21.34	4.910	.694	19.94	22.74	12	35
high dose	50	35.92	4.882	.690	34.53	37.31	21	44
low dose	50	30.24	9.395	1.329	27.57	32.91	10	46
Total	150	29.17	9.002	.735	27.71	30.62	10	46

Test of Homogeneity of Variances

Levene Statistic	df1	df2	Sig.
18.395	2	147	.000

ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5400.813	2	2700.407	59.478	.000
Within Groups	6674.020	147	45.401		
Total	12074.833	149			

Robust Tests of Equality of Means

	Statistic[a]	df1	df2	Sig.
Brown-Forsythe	59.478	2	101.679	.000

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
control	high dose	-14.580*	1.348	.000	-17.24	-11.92
	low dose	-8.900*	1.348	.000	-11.56	-6.24
high dose	control	14.580*	1.348	.000	11.92	17.24
	low dose	5.680*	1.348	.000	3.02	8.34
low dose	control	8.900*	1.348	.000	6.24	11.56
	high dose	-5.680*	1.348	.000	-8.34	-3.02

Oneway ANOVA of C.B.C. in control group at all time points.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
R.B.C	2 hr	5	5.36E6	260768.096	1.166E5	5036213.64	5683786.36	5200000	5800000
	8 hr	5	4.90E6	141421.356	6.325E4	4724402.19	5075597.81	4700000	5100000
	24 hr	5	5.09E6	187936.159	8.405E4	4854646.44	5321353.56	4800000	5300000
	Total	15	5.12E6	270998.155	6.997E4	4965926.22	5266073.78	4700000	5800000
hemoglobin	2 hr	5	13.40	.548	.245	12.72	14.08	13	14
	8 hr	5	12.20	.447	.200	11.64	12.76	12	13
	24 hr	5	12.80	.447	.200	12.24	13.36	12	13
	Total	15	12.80	.676	.175	12.43	13.17	12	14
hematocrit	2 hr	5	37.40	1.949	.872	34.98	39.82	35	40
	8 hr	5	34.40	.548	.245	33.72	35.08	34	35
	24 hr	5	35.60	1.342	.600	33.93	37.27	34	37
	Total	15	35.80	1.821	.470	34.79	36.81	34	40
platelet	2 hr	5	5.24E5	38694.961	1.730E4	475553.86	571646.14	487000	576000
	8 hr	5	5.08E5	83238.813	3.723E4	404845.36	611554.64	411000	631000
	24 hr	5	4.73E5	49663.870	2.221E4	411334.16	534665.84	403000	539000
	Total	15	5.02E5	59939.017	1.548E4	468406.88	534793.12	403000	631000
W.B.C	2 hr	5	4780.00	554.076	247.790	4092.02	5467.98	3800	5100
	8 hr	5	5120.00	563.028	251.794	4420.91	5819.09	4400	5900
	24 hr	5	3520.00	756.307	338.231	2580.92	4459.08	2500	4300
	Total	15	4473.33	921.541	237.941	3963.00	4983.67	2500	5900
N	2 hr	5	21.00	3.536	1.581	16.61	25.39	15	24
	8 hr	5	27.40	5.273	2.358	20.85	33.95	22	33
	24 hr	5	15.20	2.588	1.158	11.99	18.41	12	19
	Total	15	21.20	6.327	1.634	17.70	24.70	12	33
E	2 hr	5	1.40	1.342	.600	-.27	3.07	0	3
	8 hr	5	2.00	1.581	.707	.04	3.96	0	4
	24 hr	5	.80	.447	.200	.24	1.36	0	1
	Total	15	1.40	1.242	.321	.71	2.09	0	4

L	2 hr	5	75.80	3.701	1.655	71.20	80.40	73	82
	8 hr	5	69.40	4.159	1.860	64.24	74.56	64	74
	24 hr	5	82.20	1.924	.860	79.81	84.59	79	84
	Total	15	75.80	6.259	1.616	72.33	79.27	64	84
M	2 hr	5	1.80	1.095	.490	.44	3.16	1	3
	8 hr	5	1.20	.447	.200	.64	1.76	1	2
	24 hr	5	1.60	.894	.400	.49	2.71	1	3
	Total	15	1.53	.834	.215	1.07	2.00	1	3

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
R.B.C	.979	2	12	.404
hemoglobin	.821	2	12	.463
hematocrit	3.571	2	12	.061
platelet	.914	2	12	.427
W.B.C	.454	2	12	.645
N	2.073	2	12	.169
E	3.538	2	12	.062
L	1.313	2	12	.305
M	5.765	2	12	.018

Oneway ANOVA of Kupffer cells in control group at all time points(cont.).

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
R.B.C	Between Groups	5.349E11	2	2.674E11	6.506	.012
	Within Groups	4.933E11	12	4.111E10		
	Total	1.028E12	14			
hemoglobin	Between Groups	3.600	2	1.800	7.714	.007
	Within Groups	2.800	12	.233		
	Total	6.400	14			
hematocrit	Between Groups	22.800	2	11.400	5.797	.017
	Within Groups	23.600	12	1.967		
	Total	46.400	14			
platelet	Between Groups	6.728E9	2	3.364E9	.926	.423
	Within Groups	4.357E10	12	3.631E9		
	Total	5.030E10	14			
W.B.C	Between Groups	7105333.333	2	3552666.667	8.911	.004
	Within Groups	4784000.000	12	398666.667		
	Total	1.189E7	14			
N	Between Groups	372.400	2	186.200	11.885	.001
	Within Groups	188.000	12	15.667		
	Total	560.400	14			
E	Between Groups	3.600	2	1.800	1.200	.335
	Within Groups	18.000	12	1.500		
	Total	21.600	14			
L	Between Groups	409.600	2	204.800	17.706	.000
	Within Groups	138.800	12	11.567		
	Total	548.400	14			
M	Between Groups	.933	2	.467	.636	.546
	Within Groups	8.800	12	.733		
	Total	9.733	14			

Oneway ANOVA of Kupffer cells in control group at all time points (cont.).

Robust Tests of Equality of Means[b,c]

		Statistic[a]	df1	df2	Sig.
R.B.C	Brown-Forsythe	6.506	2	9.700	.016
hemoglobin	Brown-Forsythe	7.714	2	11.529	.007
hematocrit	Brown-Forsythe	5.797	2	7.836	.029
platelet	Brown-Forsythe	.926	2	8.425	.433
W.B.C	Brown-Forsythe	8.911	2	10.963	.005
N	Brown-Forsythe	11.885	2	9.072	.003
E	Brown-Forsythe	1.200	2	8.499	.348
L	Brown-Forsythe	17.706	2	9.620	.001
M	Brown-Forsythe	.636	2	9.132	.551

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
R.B.C	2 hr	8 hr	460000.000*	1.282E5	.004	180613.09	739386.91
		24 hr	272000.000	1.282E5	.055	-7386.91	551386.91
	8 hr	2 hr	-460000.000*	1.282E5	.004	-739386.91	-180613.09
		24 hr	-188000.000	1.282E5	.168	-467386.91	91386.91
	24 hr	2 hr	-272000.000	1.282E5	.055	-551386.91	7386.91
		8 hr	188000.000	1.282E5	.168	-91386.91	467386.91
hemoglobin	2 hr	8 hr	1.200*	.306	.002	.53	1.87
		24 hr	.600	.306	.073	-.07	1.27
	8 hr	2 hr	-1.200*	.306	.002	-1.87	-.53
		24 hr	-.600	.306	.073	-1.27	.07
	24 hr	2 hr	-.600	.306	.073	-1.27	.07
		8 hr	.600	.306	.073	-.07	1.27
hematocrit	2 hr	8 hr	3.000*	.887	.005	1.07	4.93
		24 hr	1.800	.887	.065	-.13	3.73
	8 hr	2 hr	-3.000*	.887	.005	-4.93	-1.07
		24 hr	-1.200	.887	.201	-3.13	.73
	24 hr	2 hr	-1.800	.887	.065	-3.73	.13
		8 hr	1.200	.887	.201	-.73	3.13
W.B.C	2 hr	8 hr	-340.000	399.333	.411	-1210.07	530.07
		24 hr	1260.000*	399.333	.008	389.93	2130.07
	8 hr	2 hr	340.000	399.333	.411	-530.07	1210.07
		24 hr	1600.000*	399.333	.002	729.93	2470.07
	24 hr	2 hr	-1260.000*	399.333	.008	-2130.07	-389.93
		8 hr	-1600.000*	399.333	.002	-2470.07	-729.93

L	2 hr	8 hr	6.400*	2.151	.012	1.71	11.09
		24 hr	-6.400*	2.151	.012	-11.09	-1.71
	8 hr	2 hr	-6.400*	2.151	.012	-11.09	-1.71
		24 hr	-12.800*	2.151	.000	-17.49	-8.11
	24 hr	2 hr	6.400*	2.151	.012	1.71	11.09
		8 hr	12.800*	2.151	.000	8.11	17.49
E	2 hr	8 hr	-.600	.775	.454	-2.29	1.09
		24 hr	.600	.775	.454	-1.09	2.29
	8 hr	2 hr	.600	.775	.454	-1.09	2.29
		24 hr	1.200	.775	.147	-.49	2.89
	24 hr	2 hr	-.600	.775	.454	-2.29	1.09
		8 hr	-1.200	.775	.147	-2.89	.49
N	2 hr	8 hr	-6.400*	2.503	.025	-11.85	-.95
		24 hr	5.800*	2.503	.039	.35	11.25
	8 hr	2 hr	6.400*	2.503	.025	.95	11.85
		24 hr	12.200*	2.503	.000	6.75	17.65
	24 hr	2 hr	-5.800*	2.503	.039	-11.25	-.35
		8 hr	-12.200*	2.503	.000	-17.65	-6.75

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of C.B.C. in group treated with 0.25 mg/kg bee venom at all time points.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
R.B.C	2 hr	5	5.36E6	364691.651	1.631E5	4907175.51	5812824.49	5100000	6000000
	8 hr	5	5.54E6	313049.517	1.400E5	5151297.69	5928702.31	5200000	5800000
	24 hr	5	5.45E6	313974.521	1.404E5	5064149.14	5843850.86	5070000	5900000
	Total	15	5.45E6	316156.983	8.163E4	5276251.43	5626415.23	5070000	6000000
hemoglobin	2 hr	5	13.20	.837	.374	12.16	14.24	12	14
	8 hr	5	13.20	.837	.374	12.16	14.24	12	14
	24 hr	5	13.40	1.140	.510	11.98	14.82	12	15
	Total	15	13.27	.884	.228	12.78	13.76	12	15
hematocrit	2 hr	5	37.60	2.302	1.030	34.74	40.46	34	40
	8 hr	5	36.80	1.643	.735	34.76	38.84	35	38
	24 hr	5	36.60	2.191	.980	33.88	39.32	34	40
	Total	15	37.00	1.964	.507	35.91	38.09	34	40
platelet	2 hr	5	4.53E5	13685.759	6120.457	435606.89	469593.11	441000	475000
	8 hr	5	5.30E5	22365.151	1.000E4	502430.00	557970.00	506000	562000
	24 hr	5	3.79E5	194009.794	8.676E4	138505.02	620294.98	69000	524000
	Total	15	4.54E5	122526.071	3.164E4	386213.99	521919.34	69000	562000
W.B.C	2 hr	5	4960.00	1312.631	587.026	3330.15	6589.85	3400	6500
	8 hr	5	5880.00	1439.444	643.739	4092.69	7667.31	4400	8100
	24 hr	5	4380.00	1173.456	524.786	2922.96	5837.04	2700	5300
	Total	15	5073.33	1373.456	354.625	4312.74	5833.93	2700	8100
N	2 hr	5	37.60	7.335	3.280	28.49	46.71	27	47
	8 hr	5	43.60	8.905	3.982	32.54	54.66	30	53
	24 hr	5	20.20	6.760	3.023	11.81	28.59	11	30
	Total	15	33.80	12.514	3.231	26.87	40.73	11	53
E	2 hr	5	.40	.548	.245	-.28	1.08	0	1
	8 hr	5	.60	.894	.400	-.51	1.71	0	2
	24 hr	5	1.20	1.304	.583	-.42	2.82	0	3
	Total	15	.73	.961	.248	.20	1.27	0	3
L	2 hr	5	58.60	6.656	2.977	50.34	66.86	52	69
	8 hr	5	53.80	8.198	3.666	43.62	63.98	46	67
	24 hr	5	78.60	8.503	3.803	68.04	89.16	65	87
	Total	15	63.67	13.270	3.426	56.32	71.02	46	87
M	2 hr	5	3.00	1.581	.707	1.04	4.96	1	5
	8 hr	5	2.00	1.000	.447	.76	3.24	1	3
	24 hr	5	2.00	1.000	.447	.76	3.24	1	3
	Total	15	2.33	1.234	.319	1.65	3.02	1	5

Oneway ANOVA of C.B.C. in group treated with 0.25 mg/kg bee venom at all time points (cont.).

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
R.B.C	.055	2	12	.947
hemoglobin	.407	2	12	.675
hematocrit	.059	2	12	.943
platelet	10.239	2	12	.003
W.B.C	.039	2	12	.962
N	.227	2	12	.800
E	2.313	2	12	.141
L	.053	2	12	.949
M	.727	2	12	.503

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
R.B.C	Between Groups	8.105E10	2	4.053E10	.369	.699
	Within Groups	1.318E12	12	1.099E11		
	Total	1.399E12	14			
hemoglobin	Between Groups	.133	2	.067	.074	.929
	Within Groups	10.800	12	.900		
	Total	10.933	14			
hematocrit	Between Groups	2.800	2	1.400	.328	.727
	Within Groups	51.200	12	4.267		
	Total	54.000	14			
platelet	Between Groups	5.687E10	2	2.843E10	2.226	.151
	Within Groups	1.533E11	12	1.278E10		
	Total	2.102E11	14			
W.B.C	Between Groups	5721333.333	2	2860666.667	1.659	.231
	Within Groups	2.069E7	12	1724000.000		
	Total	2.641E7	14			
N	Between Groups	1477.200	2	738.600	12.393	.001
	Within Groups	715.200	12	59.600		
	Total	2192.400	14			
E	Between Groups	1.733	2	.867	.929	.422
	Within Groups	11.200	12	.933		
	Total	12.933	14			
L	Between Groups	1730.133	2	865.067	14.120	.001
	Within Groups	735.200	12	61.267		
	Total	2465.333	14			
M	Between Groups	3.333	2	1.667	1.111	.361
	Within Groups	18.000	12	1.500		
	Total	21.333	14			

Oneway ANOVA of C.B.C. in group treated with 0.25 mg/kg bee venomat all time points (cont.).

Robust Tests of Equality of Means[b,c,d]

		Statistic[a]	df1	df2	Sig.
R.B.C	Brown-Forsythe	.369	2	11.740	.699
hemoglobin	Brown-Forsythe	.074	2	10.921	.929
hematocrit	Brown-Forsythe	.328	2	11.218	.727
platelet	Brown-Forsythe	2.226	2	4.147	.220
W.B.C	Brown-Forsythe	1.659	2	11.684	.232
N	Brown-Forsythe	12.393	2	11.345	.001
E	Brown-Forsythe	.929	2	8.663	.431
L	Brown-Forsythe	14.120	2	11.544	.001
M	Brown-Forsythe	1.111	2	9.818	.367

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
N	2 hr	8 hr	-6.000	4.883	.243	-16.64	4.64
		24 hr	17.400*	4.883	.004	6.76	28.04
	8 hr	2 hr	6.000	4.883	.243	-4.64	16.64
		24 hr	23.400*	4.883	.000	12.76	34.04
	24 hr	2 hr	-17.400*	4.883	.004	-28.04	-6.76
		8 hr	-23.400*	4.883	.000	-34.04	-12.76
L	2 hr	8 hr	4.800	4.950	.351	-5.99	15.59
		24 hr	-20.000*	4.950	.002	-30.79	-9.21
	8 hr	2 hr	-4.800	4.950	.351	-15.59	5.99
		24 hr	-24.800*	4.950	.000	-35.59	-14.01
	24 hr	2 hr	20.000*	4.950	.002	9.21	30.79
		8 hr	24.800*	4.950	.000	14.01	35.59

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of C.B.C. in group treated with 0.5 mg/kg bee venom at all time points.

Descriptives

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
R.B.C	2 hr	5	5.64E6	270185.122	1.208E5	5304520.86	5975479.14	5300000	6000000
	8 hr	5	5.93E6	264801.813	1.184E5	5603205.12	6260794.88	5600000	6200000
	24 hr	5	5.36E6	444971.909	1.990E5	4807494.40	5912505.60	4800000	5900000
	Total	15	5.64E6	394838.122	1.019E5	5425345.94	5862654.06	4800000	6200000
hemoglobin	2 hr	5	13.20	1.095	.490	11.84	14.56	12	15
	8 hr	5	14.40	.548	.245	13.72	15.08	14	15
	24 hr	5	13.00	1.414	.632	11.24	14.76	11	14
	Total	15	13.53	1.187	.307	12.88	14.19	11	15
hematocrit	2 hr	5	37.60	3.050	1.364	33.81	41.39	34	42
	8 hr	5	40.00	1.225	.548	38.48	41.52	38	41
	24 hr	5	35.60	2.608	1.166	32.36	38.84	32	38
	Total	15	37.73	2.915	.753	36.12	39.35	32	42
platelet	2 hr	5	4.78E5	48918.299	2.188E4	417259.91	538740.09	431000	561000
	8 hr	5	4.02E5	161911.087	7.241E4	201160.83	603239.17	125000	542000
	24 hr	5	5.23E5	8786.353	3929.377	511890.30	533709.70	513000	537000
	Total	15	4.68E5	104164.476	2.690E4	409982.30	525351.03	125000	561000
W.B.C	2 hr	5	4880.00	746.324	333.766	3953.32	5806.68	4400	6100
	8 hr	5	6060.00	1593.110	712.461	4081.89	8038.11	4200	8200
	24 hr	5	4500.00	543.139	242.899	3825.60	5174.40	3700	5000
	Total	15	5146.67	1200.516	309.972	4481.84	5811.49	3700	8200
N	2 hr	5	39.00	12.329	5.514	23.69	54.31	24	54
	8 hr	5	57.20	8.871	3.967	46.18	68.22	45	70
	24 hr	5	37.60	12.198	5.455	22.45	52.75	26	58
	Total	15	44.60	13.922	3.595	36.89	52.31	24	70
E	2 hr	5	1.00	1.000	.447	-.24	2.24	0	2
	8 hr	5	.80	.837	.374	-.24	1.84	0	2
	24 hr	5	.60	.894	.400	-.51	1.71	0	2
	Total	15	.80	.862	.223	.32	1.28	0	2
L	2 hr	5	57.80	13.330	5.962	41.25	74.35	42	75
	8 hr	5	38.40	7.503	3.356	29.08	47.72	28	48
	24 hr	5	60.20	11.212	5.014	46.28	74.12	41	70
	Total	15	52.13	14.312	3.695	44.21	60.06	28	75
M	2 hr	5	1.80	.837	.374	.76	2.84	1	3
	8 hr	5	1.20	1.095	.490	-.16	2.56	0	3
	24 hr	5	1.60	.894	.400	.49	2.71	1	3
	Total	15	1.53	.915	.236	1.03	2.04	0	3

Oneway ANOVA of C.B.C. in group treated with 0.5 mg/kg bee venom at all time points (cont.).

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
R.B.C	1.350	2	12	.296
hemoglobin	2.639	2	12	.112
hematocrit	2.148	2	12	.159
platelet	3.748	2	12	.054
W.B.C	3.570	2	12	.061
N	.609	2	12	.560
E	.178	2	12	.839
L	.884	2	12	.438
M	.036	2	12	.965

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
R.B.C	Between Groups	8.181E11	2	4.090E11	3.597	.060
	Within Groups	1.364E12	12	1.137E11		
	Total	2.183E12	14			
hemoglobin	Between Groups	5.733	2	2.867	2.457	.128
	Within Groups	14.000	12	1.167		
	Total	19.733	14			
hematocrit	Between Groups	48.533	2	24.267	4.136	.043
	Within Groups	70.400	12	5.867		
	Total	118.933	14			
platelet	Between Groups	3.716E10	2	1.858E10	1.943	.186
	Within Groups	1.147E11	12	9.562E9		
	Total	1.519E11	14			
W.B.C	Between Groups	6617333.333	2	3308666.667	2.928	.092
	Within Groups	1.356E7	12	1130000.000		
	Total	2.018E7	14			
N	Between Groups	1195.600	2	597.800	4.726	.031
	Within Groups	1518.000	12	126.500		
	Total	2713.600	14			
E	Between Groups	.400	2	.200	.240	.790
	Within Groups	10.000	12	.833		
	Total	10.400	14			
L	Between Groups	1428.933	2	714.467	5.959	.016
	Within Groups	1438.800	12	119.900		
	Total	2867.733	14			
M	Between Groups	.933	2	.467	.519	.608
	Within Groups	10.800	12	.900		
	Total	11.733	14			

Oneway ANOVA of C.B.C. in group treated with 0.5 mg/kg bee venom at all time points (cont.).

Robust Tests of Equality of Means[b,c,d]

		Statistic[a]	df1	df2	Sig.
R.B.C	Brown-Forsythe	3.597	2	9.413	.069
hemoglobin	Brown-Forsythe	2.457	2	8.861	.142
hematocrit	Brown-Forsythe	4.136	2	9.179	.052
platelet	Brown-Forsythe	1.943	2	4.750	.242
W.B.C	Brown-Forsythe	2.928	2	6.722	.122
N	Brown-Forsythe	4.726	2	11.199	.033
E	Brown-Forsythe	.240	2	11.737	.790
L	Brown-Forsythe	5.959	2	10.239	.019
M	Brown-Forsythe	.519	2	11.346	.609

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

Dependent Variable	(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
hematocrit	2 hr	8 hr	-2.400	1.532	.143	-5.74	.94
		24 hr	2.000	1.532	.216	-1.34	5.34
	8 hr	2 hr	2.400	1.532	.143	-.94	5.74
		24 hr	4.400*	1.532	.014	1.06	7.74
	24 hr	2 hr	-2.000	1.532	.216	-5.34	1.34
		8 hr	-4.400*	1.532	.014	-7.74	-1.06
N	2 hr	8 hr	-18.200*	7.113	.025	-33.70	-2.70
		24 hr	1.400	7.113	.847	-14.10	16.90
	8 hr	2 hr	18.200*	7.113	.025	2.70	33.70
		24 hr	19.600*	7.113	.017	4.10	35.10
	24 hr	2 hr	-1.400	7.113	.847	-16.90	14.10
		8 hr	-19.600*	7.113	.017	-35.10	-4.10
L	2 hr	8 hr	19.400*	6.925	.016	4.31	34.49
		24 hr	-2.400	6.925	.735	-17.49	12.69
	8 hr	2 hr	-19.400*	6.925	.016	-34.49	-4.31
		24 hr	-21.800*	6.925	.008	-36.89	-6.71
	24 hr	2 hr	2.400	6.925	.735	-12.69	17.49
		8 hr	21.800*	6.925	.008	6.71	36.89

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of C.B.C. at 2 hrs.

Descriptives

	Dose	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
R.B.C	control	5	5.36E6	260768.096	1.166E5	5036213.64	5683786.36	5200000	5800000
	high	5	5.64E6	270185.122	1.208E5	5304520.86	5975479.14	5300000	6000000
	low	5	5.36E6	364691.651	1.631E5	4907175.51	5812824.49	5100000	6000000
	Total	15	5.45E6	311371.773	8.040E4	5280901.39	5625765.27	5100000	6000000
hemoglobin	control	5	13.40	.548	.245	12.72	14.08	13	14
	high	5	13.20	1.095	.490	11.84	14.56	12	15
	low	5	13.20	.837	.374	12.16	14.24	12	14
	Total	15	13.27	.799	.206	12.82	13.71	12	15
hematocrit	control	5	37.40	1.949	.872	34.98	39.82	35	40
	high	5	37.60	3.050	1.364	33.81	41.39	34	42
	low	5	37.60	2.302	1.030	34.74	40.46	34	40
	Total	15	37.53	2.295	.593	36.26	38.80	34	42
platelet	control	5	5.24E5	38694.961	1.730E4	475553.86	571646.14	487000	576000
	high	5	4.78E5	48918.299	2.188E4	417259.91	538740.09	431000	561000
	low	5	4.53E5	13685.759	6120.457	435606.89	469593.11	441000	475000
	Total	15	4.85E5	45710.997	1.180E4	459419.43	510047.24	431000	576000
W.B.C	control	5	4780.00	554.076	247.790	4092.02	5467.98	3800	5100
	high	5	4880.00	746.324	333.766	3953.32	5806.68	4400	6100
	low	5	4960.00	1312.631	587.026	3330.15	6589.85	3400	6500
	Total	15	4873.33	863.106	222.853	4395.36	5351.31	3400	6500
N	control	5	21.00	3.536	1.581	16.61	25.39	15	24
	high	5	39.00	12.329	5.514	23.69	54.31	24	54
	low	5	37.60	7.335	3.280	28.49	46.71	27	47
	Total	15	32.53	11.575	2.989	26.12	38.94	15	54
E	control	5	1.40	1.342	.600	-.27	3.07	0	3
	high	5	1.00	1.000	.447	-.24	2.24	0	2
	low	5	.40	.548	.245	-.28	1.08	0	1
	Total	15	.93	1.033	.267	.36	1.51	0	3
L	control	5	75.80	3.701	1.655	71.20	80.40	73	82
	high d	5	57.80	13.330	5.962	41.25	74.35	42	75
	low	5	58.60	6.656	2.977	50.34	66.86	52	69
	Total	15	64.07	11.883	3.068	57.49	70.65	42	82
M	control	5	1.80	1.095	.490	.44	3.16	1	3
	high dose	5	1.80	.837	.374	.76	2.84	1	3
	low dose	5	3.00	1.581	.707	1.04	4.96	1	5
	Total	15	2.20	1.265	.327	1.50	2.90	1	5

Oneway ANOVA of C.B.C. at 2 hours (cont.).

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
R.B.C	.181	2	12	.837
hemoglobin	.298	2	12	.748
hematocrit	.511	2	12	.612
platelet	2.260	2	12	.147
W.B.C	3.898	2	12	.050
N	4.309	2	12	.039
E	3.459	2	12	.065
L	4.524	2	12	.034
M	1.265	2	12	.317

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
R.B.C	Between Groups	2.613E11	2	1.307E11	1.431	.277
	Within Groups	1.096E12	12	9.133E10		
	Total	1.357E12	14			
hemoglobin	Between Groups	.133	2	.067	.091	.914
	Within Groups	8.800	12	.733		
	Total	8.933	14			
hematocrit	Between Groups	.133	2	.067	.011	.989
	Within Groups	73.600	12	6.133		
	Total	73.733	14			
platelet	Between Groups	1.294E10	2	6.471E9	4.761	.030
	Within Groups	1.631E10	12	1.359E9		
	Total	2.925E10	14			
W.B.C	Between Groups	81333.333	2	40666.667	.047	.954
	Within Groups	1.035E7	12	862333.333		
	Total	1.043E7	14			
N	Between Groups	1002.533	2	501.267	6.889	.010
	Within Groups	873.200	12	72.767		
	Total	1875.733	14			
E	Between Groups	2.533	2	1.267	1.226	.328
	Within Groups	12.400	12	1.033		
	Total	14.933	14			
L	Between Groups	1034.133	2	517.067	6.581	.012
	Within Groups	942.800	12	78.567		
	Total	1976.933	14			
M	Between Groups	4.800	2	2.400	1.636	.235
	Within Groups	17.600	12	1.467		
	Total	22.400	14			

Oneway ANOVA of C.B.C. at 2 hours (cont.).

Robust Tests of Equality of Means[b,c,d]

		Statistic[a]	df1	df2	Sig.
R.B.C	Brown-Forsythe	1.431	2	10.864	.281
hemoglobin	Brown-Forsythe	.091	2	9.584	.914
hematocrit	Brown-Forsythe	.011	2	10.496	.989
platelet	Brown-Forsythe	4.761	2	8.310	.042
W.B.C	Brown-Forsythe	.047	2	7.936	.954
N	Brown-Forsythe	6.889	2	7.288	.021
E	Brown-Forsythe	1.226	2	8.878	.339
L	Brown-Forsythe	6.581	2	6.589	.027
M	Brown-Forsythe	1.636	2	9.467	.245

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

Dependent Variable	(I) type	(J) type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
platelet	control	high dose	45600.000	2.332E4	.074	-5203.26	96403.26
		low dose	71000.000*	2.332E4	.010	20196.74	121803.26
	high dose	control	-45600.000	2.332E4	.074	-96403.26	5203.26
		low dose	25400.000	2.332E4	.297	-25403.26	76203.26
	low dose	control	-71000.000*	2.332E4	.010	-121803.26	-20196.74
		high dose	-25400.000	2.332E4	.297	-76203.26	25403.26
N	control	high dose	-18.000*	5.395	.006	-29.75	-6.25
		low dose	-16.600*	5.395	.010	-28.35	-4.85
	high dose	control	18.000*	5.395	.006	6.25	29.75
		low dose	1.400	5.395	.800	-10.35	13.15
	low dose	control	16.600*	5.395	.010	4.85	28.35
		high dose	-1.400	5.395	.800	-13.15	10.35
L	control	high dose	18.000*	5.606	.007	5.79	30.21
		low dose	17.200*	5.606	.010	4.99	29.41
	high dose	control	-18.000*	5.606	.007	-30.21	-5.79
		low dose	-.800	5.606	.889	-13.01	11.41
	low dose	control	-17.200*	5.606	.010	-29.41	-4.99
		high dose	.800	5.606	.889	-11.41	13.01

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of C.B.C. at 8 hrs.

Descriptives

	Dose	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
R.B.C	control	5	4.90E6	141421.356	6.325E4	4724402.19	5075597.81	4700000	5100000
	high	5	5.93E6	264801.813	1.184E5	5603205.12	6260794.88	5600000	6200000
	low	5	5.54E6	313049.517	1.400E5	5151297.69	5928702.31	5200000	5800000
	Total	15	5.46E6	497586.556	1.285E5	5181779.08	5732887.58	4700000	6200000
hemoglobin	control	5	12.20	.447	.200	11.64	12.76	12	13
	high	5	14.40	.548	.245	13.72	15.08	14	15
	low	5	13.20	.837	.374	12.16	14.24	12	14
	Total	15	13.27	1.100	.284	12.66	13.88	12	15
hematocrit	control	5	34.40	.548	.245	33.72	35.08	34	35
	high	5	40.00	1.225	.548	38.48	41.52	38	41
	low	5	36.80	1.643	.735	34.76	38.84	35	38
	Total	15	37.07	2.631	.679	35.61	38.52	34	41
platelet	control	5	5.08E5	83238.813	3.723E4	404845.36	611554.64	411000	631000
	high	5	4.02E5	161911.087	7.241E4	201160.83	603239.17	125000	542000
	low	5	5.30E5	22365.151	1.000E4	502430.00	557970.00	506000	562000
	Total	15	4.80E5	113834.592	2.939E4	417160.50	543239.50	125000	631000
W.B.C	control	5	5120.00	563.028	251.794	4420.91	5819.09	4400	5900
	high	5	6060.00	1593.110	712.461	4081.89	8038.11	4200	8200
	low	5	5880.00	1439.444	643.739	4092.69	7667.31	4400	8100
	Total	15	5686.67	1259.176	325.118	4989.36	6383.98	4200	8200
N	control	5	27.40	5.273	2.358	20.85	33.95	22	33
	high	5	57.20	8.871	3.967	46.18	68.22	45	70
	low	5	43.60	8.905	3.982	32.54	54.66	30	53
	Total	15	42.73	14.563	3.760	34.67	50.80	22	70
E	control	5	2.00	1.581	.707	.04	3.96	0	4
	high	5	.80	.837	.374	-.24	1.84	0	2
	low	5	.60	.894	.400	-.51	1.71	0	2
	Total	15	1.13	1.246	.322	.44	1.82	0	4
L	control	5	69.40	4.159	1.860	64.24	74.56	64	74
	high	5	38.40	7.503	3.356	29.08	47.72	28	48
	low	5	53.80	8.198	3.666	43.62	63.98	46	67
	Total	15	53.87	14.555	3.758	45.81	61.93	28	74
M	control	5	1.20	.447	.200	.64	1.76	1	2
	high	5	1.20	1.095	.490	-.16	2.56	0	3
	low	5	2.00	1.000	.447	.76	3.24	1	3
	Total	15	1.47	.915	.236	.96	1.97	0	3

Oneway ANOVA of C.B.C. at 8 hours (cont.).

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
R.B.C	6.267	2	12	.014
hemoglobin	1.412	2	12	.281
hematocrit	4.371	2	12	.038
platelet	2.420	2	12	.131
W.B.C	2.078	2	12	.168
N	.199	2	12	.822
E	1.323	2	12	.303
L	.463	2	12	.640
M	1.204	2	12	.334

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
R.B.C	Between Groups	2.714E12	2	1.357E12	21.639	.000
	Within Groups	7.525E11	12	6.271E10		
	Total	3.466E12	14			
hemoglobin	Between Groups	12.133	2	6.067	15.167	.001
	Within Groups	4.800	12	.400		
	Total	16.933	14			
hematocrit	Between Groups	78.933	2	39.467	26.311	.000
	Within Groups	18.000	12	1.500		
	Total	96.933	14			
platelet	Between Groups	4.684E10	2	2.342E10	2.088	.167
	Within Groups	1.346E11	12	1.121E10		
	Total	1.814E11	14			
W.B.C	Between Groups	2489333.333	2	1244666.667	.758	.490
	Within Groups	1.971E7	12	1642333.333		
	Total	2.220E7	14			
N	Between Groups	2225.733	2	1112.867	17.969	.000
	Within Groups	743.200	12	61.933		
	Total	2968.933	14			
E	Between Groups	5.733	2	2.867	2.150	.159
	Within Groups	16.000	12	1.333		
	Total	21.733	14			
L	Between Groups	2402.533	2	1201.267	25.595	.000
	Within Groups	563.200	12	46.933		
	Total	2965.733	14			
M	Between Groups	2.133	2	1.067	1.333	.300
	Within Groups	9.600	12	.800		
	Total	11.733	14			

Oneway ANOVA of C.B.C. at 8 hours (cont.).

Robust Tests of Equality of Means[b,c,d]

		Statistic[a]	df1	df2	Sig.
R.B.C	Brown-Forsythe	21.639	2	9.487	.000
hemoglobin	Brown-Forsythe	15.167	2	9.290	.001
hematocrit	Brown-Forsythe	26.311	2	8.411	.000
platelet	Brown-Forsythe	2.088	2	6.156	.203
W.B.C	Brown-Forsythe	.758	2	8.962	.496
N	Brown-Forsythe	17.969	2	10.418	.000
E	Brown-Forsythe	2.150	2	8.672	.174
L	Brown-Forsythe	25.595	2	9.931	.000
M	Brown-Forsythe	1.333	2	9.290	.310

a. Asymptotically F distributed.

Oneway ANOVA of C.B.C. at 8 hours (cont.).

Post Hoc Tests

Multiple Comparisons

LSD

Dependent Variable	(I) type	(J) type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
hemoglobin	control	high dose	-2.200*	.400	.000	-3.07	-1.33
		low dose	-1.000*	.400	.028	-1.87	-.13
	high dose	control	2.200*	.400	.000	1.33	3.07
		low dose	1.200*	.400	.011	.33	2.07
	low dose	control	1.000*	.400	.028	.13	1.87
		high dose	-1.200*	.400	.011	-2.07	-.33
N	control	high dose	-29.800*	4.977	.000	-40.64	-18.96
		low dose	-16.200*	4.977	.007	-27.04	-5.36
	high dose	control	29.800*	4.977	.000	18.96	40.64
		low dose	13.600*	4.977	.018	2.76	24.44
	low dose	control	16.200*	4.977	.007	5.36	27.04
		high dose	-13.600*	4.977	.018	-24.44	-2.76
L	control	high dose	31.000*	4.333	.000	21.56	40.44
		low dose	15.600*	4.333	.004	6.16	25.04
	high dose	control	-31.000*	4.333	.000	-40.44	-21.56
		low dose	-15.400*	4.333	.004	-24.84	-5.96
	low dose	control	-15.600*	4.333	.004	-25.04	-6.16
		high dose	15.400*	4.333	.004	5.96	24.84
hematocrit	control	high dose	-5.600*	.775	.000	-7.29	-3.91
		low dose	-2.400*	.775	.009	-4.09	-.71
	high dose	control	5.600*	.775	.000	3.91	7.29
		low dose	3.200*	.775	.001	1.51	4.89
	low dose	control	2.400*	.775	.009	.71	4.09
		high dose	-3.200*	.775	.001	-4.89	-1.51
R.B.C	control	high dose	-1.032E6*	1.584E5	.000	-1377069.66	-686930.34
		low dose	-640000.000*	1.584E5	.002	-985069.66	-294930.34
	high dose	control	1032000.000*	1.584E5	.000	686930.34	1377069.66
		low dose	392000.000*	1.584E5	.029	46930.34	737069.66
	low dose	control	640000.000*	1.584E5	.002	294930.34	985069.66
		high dose	-392000.000*	1.584E5	.029	-737069.66	-46930.34

*. The mean difference is significant at the 0.05 level.

Oneway ANOVA of C.B.C. at 24 hrs.

Descriptives

	Dose	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
						Lower Bound	Upper Bound		
R.B.C	control	5	5.09E6	187936.159	8.405E4	4854646.44	5321353.56	4800000	5300000
	high	5	5.36E6	444971.909	1.990E5	4807494.40	5912505.60	4800000	5900000
	low	5	5.45E6	313974.521	1.404E5	5064149.14	5843850.86	5070000	5900000
	Total	15	5.30E6	347325.839	8.968E4	5108324.03	5493009.30	4800000	5900000
hemoglobin	control	5	12.80	.447	.200	12.24	13.36	12	13
	high	5	13.00	1.414	.632	11.24	14.76	11	14
	low	5	13.40	1.140	.510	11.98	14.82	12	15
	Total	15	13.07	1.033	.267	12.49	13.64	11	15
hematocrit	control	5	35.60	1.342	.600	33.93	37.27	34	37
	high	5	35.60	2.608	1.166	32.36	38.84	32	38
	low	5	36.60	2.191	.980	33.88	39.32	34	40
	Total	15	35.93	2.017	.521	34.82	37.05	32	40
platelet	control	5	4.73E5	49663.870	2.221E4	411334.16	534665.84	403000	539000
	high	5	5.23E5	8786.353	3929.377	511890.30	533709.70	513000	537000
	low	5	3.79E5	194009.794	8.676E4	138505.02	620294.98	69000	524000
	Total	15	4.58E5	123560.743	3.190E4	389974.34	526825.66	69000	539000
W.B.C	control	5	3520.00	756.307	338.231	2580.92	4459.08	2500	4300
	high	5	4500.00	543.139	242.899	3825.60	5174.40	3700	5000
	low	5	4380.00	1173.456	524.786	2922.96	5837.04	2700	5300
	Total	15	4133.33	919.368	237.380	3624.20	4642.46	2500	5300
N	control	5	15.20	2.588	1.158	11.99	18.41	12	19
	high	5	37.60	12.198	5.455	22.45	52.75	26	58
	low	5	20.20	6.760	3.023	11.81	28.59	11	30
	Total	15	24.33	12.500	3.227	17.41	31.26	11	58
E	control	5	.80	.447	.200	.24	1.36	0	1
	high	5	.60	.894	.400	-.51	1.71	0	2
	low	5	1.20	1.304	.583	-.42	2.82	0	3
	Total	15	.87	.915	.236	.36	1.37	0	3
L	control	5	82.20	1.924	.860	79.81	84.59	79	84
	high	5	60.20	11.212	5.014	46.28	74.12	41	70
	low	5	78.60	8.503	3.803	68.04	89.16	65	87
	Total	15	73.67	12.534	3.236	66.73	80.61	41	87
M	control	5	1.60	.894	.400	.49	2.71	1	3
	high	5	1.60	.894	.400	.49	2.71	1	3
	low	5	2.00	1.000	.447	.76	3.24	1	3
	Total	15	1.73	.884	.228	1.24	2.22	1	3

Oneway ANOVA of C.B.C. at 24 hours (cont.).

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
R.B.C	2.159	2	12	.158
hemoglobin	4.927	2	12	.027
hematocrit	.986	2	12	.401
platelet	8.804	2	12	.004
W.B.C	3.262	2	12	.074
N	1.830	2	12	.202
E	3.413	2	12	.067
L	2.038	2	12	.173
M	.063	2	12	.939

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
R.B.C	Between Groups	3.613E11	2	1.806E11	1.633	.236
	Within Groups	1.328E12	12	1.106E11		
	Total	1.689E12	14			
hemoglobin	Between Groups	.933	2	.467	.400	.679
	Within Groups	14.000	12	1.167		
	Total	14.933	14			
hematocrit	Between Groups	3.333	2	1.667	.373	.696
	Within Groups	53.600	12	4.467		
	Total	56.933	14			
platelet	Between Groups	5.301E10	2	2.650E10	1.979	.181
	Within Groups	1.607E11	12	1.339E10		
	Total	2.137E11	14			
W.B.C	Between Groups	2857333.333	2	1428666.667	1.910	.190
	Within Groups	8976000.000	12	748000.000		
	Total	1.183E7	14			
N	Between Groups	1382.533	2	691.267	10.307	.002
	Within Groups	804.800	12	67.067		
	Total	2187.333	14			
E	Between Groups	.933	2	.467	.519	.608
	Within Groups	10.800	12	.900		
	Total	11.733	14			
L	Between Groups	1392.533	2	696.267	10.356	.002
	Within Groups	806.800	12	67.233		
	Total	2199.333	14			
M	Between Groups	.533	2	.267	.308	.741
	Within Groups	10.400	12	.867		
	Total	10.933	14			

Oneway ANOVA of C.B.C. at 24 hours (cont.).

Robust Tests of Equality of Means[b,c,d]

		Statistic[a]	df1	df2	Sig.
R.B.C	Brown-Forsythe	1.633	2	8.783	.249
hemoglobin	Brown-Forsythe	.400	2	8.551	.682
hematocrit	Brown-Forsythe	.373	2	9.904	.698
platelet	Brown-Forsythe	1.979	2	4.539	.241
W.B.C	Brown-Forsythe	1.910	2	8.718	.205
N	Brown-Forsythe	10.307	2	6.671	.009
E	Brown-Forsythe	.519	2	8.168	.614
L	Brown-Forsythe	10.356	2	7.734	.006
M	Brown-Forsythe	.308	2	11.860	.741

a. Asymptotically F distributed.

Post Hoc Tests

Multiple Comparisons

LSD

Dependent Variable	(I) type	(J) type	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
N	control	high dose	-22.400*	5.179	.001	-33.69	-11.11
		low dose	-5.000	5.179	.353	-16.29	6.29
	high dose	control	22.400*	5.179	.001	11.11	33.69
		low dose	17.400*	5.179	.006	6.11	28.69
	low dose	control	5.000	5.179	.353	-6.29	16.29
		high dose	-17.400*	5.179	.006	-28.69	-6.11
L	control	high dose	22.000*	5.186	.001	10.70	33.30
		low dose	3.600	5.186	.501	-7.70	14.90
	high dose	control	-22.000*	5.186	.001	-33.30	-10.70
		low dose	-18.400*	5.186	.004	-29.70	-7.10
	low dose	control	-3.600	5.186	.501	-14.90	7.70
		high dose	18.400*	5.186	.004	7.10	29.70

*. The mean difference is significant at the 0.05 level.

BIOGRAPHY

Mrs Saichon Chaiwongwattanakul was born on January 16, 1980 in Samutprakarn Province, Thailand. She graduated with Bachelor degree of Science in Biology from Burapha University in 2002 and has studied for Master degree of Zoology at Department of Biology, Chulalongkorn University since 2006.