องค์ประกอบของพิษผึ้งหลวง *Apis dorsata* และผลที่มีต่อหนู *Rattus norvegicus*

นางสาวธัญลักษณ์ ตะโกดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีชีวภาพ

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ปีการศึกษา 2551

COMPONENTS OF BEE VENOM IN *Apis dorsata* AND THEIR EFFECTS ON RAT *Rattus norvegicus*

Miss Thanyalak Thakodee

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	COMPONENTS OF BEE VENOM IN *Apis dorsata* AND THEIR EFFECTS ON RAT *Rattus norvegicus*
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ธัญลักษณ์ ตะโกดี : องค์ประกอบของพิษผึ้งหลวง *Apis dorsata* และผลที่มีต่อหนู *Rattus norvegicus*. (COMPONENTS OF BEE VENOM IN *Apis dorsata* AND THEIR EFFECTS ON RAT *Rattus norvegicus*) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. สุรีรัตน์ เดี่ยววาณิชย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ผศ. ดร. วรินทร ชวศิริ, 93 หน้า.

จากการทดลองวัดปริมาณของพิษผึ้งแห้งของผึ้งหลวงต่อตัวเท่ากับ 0.170 ± 0.016 มิลลิกรัม และนำมาหาองค์ประกอบของพิษผึ้งโดยใช้โครมาโทรกราฟีของเหลวสมรรถนะสูงแบบรีเวอร์สเฟส (reversed phase high performance liquid chromatography) โดยเทียบกับสารตัวอย่าง พบว่า พิษผึ้งหลวงประกอบไปด้วย apamine, phospholipase A_2, melittin และยังพบ glycosyltransferase ที่ได้จากเทคนิคเปปไทด์แมสแมพพ์ (peptide mass fingerprinting) ส่วนผลของพิษผึ้งที่มีต่อเนื้อเยื่อตับ ไตของหนูขาว *Rattus norvegicus* เป็นเพศผู้ โดยแบ่งหนูออกเป็น 3 กลุ่ม ๆ ย่อยละ 5 ตัว กลุ่มที่ 1. ฉีดด้วย 0.1 ml PBS ส่วนกลุ่มที่ 2 และกลุ่มที่ 3 นั้น คือ กลุ่มที่ทดสอบด้วยพิษของผึ้งหลวง 0.25 และ 0.5 mgใน 0.1 ml PBS เข้าทางผิวหนังแล้วทิ้งไว้ 2, 8 และ 24 ชั่วโมง พบว่า พิษผึ้งมีผลต่อเนื้อเยื่อตับคือ พบว่าเกิดอาการเลือดคลั่ง (blood congestion), การขยายตัวของช่องsinusoid (sinusoidal space dilation) และการเกิดไขมันในเซลล์ตับ (lipid accumulation in hepatocyte) โดยพบปริมาณเพิ่มขึ้นตามจำนวนชั่วโมงที่เพิ่มขึ้น และยังพบว่าพิษผึ้งก่อให้เกิดการอักเสบ ซึ่งพบว่ามีปริมาณของ Kupffer cells เพิ่มมากขึ้นอย่างมีนัยสำคัญที่ 2, 8 และ 24 ชั่วโมงที่ $p<0.05$ โดยเริ่มพบจากที่ 2 ชั่วโมงในปริมาณน้อยและพบมากที่สุดที่ 24 ชั่วโมง ส่วนผลของพิษผึ้งที่มีต่อเนื้อเยื่อไตชั้นนอก (renal cortex) อาการที่ตรวจพบ คือ ท่อไตชั้นนอกเสียสภาพ (proximal tubular degeneration) และภาวะเลือดคลั่งที่ท่อไตชั้นนอก (haemorrhage) ส่วนอาการที่ตรวจพบที่ท่อไตชั้นใน(renal medulla) อาการที่พบคือ ท่อไตชั้นในเสียสภาพ (collecting duct degeneration), ภาวะเลือดคลั่งที่ท่อไตชั้นใน(haemorrhage) และพบปริมาณของเม็ดเลือดขาว(white blood cell) แทรกตัวอยู่บริเวณท่อไตชั้นใน(collecting duct) มากขึ้น และพบว่ามีแตกต่างกันอย่างมีนัยสำคัญ ที่ 2, 8 และ 24 ชั่วโมงที่ $p<0.05$ นอกจากนี้ยังพบว่า การเพิ่มจำนวนของนิวโตรฟิว (neutrophil) และการลดจำนวนลงของลิมโฟไซต์ (lymphocyte) ที่แตกต่างจากกลุ่มควบคุมอย่างมีนัยสำคัญ จึงสรุปได้ว่า พิษผึ้งมีผลต่อเนื้อเยื่อตับ ไต และเลือดของหนูขาว

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต.....

ปีการศึกษา 2551 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

#4872314823 : MAJOR BIOTECHNOLOGY
KEYWORDS: *Apis dorsata* / BEE VENOM / RAT / RH-HPLC

THANYALAK THAKODEE : COMPONENTS OF BEE VENOM IN *Apis dorsata* AND THEIR EFFECTS ON RAT *Rattus norvegicus*. THESIS ADVISOR: ASST. PROF. SUREERAT DEOWANISH, D. Agr. THESIS CO-ADVISOR : ASST. PROF. WARITORN CHAVASIRI Ph.D. 93 pp.

The amount of dried weight venom of *A. dorsata* is about 0.170 ± 0.016 mg per bee. Three of bee venom (BV) component (apamine, phospholipase A_2, melittin) were found from RP-HPLC whereas the enzyme glycosyltransferease is a enzyme that found in BV component from MALDI-TOF. Three groups of rats were used in the experiment. Control group was injected with 0.1 ml in PBS into subcutaneous. Two treatment groups were injected with venom at concentration of 0.25 mg and 0.50 mg in 0.1 ml PBS respectively. After 2, 8 and 24 hours the rats were euthanized and liver, kidney and blood were collected for histological process. Liver cell show three majors respond to BV including blood congestion, sinusoidal space dilation and lipid accumulation in hepatocyte. BV cause local inflammation of liver cell. The result support by the increasing in amount of Kupffer cells. The renal cortex shows two major responds (proximal tubular degeneration and haemorrhage) and the renal medulla shows three major responds (collecting duct degeneration, haemorrhage and white blood cell infiltration). Increasing of neutrophils and decreasing of lymphocytes were found significant difference from control group. Suggest that BV has responding to immune of mammalian. These results suggested that BV was toxic to liver, kidney and blood tissue of rat.

Field of study : Biotechnology Student's singnature .....
Academic year : 2008 Advisor's singnature .....
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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
BG	blood congestion
BV	bee venom
C	degree Celsius
CD	collecting duct
CDD	collecting duct degeneration
cm	centimeter
CV	central vain
Da	Dalton
DT	Distal tubule
DTD	Distal tubule degeneration
g	gram
HC	hepatocyte
HM	haemorrhage
Hrs	hours
H&E	haematoxylin and eosin
LP	lipid accumulation
KDa	kilodalton
M	meter
MALDI	Matrix-Assisted Laser Desorption/ionization

MALDI-TOF	Matrix-Assisted Laser Desorption/ionization/time of flight analyzer
MCDP	mast cell degranulating peptide
MeOH	methanol
Mg	milligram
Ml	milliliter
Mm	millimeter
MS	mass spectrometry
M/Z	mass/charge
N	number
No.	number
PD	proximal tubular degeneration
PLA2	phopholipase A_2
PMF	peptide mass fingerprinting
RP-HPLC	Reverse phase High Performance liquid Chromatography
PT	proximal tubule
PTD	proximal tubule degeneration
spp.	species
SS	sinusoidal space dilation
TFA	trifluoroacetic acid
TOF	time of flight analyzer
µm	micrometer
UV	ultraviolet
WBS	white blood cell
WxLxH	width x long x high
2D-gel	two dimention gel electrophoresis

CHAPTER I
INTRODUCTION

The medically important groups of Hymenoptera are the Apoidea (bees), Vespoidea (wasps, hornets and yellow jackets), and Formicidea (ants). These insects deliver their venom by stinging their victims. Bees lose their barbed stinger after stinging and die whereas wasps, hornets and yellow jackets can sting several times without dying.

The morphology of the sting apparatus of honey bees in the genus *Apis* (*Apis dorsata*, *A. mellifera*, *A. cerana*, *A. florea*) are different under scanning electron microscope. This result suggested that the structure of sting apparatus related in their sizes to the exterior proportions of the respective species. The highly specialized barbs of stylet and lancets showed a diverging situation and this character can be used to identify the honey bees genus *Apis* from each others. (Weiss, 1978; Jayasvasti and Limbipichai, 1988)

Honeybees use bee venom as a defensive agent against predators, primarily large mammals and other vertebrate predators. Honeybees can only sting once because a barbed stinger that stays behind in the victim's skin after stinging. The stinger and venom sac are pulled out of the bee's abdomen and soon after the bee dies. In order to defensive value the bee venom induces pain, altered sensory activity in the potential predator (Schmidt, 1990; Goddard, 2000).

Bee venom composed of protein, peptides, active amines, and other compounds which variety of activities (Bank and Shipolini, 1986). The major chemical component appears to be melittin (Schmidt, 1995) and this component might be responsible for much of the activity of bee venom in apitherapy use.

The composition and pharmacological properties of *A. mellifera* venom, commercially available, is well characterized but the study on *A. dorsata* venom is still little limited (Kreil, 1973, 1975). Therefore, this research to planed to study amount of venom, components and the effect of *A. dorsata* venom. High performance liquid chromatography (HPLC) method was used for separation and purification. Mass spectrometry method was used to analyze and find molecular weight of venom. The final part was to study the effect of *A. dorsata* venom on liver, kidney and blood tissues in rats by using histological techniques.

The objectives of this research were:

1. To study the amount of *A. dorsata* venom per bee.
2. To study the components of *A. dorsata* venom.
3. To study the effect of *A. dorsata* venom on liver, kidney and blood of rat, *Rattus norvegicus.*

CHAPTER II
LITERATURE REVIEW

There are five species of honey bees in Thailand. Giant or rock honey bee: *A. dorsata*, red dwarf honey bee: *A. florea*, black dwarf honey bee: *A. andreniformis*, and eastern honey bee: *Apis cerana* are native species whereas European honey bee, *A. mellifera* is an introduced species in Thailand. Only *A. mellifera* and *A. cerana* can be kept and managed in hives for commercial beekeeping. (Ruttner, 1988; Wongsiri *et al*., 1990, 1996b)

2.1 Classification *of A. dorsata*

A. dorsata is the largest honey bee in terms of its nest and body size. Workers have an average body length of 0.65 inchs (16 mm), queen is about 0.72 inchs (20 mm) and drones are 0.6 inchs (15 mm). The head, thorax and abdomen are generally black and yellow or orange. The thorax and the first abdominal segment are covered with brownish setae. The wings are dusky brown and the legs are black (Fig.2.1A.) (Seeley, 1985; Ruttner, 1988).

Taxonomic hierachey of *A. dorsata* is following.

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Family Apidae

Genus *Apis*

Species *Apis dorsata*

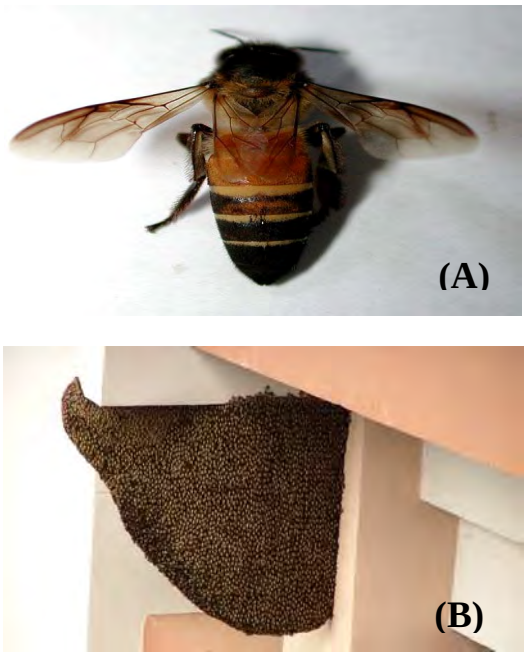


Figure 2.1 Giant honey bee, *A. dorsata*. (Photoed by Sittipong Wongvilas, 2009)

A). Worker of *A. dorsata*.

B). Single colony of *A. dorsata* on the building.

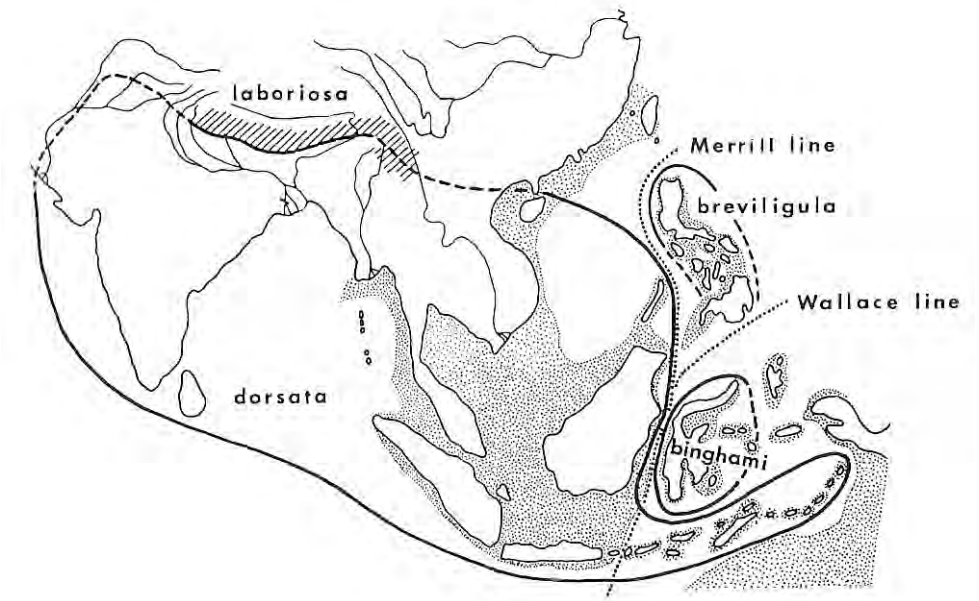


Figure 2.2 Distribution area of *A. dorsata*. (Adapted from Ruttner, 1988)

Figure 2.3 Aggreegated colonies of *A. dorsata*

(A) Colonies of *A. dorsata* on the temple. (Photoed by Sittipong Wongvilas, 2009)

(B) Aggregated colonies of *A. dorsata* on the *Koompassia exelsa* Taub. (Photoed by Siriwat Wongsiri, 2003)

2.2 Distribution and habitat of giant honey bees: *A. dorsata*

A. dorsata, giant honey bee is a native honey bee of southern and southeastern Asia. The distribution area of this bee covers from India, the Malay Peninsula, Brunei, Sumatra, the islands of Indonesia to the Philippines (Fig.2.2.) (Smith, 1991 and Wongsiri *et al.*,1996a). The giant honey bee is reported to observe in altitudes up to 1,000 m, to 1,500-1,700 m on during migration, even up to 2,000 m in different regions (Husain, 1938; Muttoo, 1956; Reddy, 1980a, 1980b; Gautam, 1984).

A. dorsata colony normally builds only a single comb on the branch of a tree, an overhang on a tall building (Fig.2.1B). The number of worker bees in a colony ranges from 5,000 to 7,000 individuals (Wongsiri *et al.*, 1996b).

Aggregated colonies (Fig 2.3) of *A. dorsata* are commonly found (Seeley, 1985; Seeley and Akratanakul, 1982; Dyer and Seeley, 1994). Colony tends to aggregate as groups and nest on a single tree (Koeniger and Koeniger, 1980). Oldroyd, Osborne and Mardan (2000) observed 120 *A. dorsata* colonies aggregated on a single tree in Alor Setar, north of peninsular Malasia. Wongsiri *et al*. (1996b) also found 69 colonies nested on a bee tree *Koompassia exelsa* Taub in Mae Hong Son province, north of Thailand. Wattanachaiyingcharoen (2001) reported that there were 14 colonies on a single tree in Nan province, north of Thailand.

2.3 Bee venom

Bee venom (BV) is synthesized by venom gland and stored in venom reservoir. Venom gland in the worker bee becomes active just after adult emergence and maximal production is achieved within two or three weeks after the emergence. Composition of worker venom changes during lifetime of bee, and these changes are believed to occur mainly due to a changing behavior from the house bee activity to field bee activity. Venom production is also higher during summer months, in which there is a peak of activity in the colony, and when the relatively young individuals are beginning their defense behavior. A different situation exists with the queen as its venom production is at maximum rate at the time of emergence (Hider, 1988)

BV unlikes many other insect venom, or chemical defenses. It is water soluble and must be injected or applied to active. This water solubility is an advantage as it allows a whole new suite of highly active defensive compounds to be used. The whole BV is composed of many chemical agents: polypeptides (melittin, apamine, mast cell degranulating peptide (MCDP); enzymes (hyaluronidase, phospholipase A_2 (PLA_2); amines (histamine, dopamine) and others (Tu, 1977). Melittin is the most characterized peptide and present to 40-50% of venom dry weight and seems to be the major responsible for intense local pain. (Edstrom, 1992; Habermann, 1972; Owen and Pfaff, 1995), The MCD peptide (mastocyte degranulating peptide), chemically similar to apamine, is probably the main venom factor responsible for a massive release of histamine (Edstrom, 1992). It represents about 2% of the venom dry weight (Gmachl and Krel, 1995). Another important peptide in BV is apamine, a

small peptide that corresponds to less than 2% of venom dry weight. It has about 2.0 kDa, only 18 amino acid residues (Bank and Shipolini, 1986)

After stinging, the sting detaches from the body of a honey bee (Fig.2.4), which contrast to other stinging insects (Mulfinger *et al*., 1992). It takes with the entire distal segment of the bee's abdomen, along with a nerve ganglion, various muscles, a venom sac, and the end of the bees digestive tract (Snodgrass, 1956). The sting itself (Fig.2.5) consists of two lancets with recurved barbs on the outer aspect of their distal end, held in grooves on a stylet. Muscular movements of the detached sting, coordinated by the attached nerve ganglion, move the stylet alternately. The barbs provide one way traction, so that the sting continues to work itself deeper into the skin. A valve and piston on the proximal ends of the moving lancets pumps venom from the venom sac between stylet and lancets, and through an opening near the tip into the wound (Visscher and Camazine, 1996).

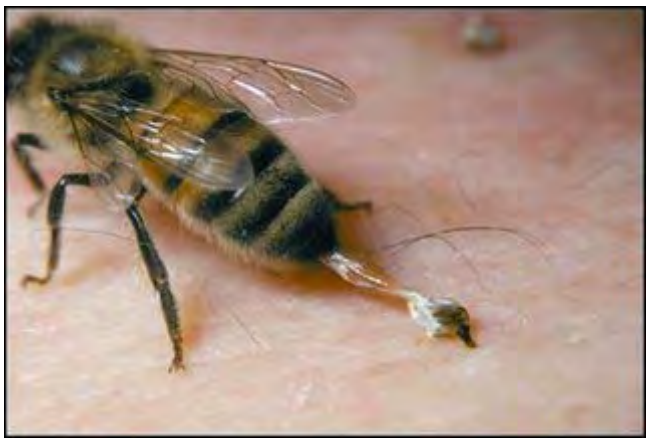


Figure 2.4 Sting of a honey bee embedded in skin. (Adapted from Auerbach, 2008)

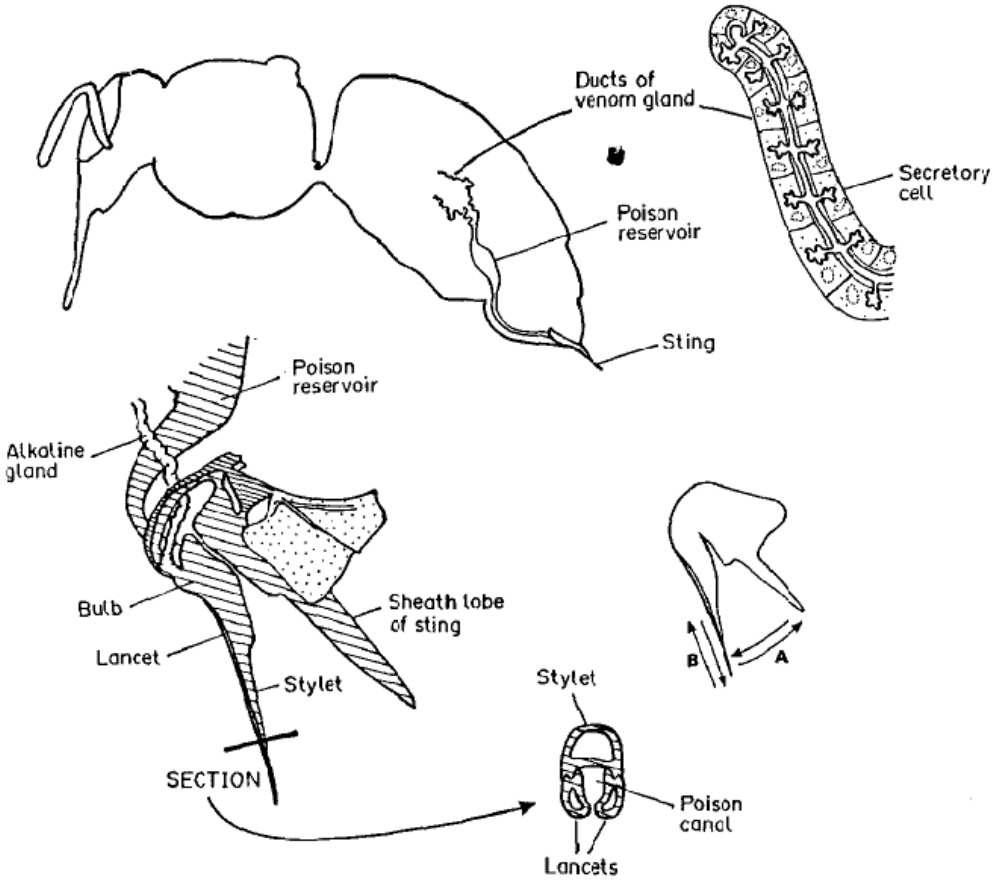


Figure 2.5 Structure of the sting of *A. mellifera* worker. (Adapted from Hider, 1988)

Stinging behavior of honey bees usually displayed to defense of the colony. Generally, they do not cause lethal effect but cause mainly local inflammatory, pain, swelling, redness, warmth, anaphylaxis or immunological reactions in the victims of their stings. Sometimes it is possible to observe the occurrence of systemic effects like liver and kidney failure (Palma, 2006). BV induced acute renal failure reported in Europe, Africa and Asia. Previous studies had been reported that massive inoculation of *A. mellifera* venom may induce acute renal failure, liver injury, hemolysis and rhabdomyolysis. The pharmacokinetics levels were decreased. (Bresolin *et al*., 2002; Daher *et al*., 2003; Gabriel *et al*., 2004;

Grisotto *et al*., 2006; Munoz-Arizpe *et al*., Daisley, 1998. and Lipps, 2002) Uapatanapanich (2005) compared the effect of venom from *A. mellifera* and *A. cerana* on liver and blood tissues of *Rattus norvegicus*. The results suggested that BV was toxic to these tissues of rat. Chen *et al*. (2006) researched on pharmacological properties of apamine, melittin, MCDP, PLA_2-related peptide. These were potent to produce thermal and mechanical hypersensitivity, local inflammation and ongoing pain-related.

2.4 Separation techniques

2.4.1 Chromatography

Chromatography is used to separate molecules on the basis of a chemical properties such as molecular mass, charge, size, binding affinity or solubility. The process divides into stationary phase and mobile phase. The stationary phase is a porous solid material with appropriate chemical properties. The mobile phase can be either liquid or gas. This phase percolates through column. The complex mixture of molecule or sample is passed over the stationary phase. Then, the mobile phase is percolated. Molecules with different physical properties are partition differently between the stationary and mobile phases, resulting in a separation. Molecules that are strongly attracted to the stationary phase will be retarded or retained in the column whereas the molecules that are not attracted to the stationary phase will be eluting out. Then, choosing the appropriate stationary and mobile phases, it is possible to obtain effective separation method of the molecules that have a slightly different properties. (Robyt and White, 1987)

2.4.2 Reversed phase high performance liquid chromatography

Reversed phase high performance liquid chromatography (RP-HPLC) is the method that used to separate molecules on the basis of hydrophobicity. The method depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase i.e. sorbent. Figure 2.6 is a schematic diagram showing the binding of a peptide or a protein to a reversed phase surface (Aguilar, 2004).

The separation method is divided in to two steps. First, the solute mixture is initially applied to sorbent in the presence of aqueous buffer. Second, solutes are eluted by the addition of organic solvent to the mobile phase. The second step is called eluted step. This step can be proceeded either by isocratic conditions where the concentration of organic solvent is constant, or by gradient elution whereby the amount of organic solvent increased over a period of time. The solutes are eluted in the order of increasing molecular hydrophobicity (Aguilar, 2004).

RP-HPLC is a very powerful technique for the analysis of peptides and proteins because of the following factors:

(1) the excellent resolution that can be achieved under a wide range of chromatography conditions for very closely related molecules as well as structurally quite distinct molecules

(2) the experimental ease with which chromatographic selectivity can be manipulated through changes in mobile phase characteristics

(3) the generally high recoveries and, hence, high productivity

(4) the excellent reproducibility of repetitive separations carried out over a long period of time, which is caused partly by the stability of the sorbent materials under a wide rang of mobile phase condition. However, RP-HPLC can cause the irreversible denaturation of protein samples thereby reducing the potential recovery of the material in a biologically active form.

The RP-HPLC experimental system for the analysis of peptides and proteins usually consists of an n-alkylsilica-based sorbent from which the solutes are eluted with gradients of increasing concentrations of organic solvent such as acetonitrile containing an ionic modifier such as trifluoroacetic acid (TFA). Complex mixtures of peptides and proteins can be routinely separated and low picomolar-femtomolar amounts of material can be collected for further characterization. Separations can be easily manipulated by changing the gradient slope, the operating temperature, the ionic modifier, or the organic solvent composition (Aguilar, 2004).

The extensive use of RP-HPLC for the purification of peptides, small polypeptides with molecular weights up to 10,000 and related compounds of pharmaceutical interest has not been replicated to the same extent for larger polypeptides (molecular mass > 10 kDa) and globular proteins. The combination of the traditionally used acidic buffering systems and the hydrophobicity of the n-alkylsilica supports which can result in low mass yields or the loss of biological activity of larger polypeptides and proteins have often discouraged practitioner from using RP-HPLC methods for large-scale protein separations. The loss of enzymatic activity, the formation of multiple peaks for compositionally pure samples, and poor yields of protein can all be attributed to the

denaturation of protein solutes during the separation process using RP-HPLC (Aguilar, 2004).

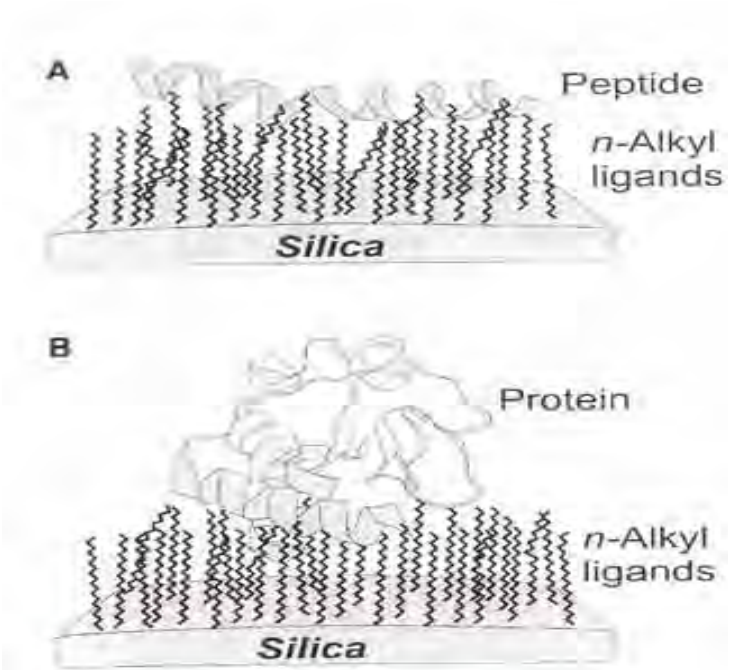


Figure 2.6 Schematic representation of the binding of (A) a peptide and (B) a protein, to an RP-HPLC silica-based sorbent. (Adapted from Aguilar, 2004)

2.5 Identification technique

2.5.1 Mass Spectrometry

Mass spectrometry is an analytic technique that measures the masses of individual molecules and atoms. The conceptual of mass spectrometry is shown in Figure 2.7. The first essential step in mass spectrometry analysis is to convert the analyzed molecules into gas-phase ionic species because the experimental can manipulate the motion of ions, and to detect them (which are not possible with neutral species). The excess energy transferred to the molecule during the ionization event

leads to fragmentation. Next, a mass analyzer separates these molecular ions and their charged fragments according to their m/z (mass/charge) ratio. The ions current due to these mass separated ions is detected by a suitable detector and displayed in the form of a mass spectrum. In order to enable the ions move freely in space without colliding or interacting with other species, each of these steps is carried out under high vacuum (10-4 – 10-8 torr)

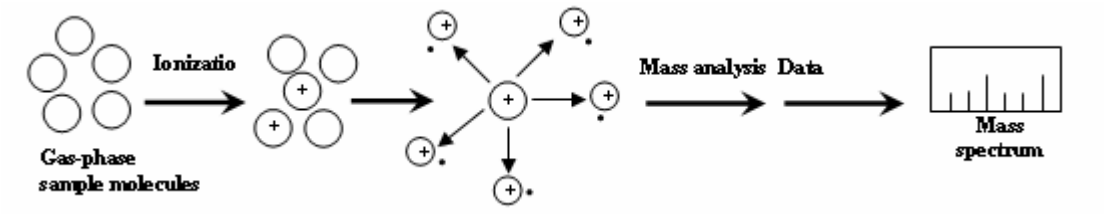


Figure 2.7 Basic concept of mass spectrometry analysis. (Adapted from Dass, 2001)

A mass spectrometry consists of several essential functional units which show in Figure 2.8 (Dass, 2001). These units composed of:

(1). An inlet system is used to transfer a sample to the ion source.

(2). A vacuum system is used to maintain a very low pressure in the mass spectrometer.

(3). An ion source is used to convert the neutral sample molecule into gas-phase ions.

(4) A mass analyzer is used to separate and mass-analyze ionic species.

(5) A detector is used to measure the relative abundance of the mass-resolved ions.

(6) Electronics is used to control the operation of various units

(7). A data system is used to record, process, store, and display the data.

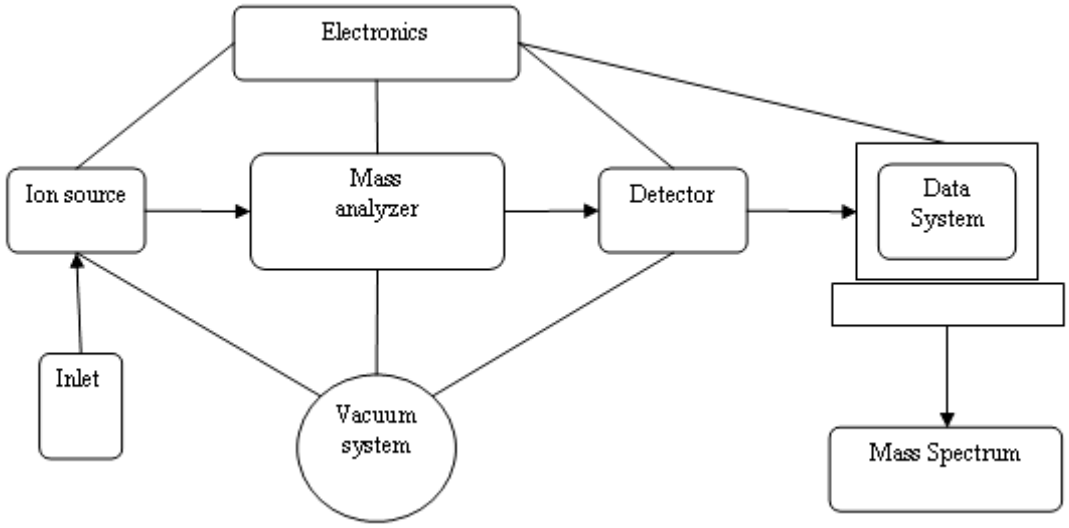


Figure 2.8 Basic components of a mass spectrometer. (Adapted from Dass, 2001)

2.6 Protein identification using data from mass spectrometry

Peptide mass fingerprinting (PMF) refers to the identification of proteins using data from intact peptide masses. The principle of the technique is that each protein can be uniquely identified by the masses of its constituent peptides, this unique signature being known at the peptide mass fingerprint. Algorithms allowing database searching on the basis of peptide mass data were developed simultaneously by several groups in the early 1990s and have been implemented in a number of software packages, many of which are available over the internet. Diagram in Figure 2.9. show a overall strategy for protein determination by mass

finger print. These method composed of six steps as described below (Twyman, 2004)

(1) The sample of interest should comprise a single protein or a simple mixture, e.g. an individual spot from a 2D-gel or a single LC fraction. The sample is digested with a specific cleavage reagent, usually trypsin.

(2) The masses of the peptides are determined, e.g. by MALDI-TOF-MS

(3) The experimenter chooses one or more protein sequence databases to be used for correlative searching. Examples include the SWISS-PROT and TrEMBL protein databases

(4) The algorithm carries out a virtual digest of each protein in the sequence database using the same cleavage specificity as trypsin (or whichever other reagent has been used experimentally) and then calculates theoretical peptide masses for each protein.

(5) The algorithm attempts to correlate the theoretical peptide masses with the experimentally determined ones.

(6) Proteins in the database are ranked in order of best correlation, usually with a significance threshold based on a minimum number of peptides matched.

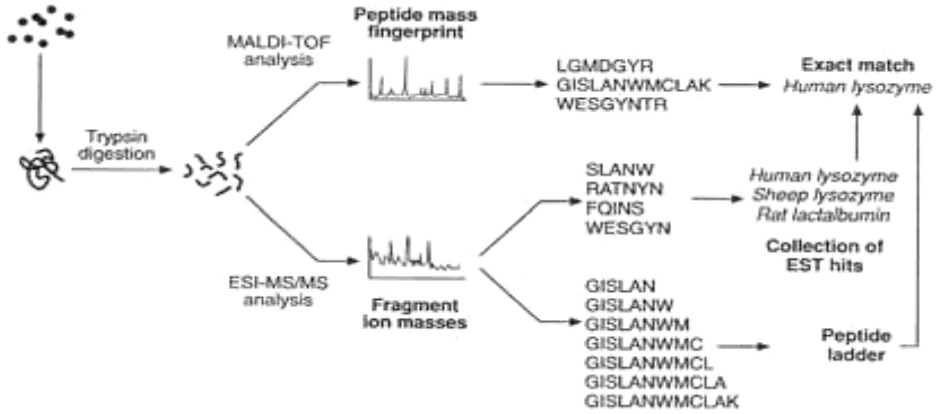


Figure 2.9 Protein identification by mass spectrometry. In a typical strategy, digested peptides are analyzed by MALDI-TOF MS in order to determine the masses of intact peptides. (Adapted from Twyman, 2004)

CHAPTER III
MATERIALS AND METHODS

3.1 Preparation of *A. dorsata* venom

3.1.1 Venom quantity in venom sac (mg)

BV was collected according to method of Funari *et al*., 2001. One hundred workers of *A. dorsata* were collected from nest and anesthetized by freezing. Then, the sting was detached from the body of a honey bee. Venom sac (reservoir) from 10 workers of *A. dorsata* were opened onto glass slide under stereo microscope and cool light. After 48 hours, the dried bee venom on the glass was scratched and weighted in the dark area. The mean amount of venom per bee was calculated. The collected crude BV was kept in the dark at – 4°C until used.

3.2 Separation and purification of BV components

3.2.1 Separation and purification

Standard solutions were prepared by dilution of 5 mg freeze-dried venom, 4.00 mg of melittin, 0.25 mg apamine, 1.00 mg phospholipase A_2. For sample, 10.00 mg of *A. dorsata* venom was dissolved in 1 ml of distilled water and then was filtered through 0.45 μm Millipore filter paper (Vertical Chromatography Co. Ltd).

Analysis of venom was performed by HPLC. The system was equipped with auto sampler (model 410). Detector was carried out with UV-VIS detector (model 335). C18 reverse phase column (Apollo, 4.5 x 250 mm, 5 μM) was used for this analysis. The separation was done by

the gradient elution of two solvent systems. The two solvents used were HPLC-grade 0.1% ACN in H_20 (solution A) and 0.05% TFA in ACN (solution B). Both solvent A and B were HPLC-grade and filtered through 0.45 μm pore size filter paper (SUN SRI). Before sample injection, the column was equilibrated with solvent B by flow the solution through the column at flow rate of 1 ml/min for at least 30 min. Then, the standard solution was injected. Next step, *A.dorsata* venom was injected into column. The experiment was done in room temperature. A linear gradient of 0% ACN to 100% ACN (0-70% mobile phase B) in 52 min running times at a flow rate of 1.2 ml/min was used to elute the sample. Chromatogram at 220 nm was monitored. The eluant was fractionated. Then collected fraction were kept at 4 °C until use.

3.2.2. Identification of BV components

Peptide mass mapping obtained from each digested protein was searched against protein database via the MASCOT program (www.matrixscience.com). Search parameters allowed for carbamidomethylation of cysteine, one missed trypsin cleavages, and 100 ppm mass accuracy. Peptides in the mass range of 800-3,500 Da were selected for database search.

3.3 Effects of *A. dorsata* venom on liver, kidney and blood tissues of rat, *Rattus norvegicus*

3.3.1 Animals

The experiments was performed on male Sprague-Dawley albino wistar rat, *Rattus norvegicus* weighing 200-250 g. The rats were provided

by the National Laboratory Animal Centre, Mahidol University. The rats were acclimatized in laboratory condition for 5 days. They were taken care in Laboratory Animal's House, Faculty of Science, Department of Biology, Chulalongkorn University. The number of animals used and their suffering have been greatly minimized. The rats were housed in stainless steel cage with solid bottom and open top under strictly hygienic conventional condition, in temperature ranging from 23-26^{O}C, and relative humidity at 50 $\pm$ 10 % and standard fluorescent at 12:12 hrs light-dark cycle. Cage size was 24x45x14 cm. (W x L x H). Sawdust bedding were changed every 3 days. Drinking fluid was tap water. Type of food was standard diet. Drinking fluid and food were available ad libitum and changed every day.

3.3.2 Toxicity test

Forty five rats were used in the experiment. The rats were randomly divided into three groups. Each group composed of 15 rats. Control group was injected with 0.1 ml PBS into subcutaneous tissue. Two treatment groups were injected with 0.25 mg and 0.50 mg of *A. dorsata* venom per kg in 0.1 ml PBS respectively. After 2, 8 and 24 hours the rats were euthanized by 99.7 % diethyl ether. Blood was collected from cardiac puncture. The liver and kidney tissues of each groups were collected at 2 (n=5) ,8 (n=5) and 24 (n=5) hours after treatment.

3.3.3 Blood collecting

Before collecting the blood, animals were grown without any food in 8 hours. When the rat was anesthesia with diethyl ether, syringe size 3 ml and Needle 21 1/2 G were used for collecting the blood from cardiac

puncture amount of 2 ml into the tube with heparin for analysis of blood chemistry. Blood was also smeared for analysis of blood tissue.

3.3.4 Histological analysis

The left lateral lobe of liver and left kidney from five rats of each group were fixed in 10% buffered formalin for 24 hours, and preserved in 70% ethanol. The tissues were processed using standard paraffin method. All paraffin blocks were sectioned at 6 μm and stained with haematoxylin and eosin (H&E). Each major histopathological alterations found in the rat livers and kidneys were scored using this following criteria:

Severe lesion = +++, 3 (the lesion found more than 70% of all area observed)

Moderate lesion = ++, 2 (the lesion found between 50-70% of all area observed)

Mild lesion = +, 1 (the lesion found between 1-50% of all area observed)

No observable lesion = -, 0 (there is no lesion found of all area observed)

3.4 Statistical analysis

The amount of venom quantity in venom sac were shown as mean ±S.D. Toxicity test of three groups (control, two treatment groups) on liver and kidney were compared by using ANOVA on Rank (Kruskal-Wallis). Complete blood count was compared by using One way ANOVA (LSD). P-value at 0.05 was considered to be statistically significant.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Venom quantity in venom sac (mg)

Funari *et al*. (2001). suggested the dried weight venom of three types of *A. mellifera* as Africanized, Italian x Africanized and Carniolan x Africanized were 0.117±0.015, 0.139±0.020, and 0.147±0.024 (mg), respectively. In this study, the amount of dried weight venom of *A. dorsata* is about 0.154 - 0.193 mg per bee with an average of 0.170 ± 0.016 mg per bee (Table 4.1). This amount of venom weight is higher than those Africanized, Italian x Africanized and Carniolan Africanized about 5.3%, 3.1%, 2.3%, respectively.

In each major peak of protein could calculate area as 6.735 % for apamine, 10.540 % for phospholipase A_2 and 20.416 % for melittin. *A. dorsata* venom showed that melittin is the highest and then are phospholipase A_2 and apamine, respectively. The quantity of each protein component in *A. mellifera* were found 3% for apamine, 10-12% for phospholipase A_2 and 40-50% for melittin (Schmidt, 1982 and Shipolini, 1985). This suggested that melittin is the main component in *A. dorsata* venom as in *A. mellifera* which Schmidt (1995) had been described that it was the main component in honeybees.

When compared to other honey bees *A. dorsata* is the biggest one (Ruttner, 1988). The high amount of *A. dorsata* venom should be caused by body size of bee which bigger than *A. mellifera* and others.

Table 4.1 Amount of dried weight venom of *A. dorsata*

No	Dried weight of *A. dorsata* venom (mg)	
	100 individuals	per individual
1	15.400	0.154
2	17.800	0.178
3	15.700	0.157
4	19.300	0.193
5	17.000	0.170
Mean	17.040	0.170
S.D	±1.590	±0.016

4.2 Analysis of venom using RP-HPLC

The crude bee venom was analyzed by RP-HPLC. Twenty microliters of crude were injected into column. The sample was eluted with di-solvent system. The chromatographic profile shows in the 30 dominate peaks Fig. 4.2. The peaks at retention time 21.807, 23.751, 30.318, 31.342, 34.511, 35.621, 40.614 were fractionated and subjected to further analysis by MALDI-TOF. The figure 4.1 shows a chromatographic profile of standard sample (apamine, phospholipase A_2 and melittin). This result suggested that apamine, Phospholipase A_2 and melittin were eluted at retention time 17.062, 29.503 and 37.739, respectively. Therefore, the peak at retention time 18.016. 31.042 and 39.392 in the venom sample solution was characterized as apamine, phospholipase A_2 and melittin, respectively. The slight difference between retention time of standard and venom sample may be caused by the different origin of samples and the use of different sampling methods.

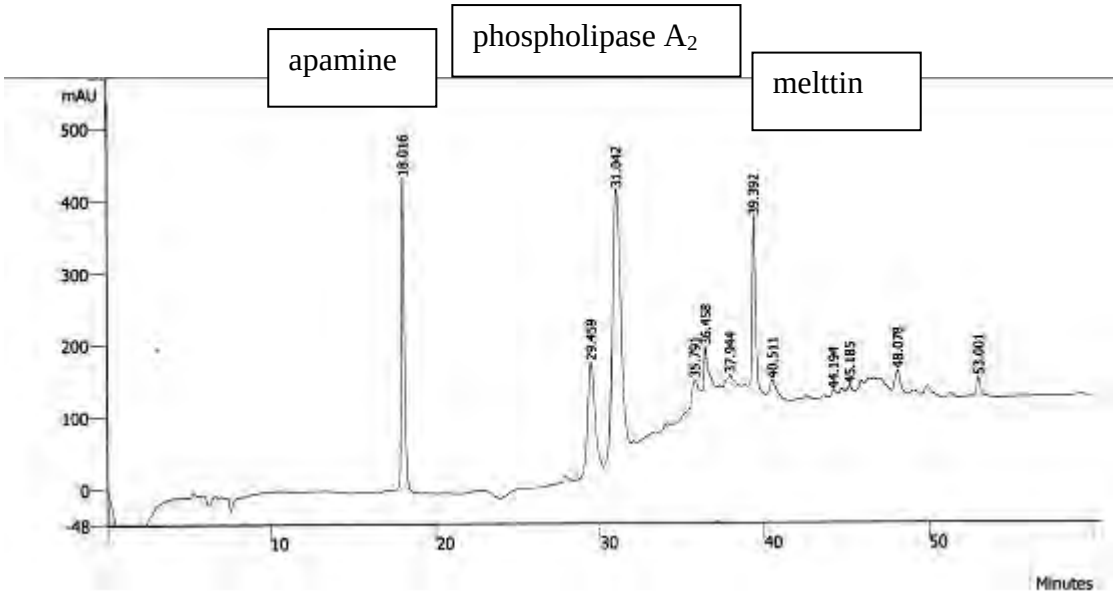


Figure 4.1 RP-HPLC fractions of standard samples: apamine, phospholipase A_2 and melittin respectively.

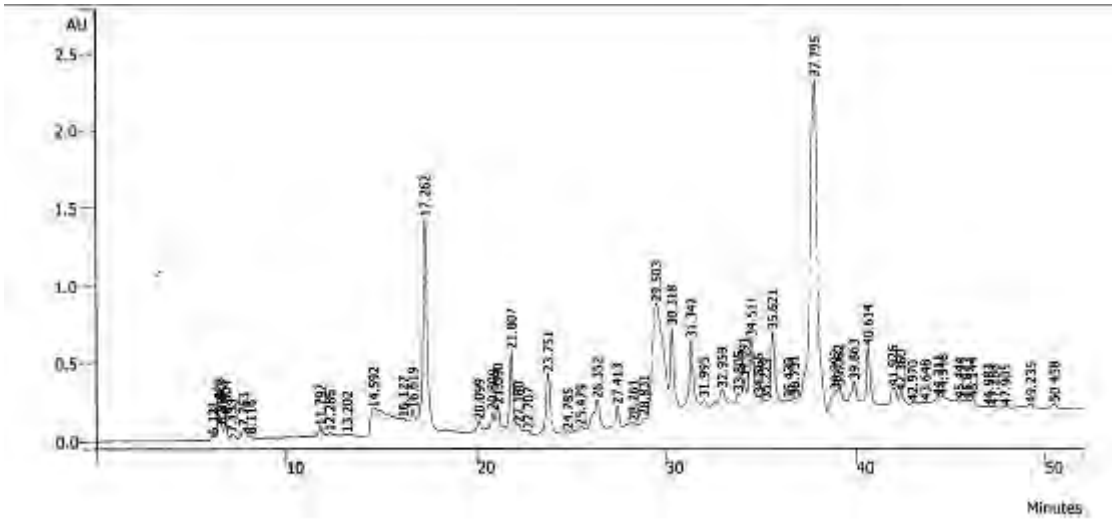


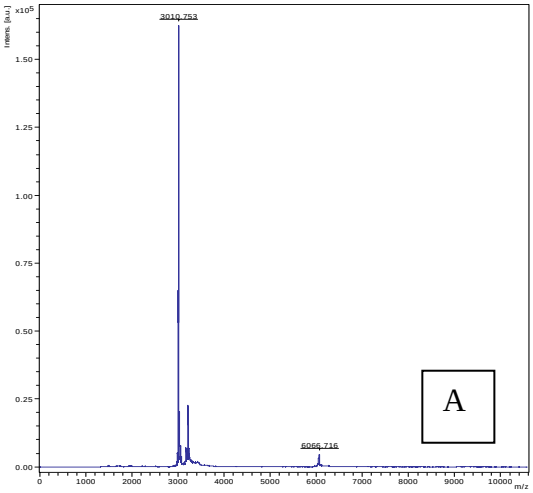
Figure 4.2 RP-HPLC fractions of crude *A. dorsata* venom.

4.3 Identification of bee venom

In order to determine the component of bee venom, seven selected peaks were subjected to analysis by peptide finger print. The result of peptide mass finger print was showed in Fig 4.3. The pattern of peptide mass finger print was searched by Mascort Searching Program based on NCBI 20090123 data based (at http://www.matrixscience.com). The result showed that peak at retention 21.807 was matched to glycosyltransferase from *Lycodes terraenovea* with 79% matching (Fig. 4.5A). The other peaks do not match to protein in searching data base (Figure 4.5B-G). Figure 4.4 showed the matching sequences of peptide finger print with glycosyltransferase.

Glycosyltransferases are the enzymes, act as a catalyst for the transfer of a monosaccharide unit from an activated sugar phosphate (known as the "glycosyl donor") to an acceptor molecule, usually an alcohol. The result of glycosyl transfer can be a monosaccharide glycoside, an oligosaccharide, or a polysaccharide, although some glycosyltransferases catalyse transfer to inorganic phosphate or water.
Glycosyl transfer can also occur to protein residues, usually to tyrosine, serine or threonine to give O-linked glycoproteins, or to asparagine to give N-linked glycoproteins. Mannosyl groups may be transferred to tryptophan to generate C-mannosyl tryptophan, which is relatively abundant in eukaryotes

The functions of glycosyltransferase in honey bees, *Apis* spp. are not well understand. However, it has been shown that phospholipase A_2 contains N-linked oligosaccharide. Therefore, the presence of glycosyltransferase may involve in biosynthesis of phospholipase A_2.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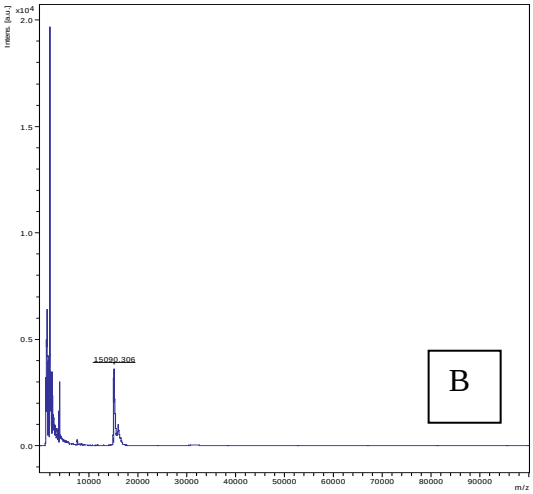
6066.716
A


15090.306
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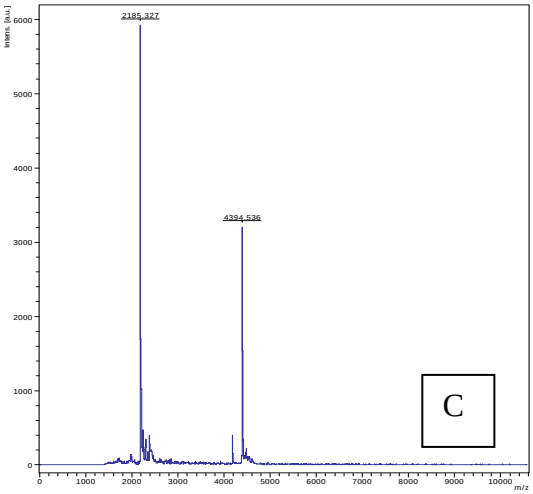
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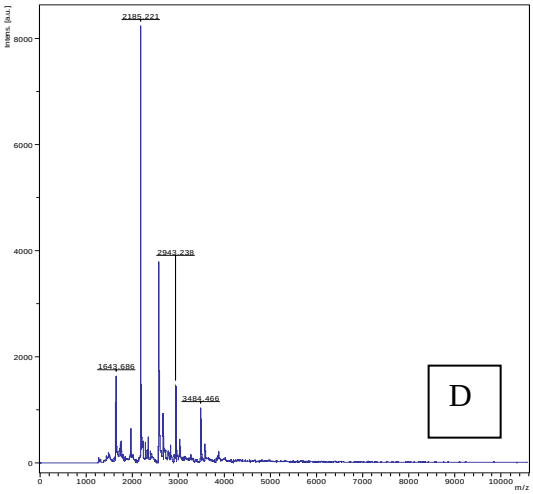
4394.536
C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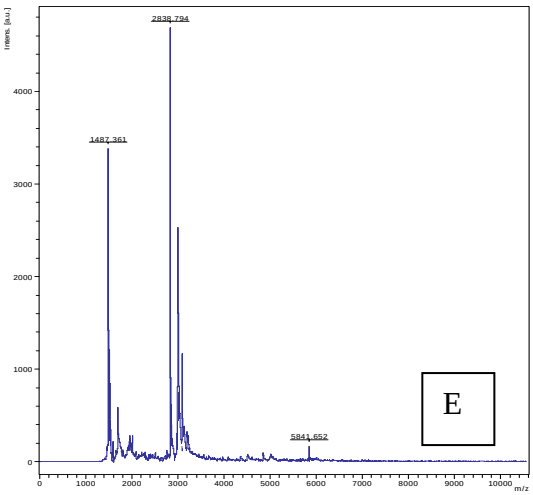
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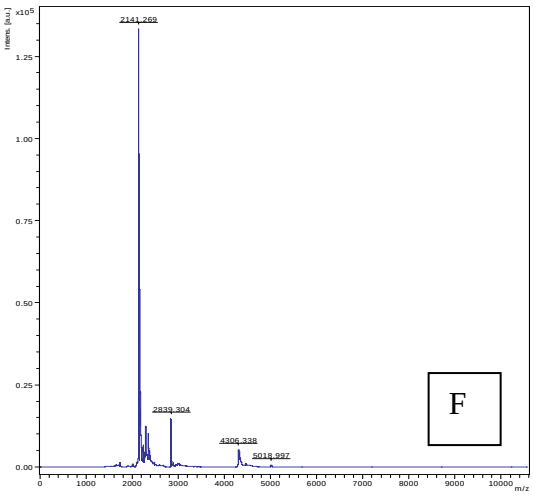
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5018.997
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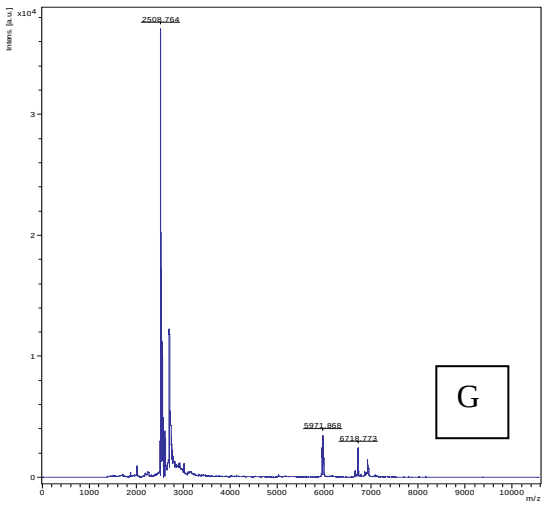

Figure 4.3 MALDI-TOF MS spectrum of seven selected peaks. A-G means to fraction from retention time at 21.807, 23.751, 30.318, 31.342, 34.511, 35.621 and 40.614 respectively.

```
  1 VPKANMLVSG NEIRQFARAL MEKMNITTNV EKEKDGGSAE DEKERKDDYV
 51 VVFSRSTTRL ILNEAELIMA LAQEFQMRVV TVSLEEQPFP SIVQVISGAS
101 VLVSMHGAQL ITSLFLPRGA AVVELFPFAV NPEQYTPYKT LATLPGMDLH
151 YISWRNTKEE NTITHPDRPW EQGGIAHLEK EEQERILESR DVPRHLCCRN
201 PEWLFRIYQD TLVDIPSFLE ALKEGMKTKP SVRKSKPAST VHPGRVRQPQ
251 CQTSVQTTNE AKLTVSWQIP WNLKYLKVRE VKYEVWIQ
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Figure 4.4 Amino acid sequence of glycosyltransferase from *Lycodes terraenovea*. Red color indicates the matched peptides.

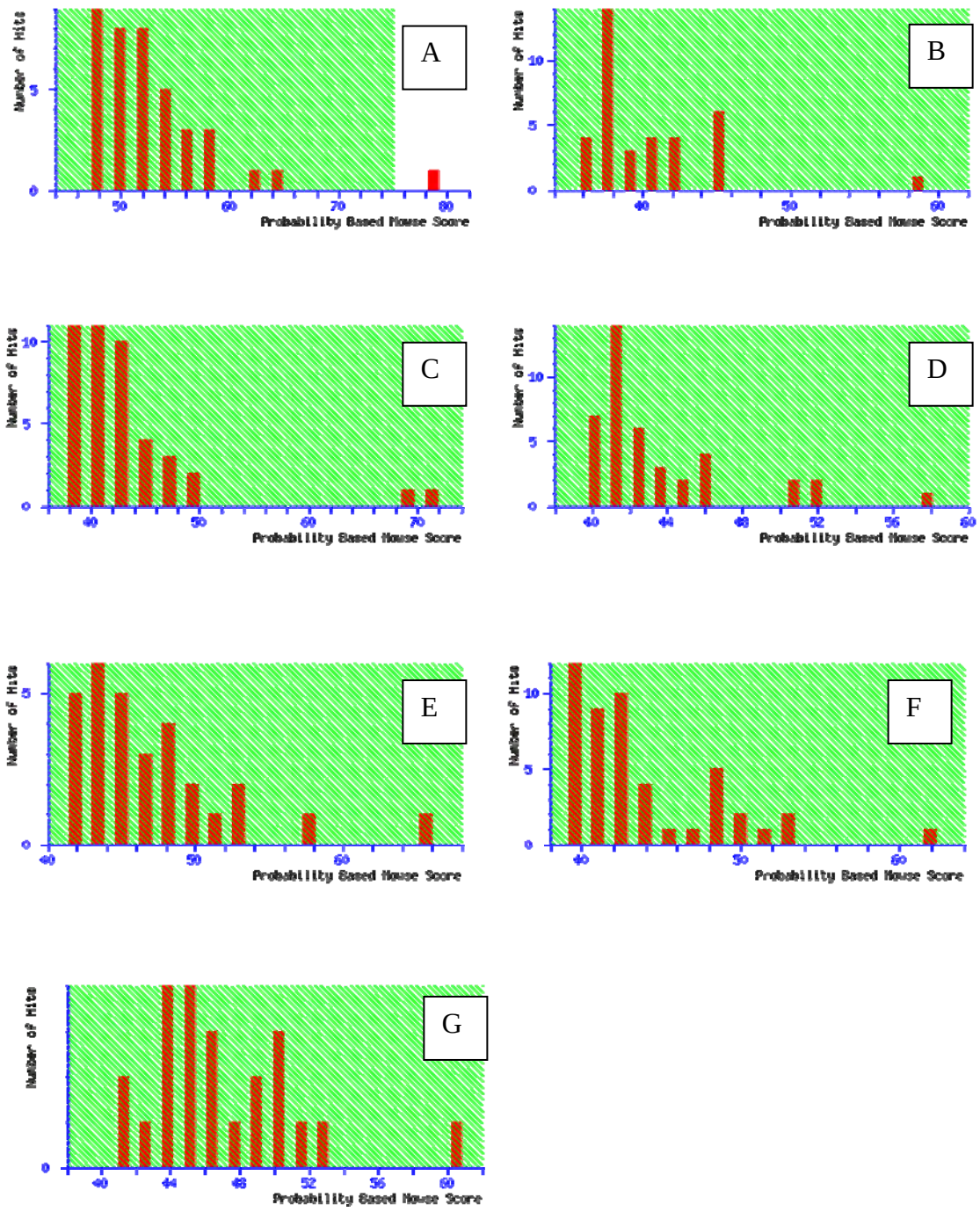


Figure 4.5 Probability Based Mowse Score of proteins. Protein score is –10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 75 are significant ($p<0.05$).

4.4 Effects of *A. dorsata* venom on liver of rat

Three major histopathological effects of bee venom on rat liver were found. The first is blood congestion in sinusoidal space. The second is sinusoidal space dilation, and the third is lipid accumulation in hepatocytes.

In the experiment, samples were divided into three groups; control (treated with 0.1 ml PBS), treatment one which treated with 0.25 mg BV in 0.1 ml PBS and treatment two which treated with 0.50 mg BV in 0.1 ml PBS. The liver samples were taken from each group in difference time interval (2, 8 and 24 hrs). The blood congestion, sinusoidal space dilation and lipid accumulation in hepatocytes were observed and scored. There are four levels of score assigned by number 0, 1, 2, and 3. Level 0 means no observable lesion (Fig 4.6). Level 1, 2, and 3 mean mild lesion, moderate lesion and severe lesion (Fig 4.7-4.9).

Blood congestion in sinusoidal space

In control group after 2, 8, and 24 hours, blood congestion was normal (no observable lesion) and found significant difference.

Treatment one which treated with 0.25 mg BV in 0.1 ml PBS was found significant difference. At 2 hours blood congestion were found mild lesion level but at 8 hours and 24 hours moderate lesion level were found.

Finally, treatment which treated with 0.50 mg bee venom in 0.1 ml PBS at time intervals was found significant difference. Blood congestion after 2 and 8 hours were found moderate lesion level but 24 hours found sever lesion level.

Table 4.2 Comparison of the response levels of BV to blood congestion on rat liver after injected with 0.1 ml PBS, 0.25 and 0.50 mg BV in 0.1 ml PBS

Time (Hours)	Control	0.25 mg BV in 0.1 ml PBS	0.50 mg BV in 0.1 ml PBS
2	0*,#	1*,#	2*,#
8	0*,#	1*,#	2*,#
24	0*,#	2*,#	3*,#

*: Significant difference in time interval ($p<0.05$)

#: Significant difference in control and both treatments ($p<0.05$)

Lipid accumulation in hepatocytes

In control group after 2, 8, and 24 hours, lipid accumulation in hepatocytes was normal (no observable lesion) but low dose, treated 0.25 mg BV in 0.1 ml PBS and high dose, treated 0.50 mg BV in 0.1 ml PBS groups were found significant difference.

Low dose which treated 0.25 mg BV in 0.1 ml PBS at 2 and 8 hours was found mild lesion of lipid accumulation in hepatocytes but at 24 hours was found moderate lesion level.

High dose which treated 0.50 mg BV in 0.1 ml PBS was found lipid accumulation in hepatocytes mild, moderate, and server lesion on time interval 2, 8, and 24 hours respectively

Table 4.3 Comparison of the response levels of BV to lipid accumulation in hepatocytes after injected with 0.1 ml PBS, 0.25 and 0.50 mg BV in 0.1 ml PBS

Time (Hours)	Control	0.25 mg BV in 0.1 ml PBS	0.50 mg BV in 0.1 ml PBS
2	$0^{\#}$	$1^{*,\#}$	$1^{*,\#}$
8	$0^{\#}$	$1^{*,\#}$	$2^{*,\#}$
24	$0^{\#}$	$2^{*,\#}$	$3^{*,\#}$

*: Significant difference in time interval ($p<0.05$)

$^{\#}$: Significant difference in control and both treatments ($p<0.05$)

Sinusoidal space dilation

In control group of sinusoidal space dilation at time interval was normal (no observable lesion) but low dose, treated 0.25 mg BV in 0.1 ml PBS and high dose, treated 0.50 mg BV in 0.1 ml PBS groups were found significant difference.

Sinusoidal space dilation was moderate lesion level and found significant difference in a low dose group after 2, 8, and 24 hours.

Mild lesion of sinusoidal space dilation at 2 hours was found in high dose group. At 8 and 24 hours, server lesions of sinusoidal space dilation were observed.

Table 4.4 Comparison of the response levels of BV to sinusoidal space dilation on rat liver after injected with 0.1 ml PBS, 0.25 and 0.50 mg BV in 0.1 ml PBS

Time (Hours)	Control	0.25 mg BV in 0.1 ml PBS	0.50 mg BV in 0.1 ml PBS
2	$0^{\#}$	$2^{*,\#}$	$2^{*,\#}$
8	$0^{\#}$	$2^{*,\#}$	$3^{*,\#}$
24	$0^{\#}$	$2^{*,\#}$	$3^{*,\#}$

*: Significant difference in time interval ($p<0.05$)

$^{\#}$: Significant difference in control and both treatments ($p<0.05$)

This experiment found sinusoidal space dilation on rat liver. In addition, lipid accumulation, blood congestion, were found increasing significantly in the treatment groups. Sinusoidal space dilation, blood congestion caused by melittin, This polypeptide is occupying over 50% of the whole honeybee venom, plays a central role in production of local inflammation, nociception and hyperalgesia (Chen *et al*, 2006). Melittin is a protein that hydrolyzes cell membranes, alters cellular permeability. Inadditon, phospholipase A_2 is the major allergenic component of BV and act in concern with melittin to cause intravascular hemolysis (Fitzgerald and Flood, 2006). Bright-field light microscopy was used to study the effect of mellitin on plasma membranes of 3T3 mouse fibroblasts, was found increased permeability as indicated by the presence of intracellular (Lo *et al*, 1996). Lipid accumulation may be related with the role of the fat storing cell in rat liver (Kuiper *et al*., 1994).

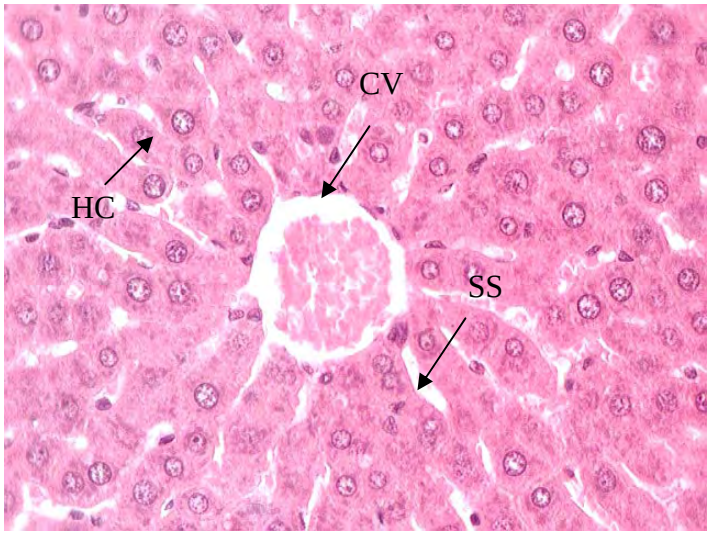
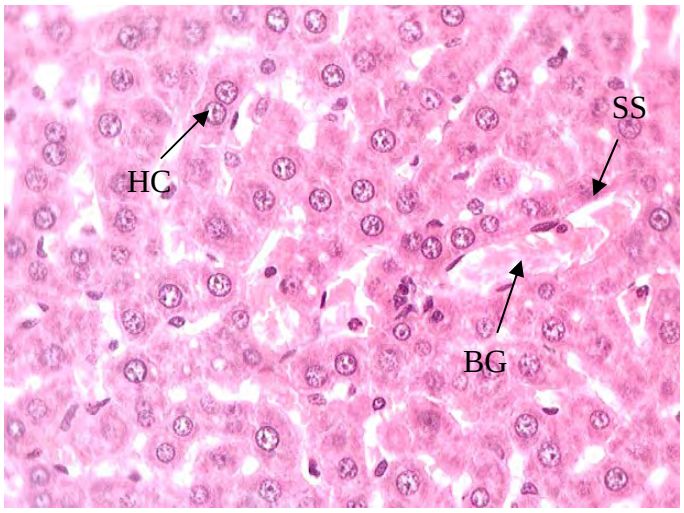


Figure 4.6. Liver tissue of a control rat shows normal histological structure of hepatic lobule. H&E, x 40. CV = central vein, HC = hepatocyte, SS = sinusoid



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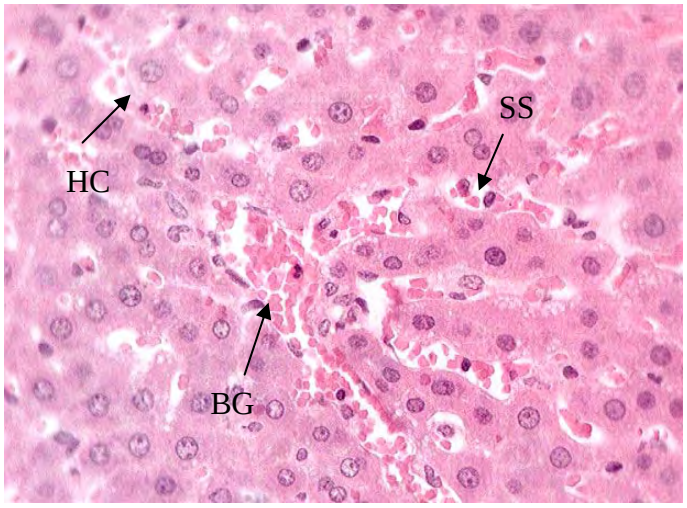

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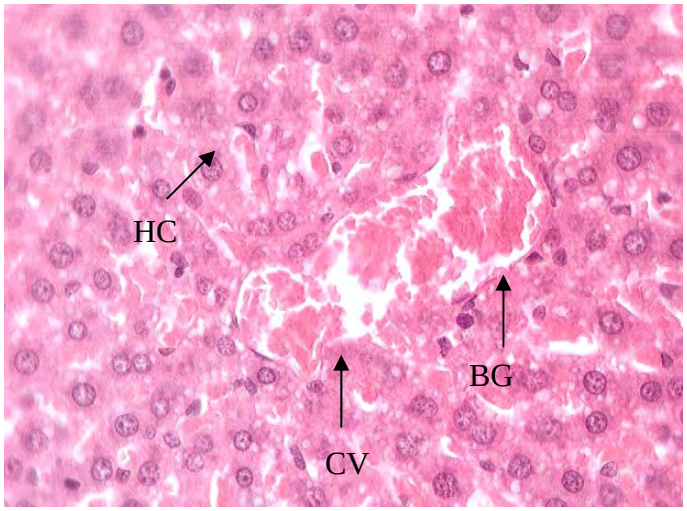
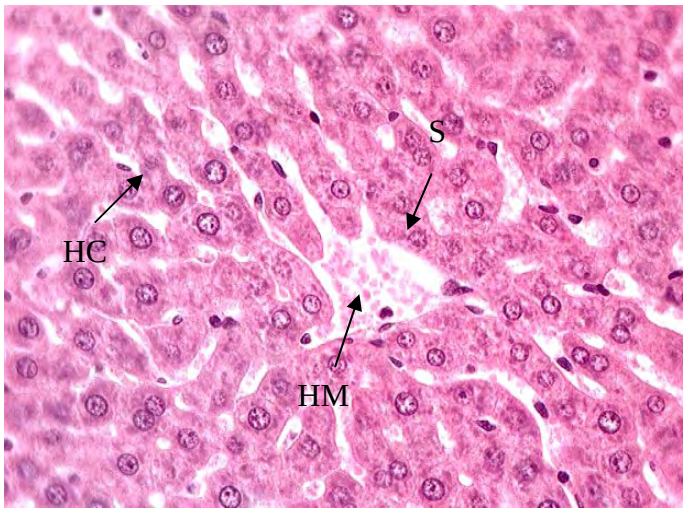


Level 3

Figure 4.7. Blood congestion occurred in the liver tissues of rat treated with BV at different levels of alteration. H&E, x 40. BG = blood congestion, CV = central vein, HC = hepatocyte, SS = sinusoid



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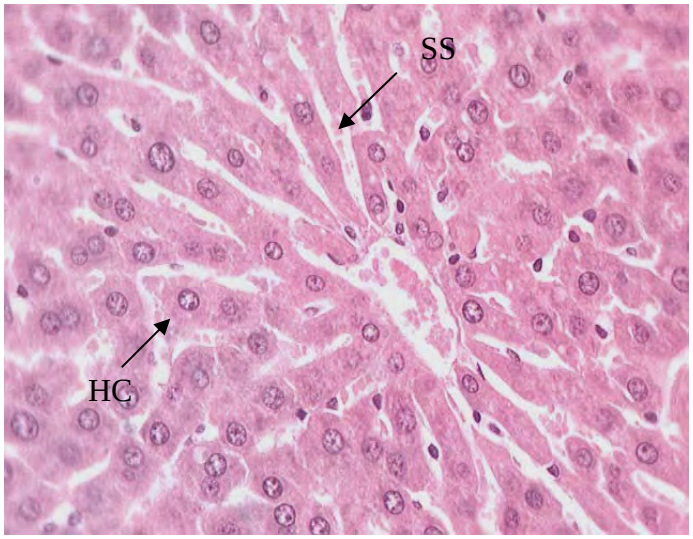

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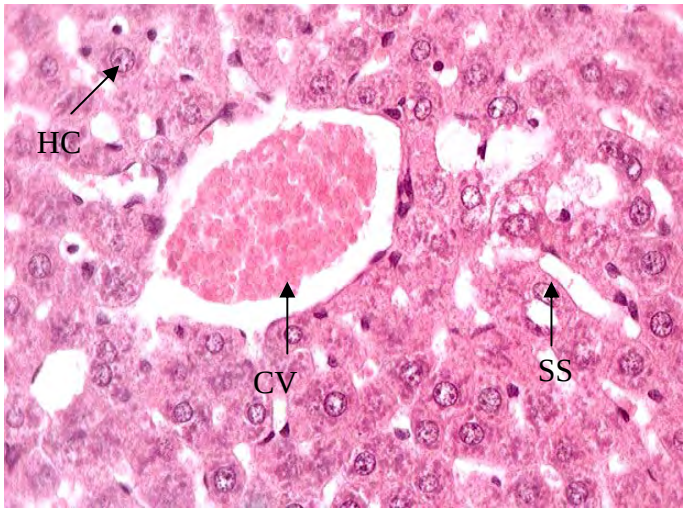


Level 3

Figure 4.8. Sinusoidal space dilations occured in the liver of rat treated with BV at different levels of alteration. H&E, x 40. CV = central vein, HC = hepatocyte, SS = sinusoid

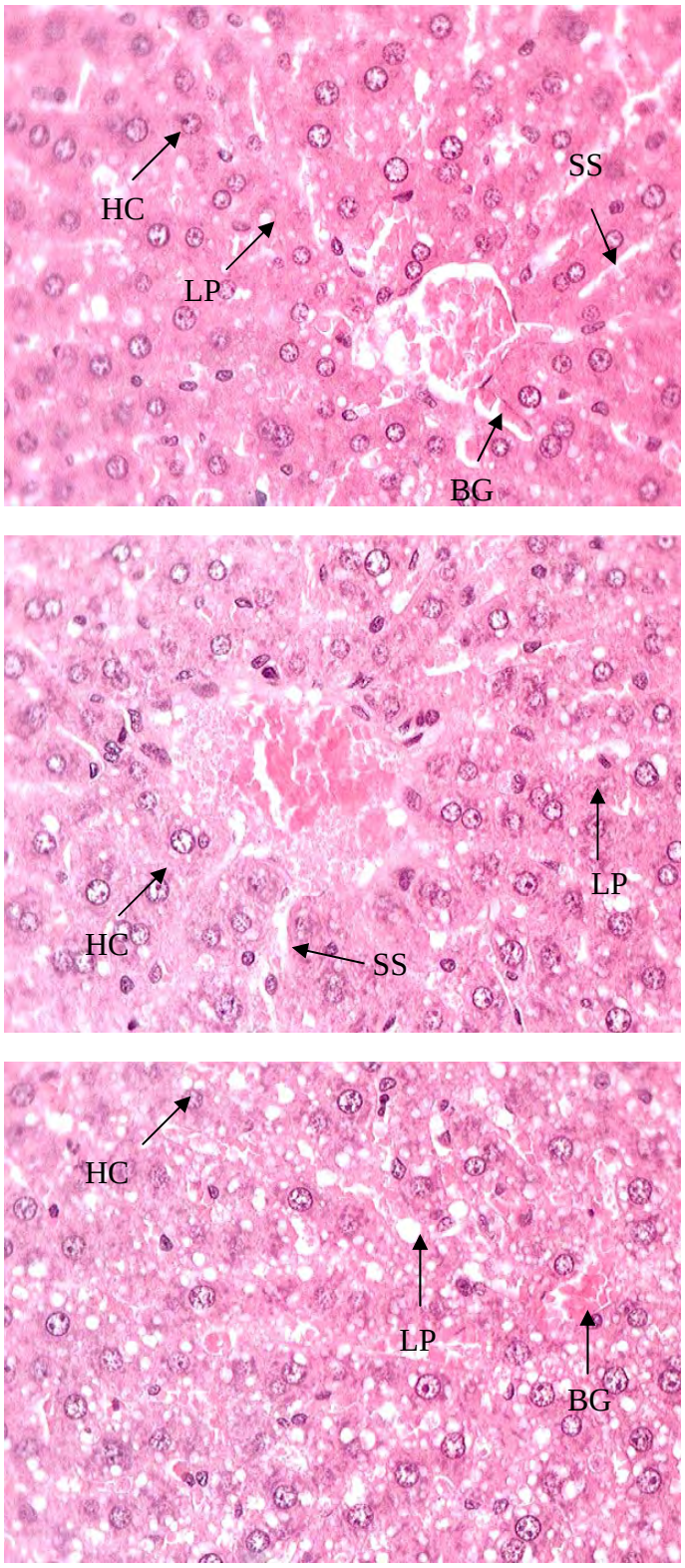


Figure 4.9. Fatty degeneration found in the liver of rat treated with BV at different levels of accumulation. H&E, x 40. CV = central vein, HC = hepatocyte, LP = lipid droplets, SS = sinusoid

4.4.1 Amount of Kupffer cell in liver

Comparison of the Kupffer cell number between control and treatment groups was done. Increased amount of Kupffer cells were found in liver tissue of both treatment groups BV 0.25 mg/kg and 0.5 mg/kg in 0.1 PBS of at 2, 8 and 24 hours respectively (Fig 4.10). These results were significantly difference between two treatment groups. Both events suggested that BV was toxic to liver tissue of rat. Increasing of Kupffer cells in both treatment groups may be due to innate (nonspecific) immunity response of the liver to local inflammation. Nonspecific phagocytosis in the liver is mediated primarily by Kupffer cells (Parker and Picut, 2005). Subcutaneous injection of BV produced local inflammation, tonic pain responses lasting from 10 min to more than 1 h (Melzack and Lariviere, 1996).

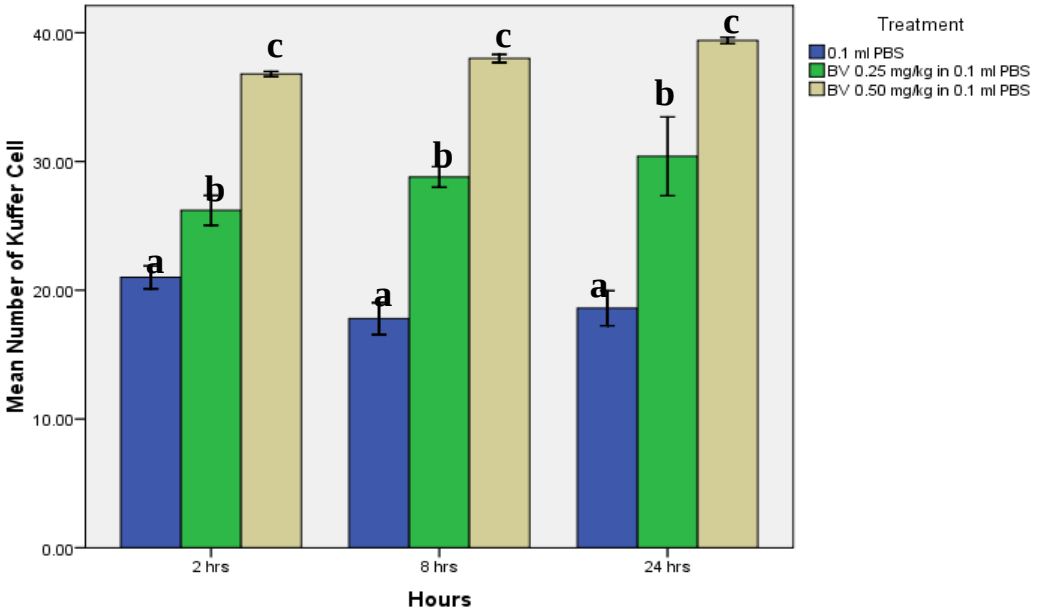


Figure 4.10. Amount of Kupffer cells in liver at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (Pink) in 0.1 ml PBS.

4.5 Effects of *A. dorsata* venom on Kidney of rat

Proximal tubular degeneration

Proximal tubular degeneration in control group for all time intervals was no observable lesion but found significant difference between 2, 8, and 24 hours after injection.

Low dose group treated with 0.25 mg BV in 0.1 ml PBS was found significant difference. Mild lesion level was found after 2 hours and moderate lesion level was found after 8 hours and 24 hours.

Moderate lesion level of proximal tubular degeneration were observed significant difference in a high dose after 2, 8, and 24 hours.

Table 4.5 Comparison of the response levels of BV to proximal tubular degeneration on rat kidney after injected with 0.1 ml PBS, 0.25 and 0.50 mg BV in 0.1 ml PBS

Time (Hours)	Control	0.25 mg BV in 0.1 ml PBS	0.50 mg BV in 0.1 ml PBS
2	$0^{*,\#}$	$1^{*,\#}$	$2^{*,\#}$
8	$0^{*,\#}$	$2^{*,\#}$	$2^{*,\#}$
24	$0^{*,\#}$	$2^{*,\#}$	$2^{*,\#}$

*: Significant difference in time interval ($p<0.05$)

$^{\#}$: Significant difference in control and both treatments ($p<0.05$)

Haemorrhage in renal cortex

In control group after 2, 8, and 24 hours, haemorrhage in renal cortex of kidney was no observable lesion. But low dose, treated 0.25 mg BV in 0.1 ml PBS and high dose, treated 0.50 mg BV in 0.1 ml PBS groups were found significant difference.

Low dose at 2 and 8 hours was found mild lesion of haemorrhage in renal cortex but 24 hours was found moderate lesion level.

High dose was found mild lesion level of haemorrhage in renal cortex and moderate lesion was found after 8 and 24 hours.

Table 4.6 Comparison of the response levels of BV to haemorrhage in renal cortex of kidney after injected with 0.1 ml PBS, 0.25 and 0.50 mg BV in 0.1 ml PBS

Time (Hours)	Control	0.25 mg BV in 0.1 ml PBS	0.50 mg BV in 0.1 ml PBS
2	$0^{\#}$	$1^{*,\#}$	$1^{*,\#}$
8	$0^{\#}$	$1^{*,\#}$	$2^{*,\#}$
24	$0^{\#}$	$2^{*,\#}$	$2^{*,\#}$

*: Significant difference in time interval ($p<0.05$)

$^{\#}$: Significant difference in control and both treatments ($p<0.05$)

Collecting duct degeneration in renal medulla

In control group after 2, 8, and 24 hours, collecting duct degeneration in renal medulla of kidney was no observable lesion. But treated with 0.25 and 0.50 mg BV in 0.1 ml PBS were found significant difference.

Treatment one which treated 0.25 mg BV in 0.1 ml PBS at 2 hours was found normal (no observable lesion) but 8 and 24 hours were found moderate lesion level of collecting duct in renal medulla.

Treatment two which treated 0.50 mg BV in 0.1 ml PBS was found mild lesion level of collecting duct in renal medulla and moderate lesion was found after 8 and 24 hours.

Table 4.7 Comparison of the response levels of BV to collecting duct degeneration in renal medulla of kidney after injected with 0.1 ml PBS, 0.25 and 0.50 mg BV in 0.1 ml PBS

Time (Hours)	Control	0.25 mg BV in 0.1 ml PBS	0.50 mg BV in 0.1 ml PBS
2	$0^{\#}$	$0^{*,\#}$	$1^{*,\#}$
8	$0^{\#}$	$2^{*,\#}$	$2^{*,\#}$
24	$0^{\#}$	$2^{*,\#}$	$2^{*,\#}$

*: Significant difference in time interval ($p<0.05$)

$^{\#}$: Significant difference in control and both treatments ($p<0.05$)

Heamorragh in renal medulla

In control group after 2, 8, and 24 hours, heamorragh in renal medulla of kidney was no observable lesion But low dose, treated 0.25 mg BV in 0.1 ml PBS and high dose, treated 0.50 mg BV in 0.1 ml PBS groups were found significant difference.

Low dose, at 2 hours was found mild lesion level but 8 hours were found semi-moderate lesion level and 24 hours were found moderate lesion level of heamorragh in renal medulla.

Mild lesion level of heamorragh in renal medulla at 2 hours was found in high dose group. After 8 and 24 hours, moderate lesion were observed.

Table 4.8 Comparison of the response levels of BV to heamorragh in renal medulla of kidney after injected with 0.1 ml PBS, 0.25 and 0.50 mg BV in 0.1 ml PBS

Time (Hours)	Control	0.25 mg BV in 0.1 ml PBS	0.50 mg BV in 0.1 ml PBS
2	$0^{\#}$	$1^{*,\#}$	$1^{*,\#}$
8	$0^{\#}$	$1.5^{*,\#}$	$2^{*,\#}$
24	$0^{\#}$	$2^{*,\#}$	$2^{*,\#}$

*: Significant difference in time interval ($p<0.05$)

$^{\#}$: Significant difference in control and both treatments ($p<0.05$)

White blood cell in renal medulla

In control group after 2, 8, and 24 hours, white blood cell in renal medulla of kidney was no observable lesion. But two treatment groups were found significant difference.

Treatment one which treated 0.25 mg BV in 0.1 ml PBS at 2 hours was found mild lesion level of white blood cell in renal medulla and moderate lesion was found after 8 and 24 hours.

Treatment two which treated 0.50 mg BV in 0.1 ml PBS was found mild lesion level of white blood cell in renal medulla and moderate lesion was found after 8 and 24 hours.

Table 4.9 Comparison of the response levels of BV to white blood cell in renal medulla on kidney after injected with 0.1 ml PBS, 0.25 and 0.50 mg BV in 0.1 ml PBS

Time (Hours)	Control	0.25 mg BV in 0.1 ml PBS	0.50 mg BV in 0.1 ml PBS
2	$0^{\#}$	$1^{*,\#}$	$1^{*,\#}$
8	$0^{\#}$	$2^{*,\#}$	$2^{*,\#}$
24	$0^{\#}$	$2^{*,\#}$	$2^{*,\#}$

*: Significant difference in time interval ($p<0.05$)

$^{\#}$: Significant difference in control and both treatments ($p<0.05$)

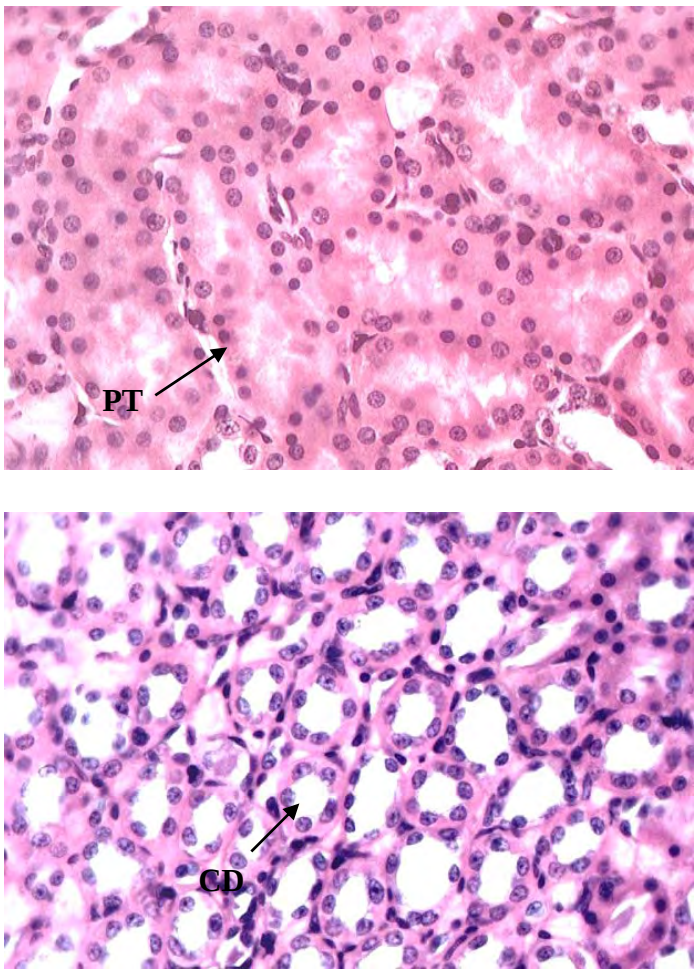
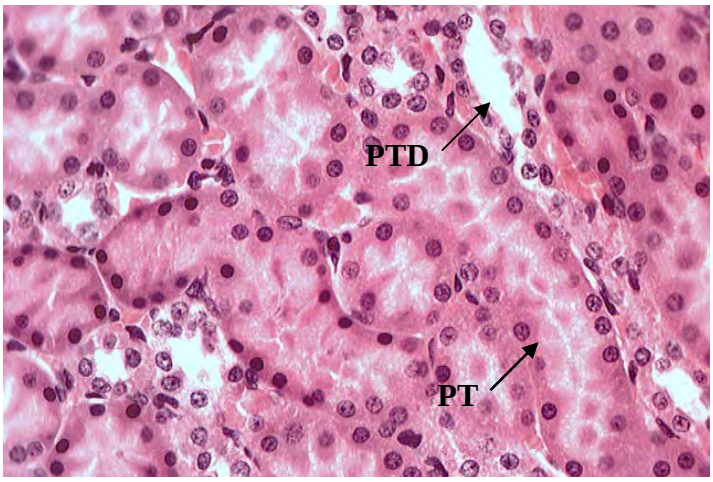


Figure 4.11. Kidney tissues of a control rat shows normal histological structure of kidney. H&E, x 40. PT = Proximal tubule, CD = Collecting duct.



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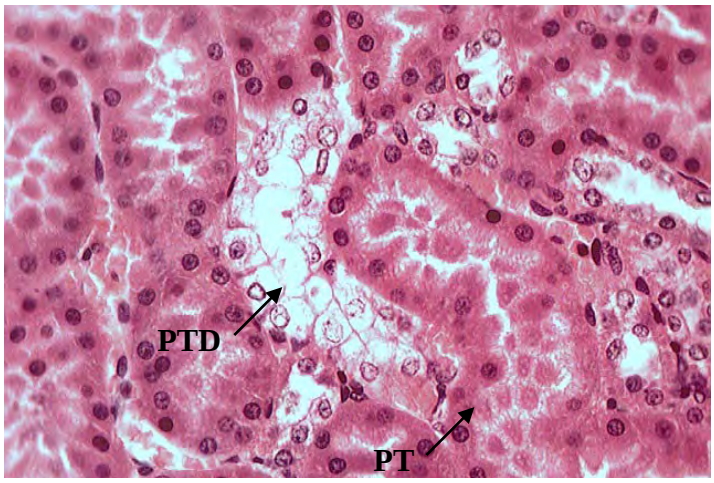

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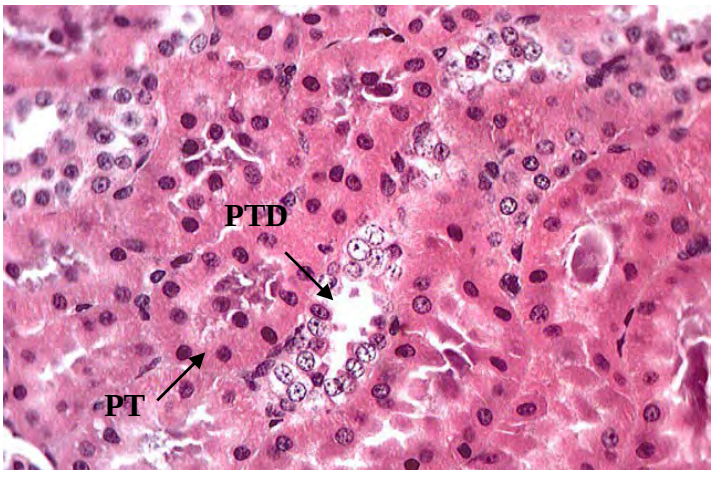
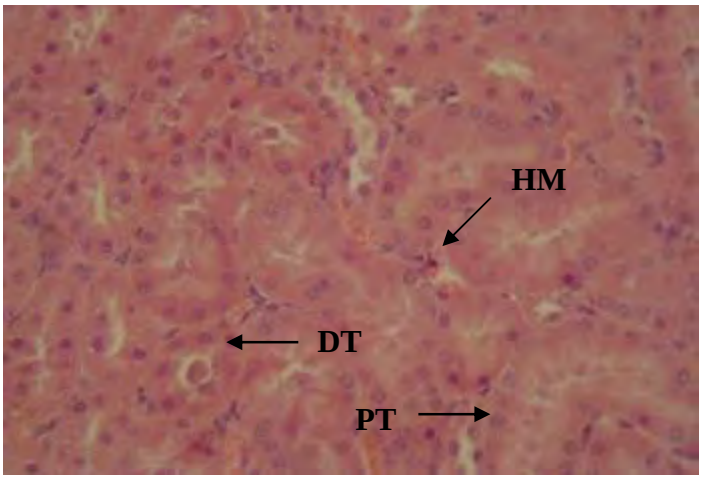


Level 3

Figure 4.12. Proximal tubular degeneration found in renal cortex of rat kidney H&E, x 40. PT = Proximal tubule, PTD = Proximal tubular degeneration



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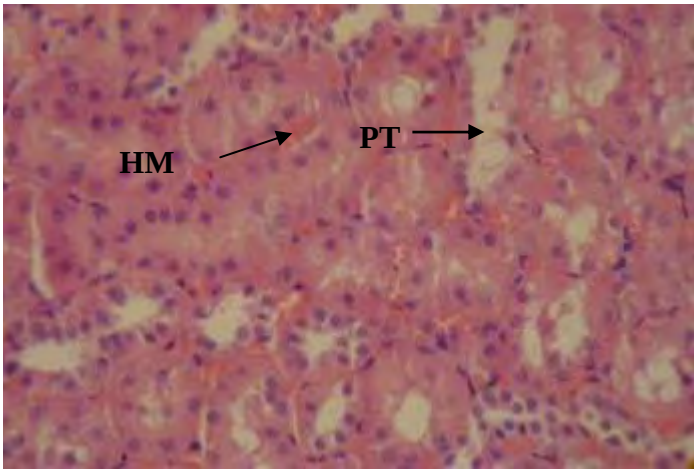

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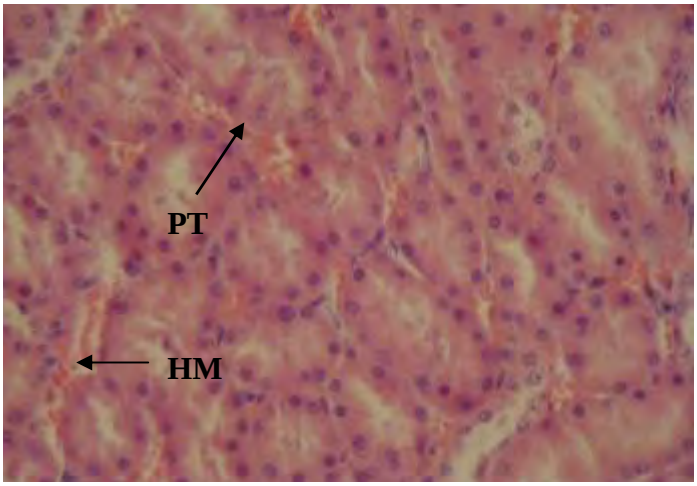
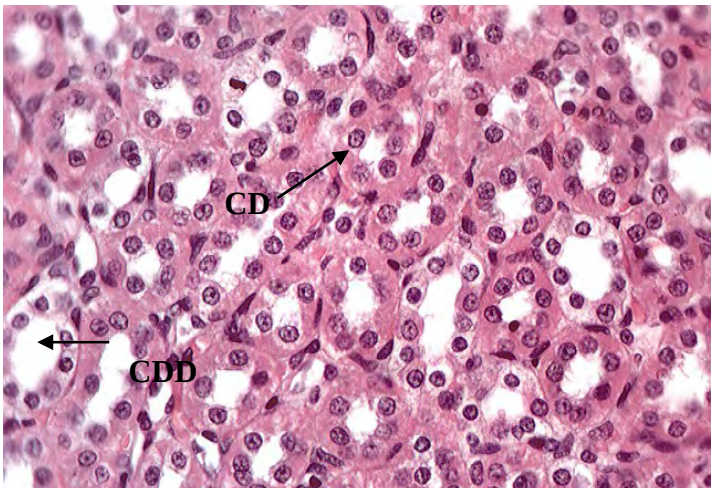


Level 3

Figure 4.13. Haemorrhage found in renal cortex of rat kidney H&E, x 40. PT = Proximal tubule, DT = Distal tubule, HM = Haemorrhage



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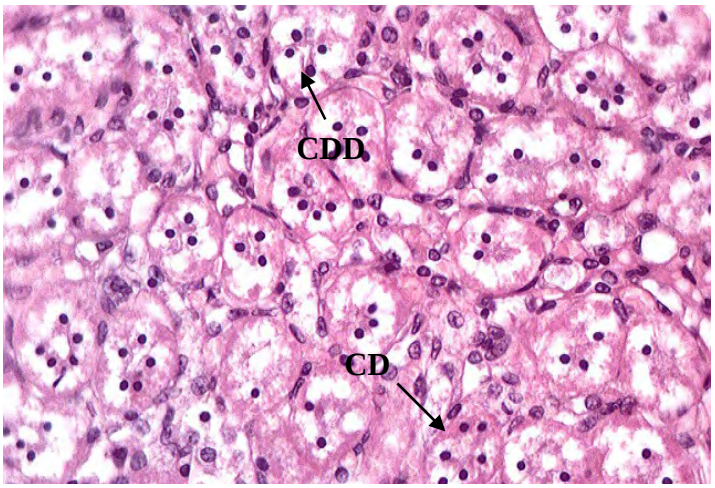

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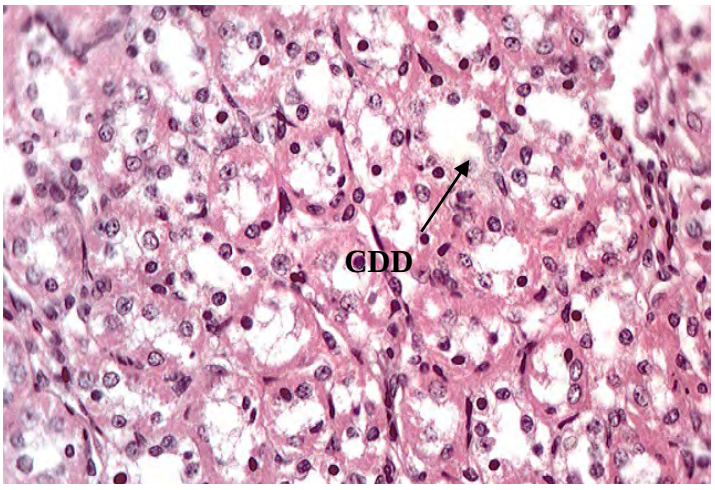
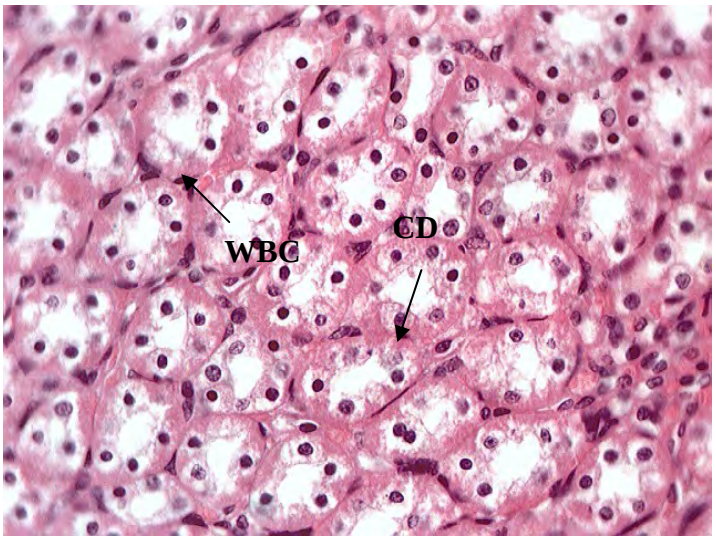


Level 3

Figure 4.14. Collecting duct degeneration found in renal medulla of rat kidney H&E, x 40.CD = Collecting duct, CDD = Collecting duct degeneration



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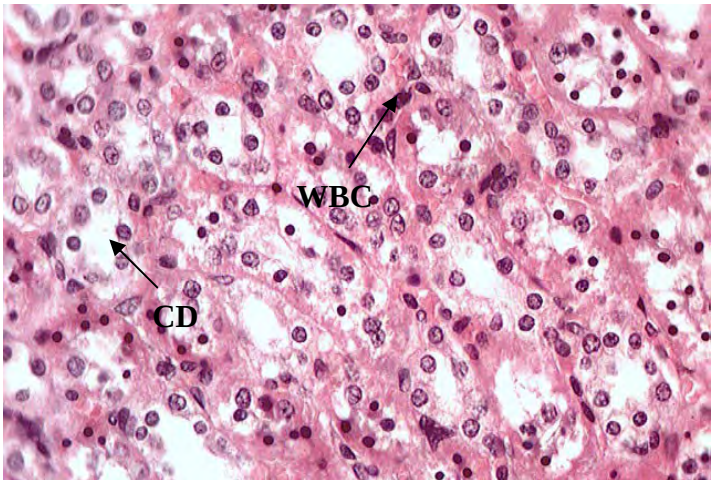

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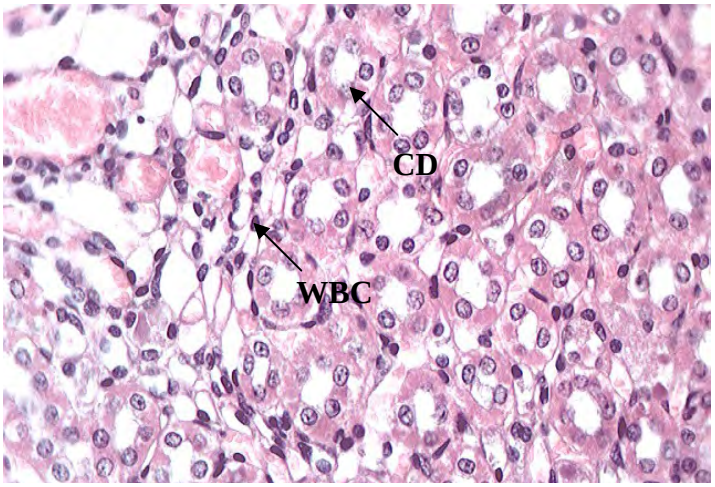

Level 3

Figure 4.15. White blood cells found in renal medulla of rat kidney H&E, x 40. CD = Collecting duct, WBC = White blood cell

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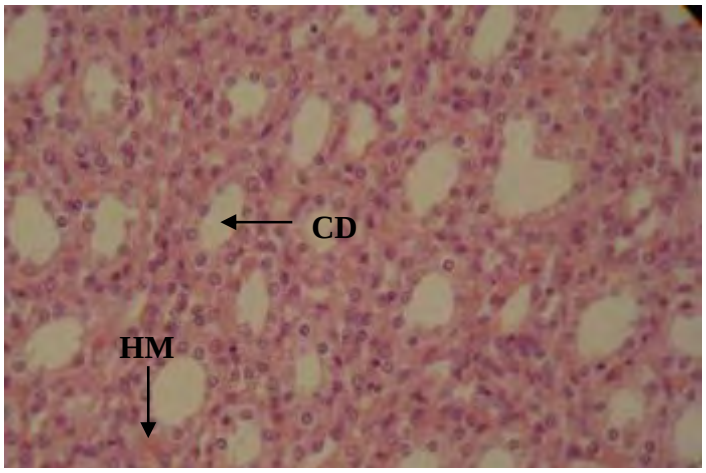

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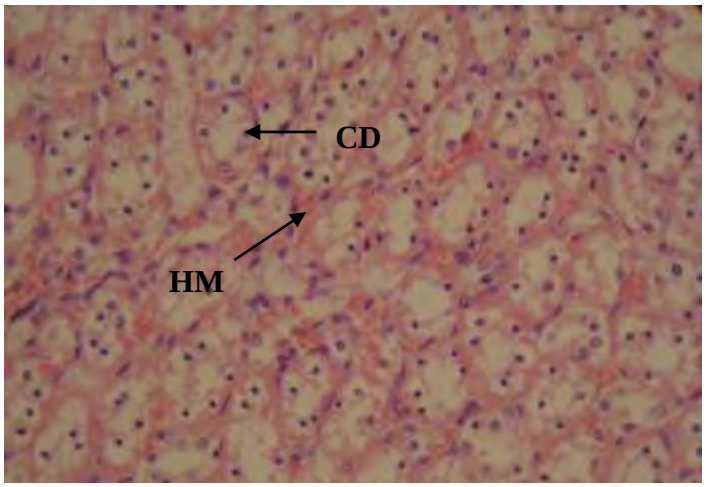

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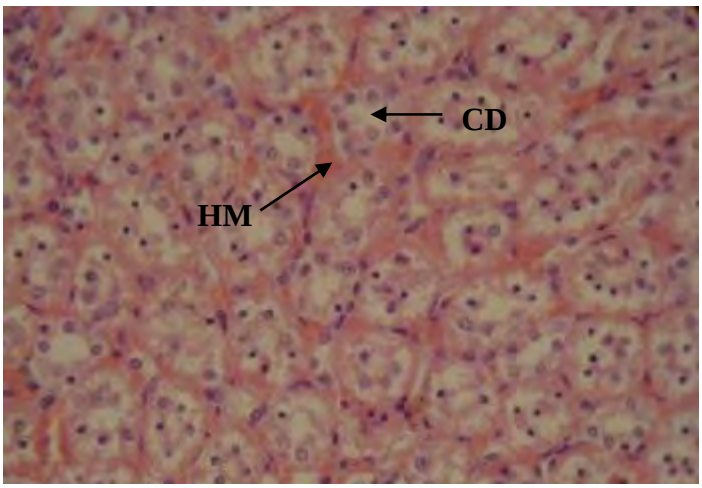

Level 3

Figure 4.16. Haemorrhage found in renal medulla of rat kidney H&E, x 40. PT = Proximal tubule, CD = Collecting duct, HM = Haemorrhage

4.6 Effects of *A. dorsata* venom on blood of rat

Red blood cell

The effects of BV on red blood cell were observed when rats were injected with differential concentrations. Amount of red blood cell were significant difference at $p<0.05$. The rats that were injected 0.5 mg BV in 0.1 ml PBS at 2 hours, had the highest level of red blood cell. Whereas they were not significant difference from the groups that were injected in lower concentrations at 8 and 24 hours, including with control group at 2 hours. In addition the control groups at 8 and 24 hours had lowest amount of red blood cell. Accordingly, the group of rats that treated with 0.25 mg BV in 0.1 ml PBS after 2, 8 and 24 hours, was not significant difference from control group at 2, 8 and 24 hours.

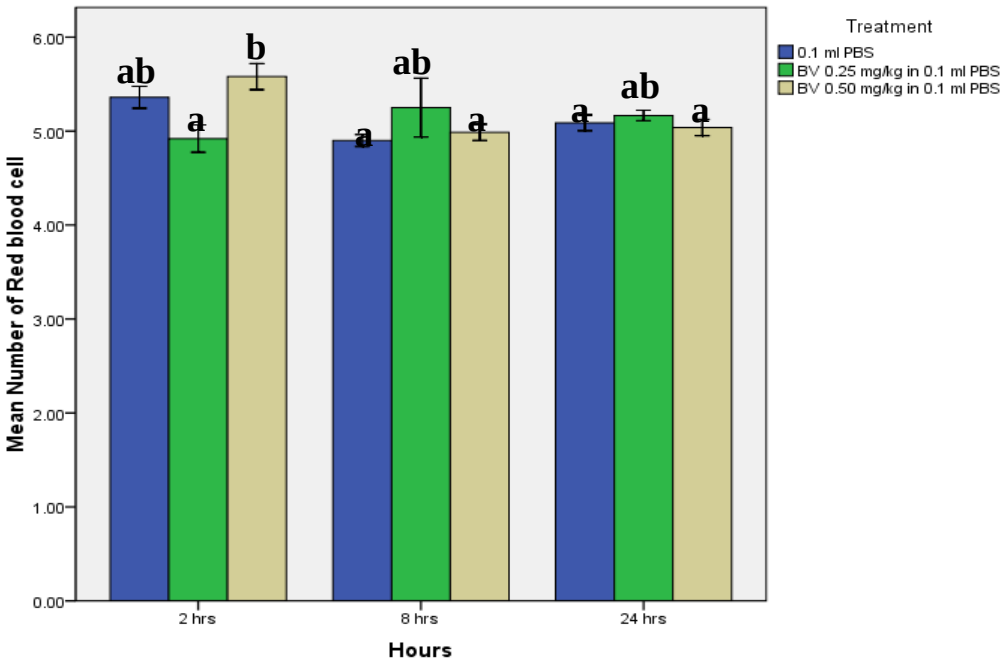

Figure 4.17. Amount of red blood cell at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

Haemoglobin

The experimental data showed that there was not significant difference in the amount of haemoglobin between both of BV injected rats at differential concentrations and control group at 2, 8 and 24 hours.

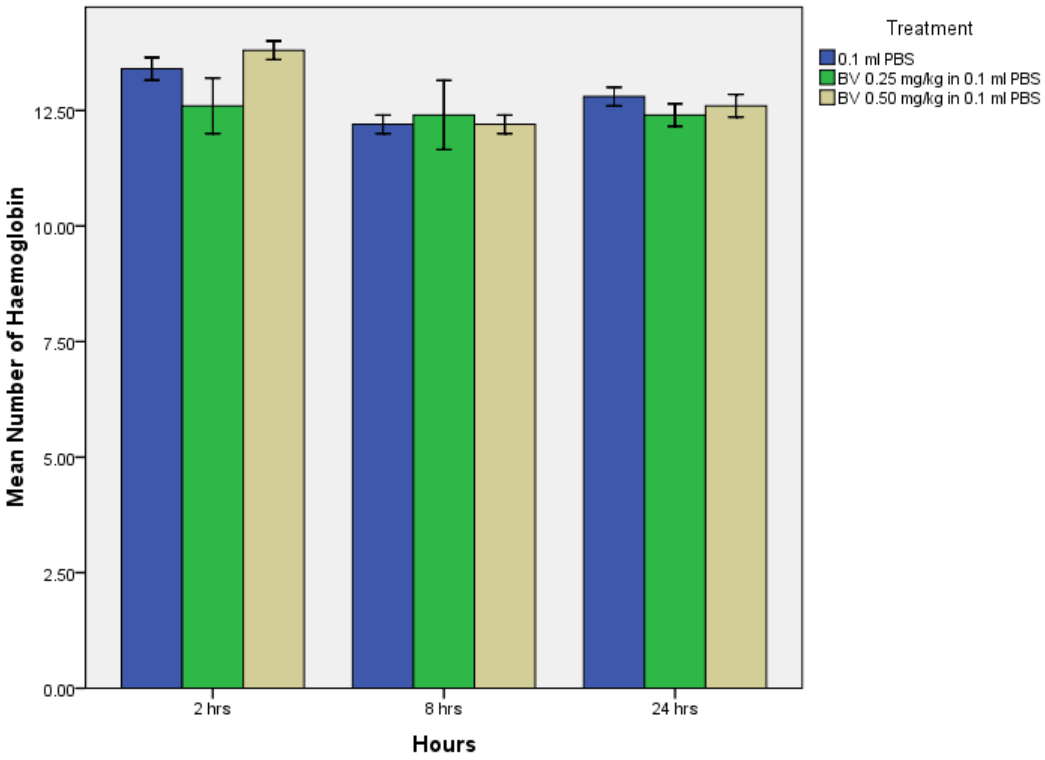

Figure 4.18. Amount of haemoglobin at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

Haematocrit

The effects of BV on haematocrit were observed when rats were injected in differential concentrations. The Level of haematocrit were significant difference at $p<0.05$. The rats that received 0.5 mg BV in 0.1 ml PBS at 2 hours had the highest level of haematocrit. Whereas they were not significant difference from the rats that injected in lower concentration at 8 and 24 hours, including with control group at 2 hours. In addition the control group at 8 and 24 hours. Showed the lowest level of haematocrit. Accordingly, the group of rats that treated with 0.25 mg BV in 0.1 ml PBS after 2, 8 and 24 hours, was not significant difference from control group at 2, 8 and 24 hours.

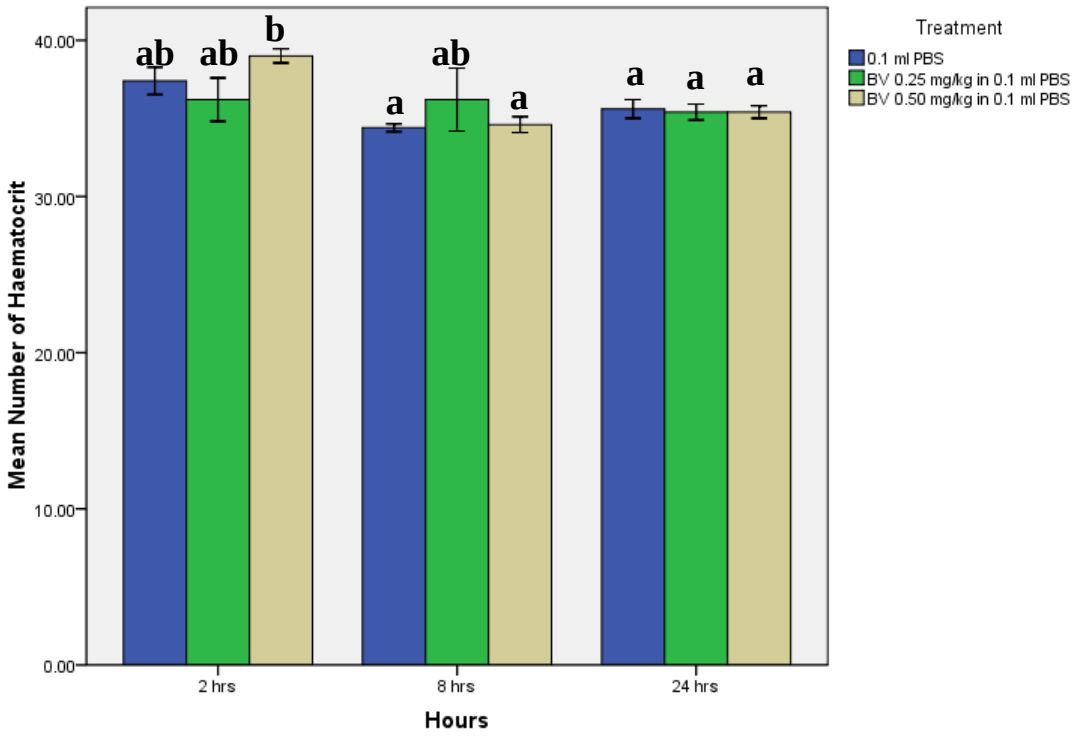

Figure 4.19. Level of haematocrit at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

Platelets

There was not significant difference in amount of platelets between control and both treatment groups at 2, 8 and 24 hours. So, *A. dorsata* venom may not related with coagulation.

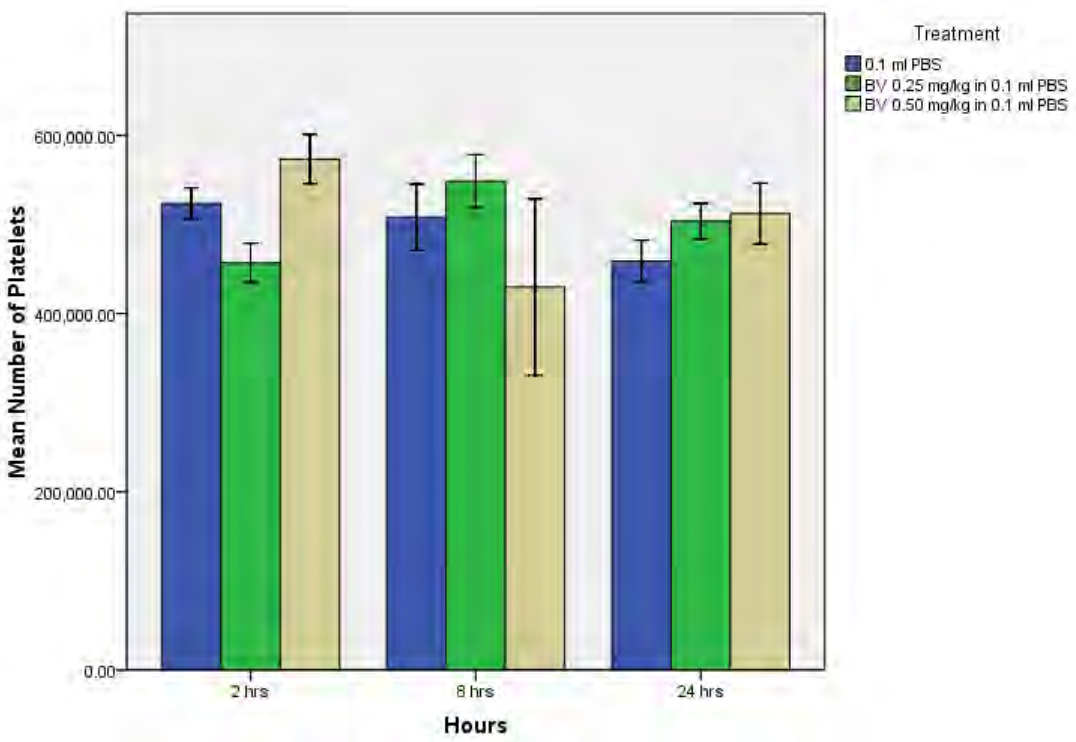

Figure 4.20. Amount of platelets at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

White blood cells

There were significant difference in amount of white blood cells between the treatment groups at 2, 8 and 24 hours ($p<0.05$). At 8 hours higher amount of white blood cells than the both treatment.

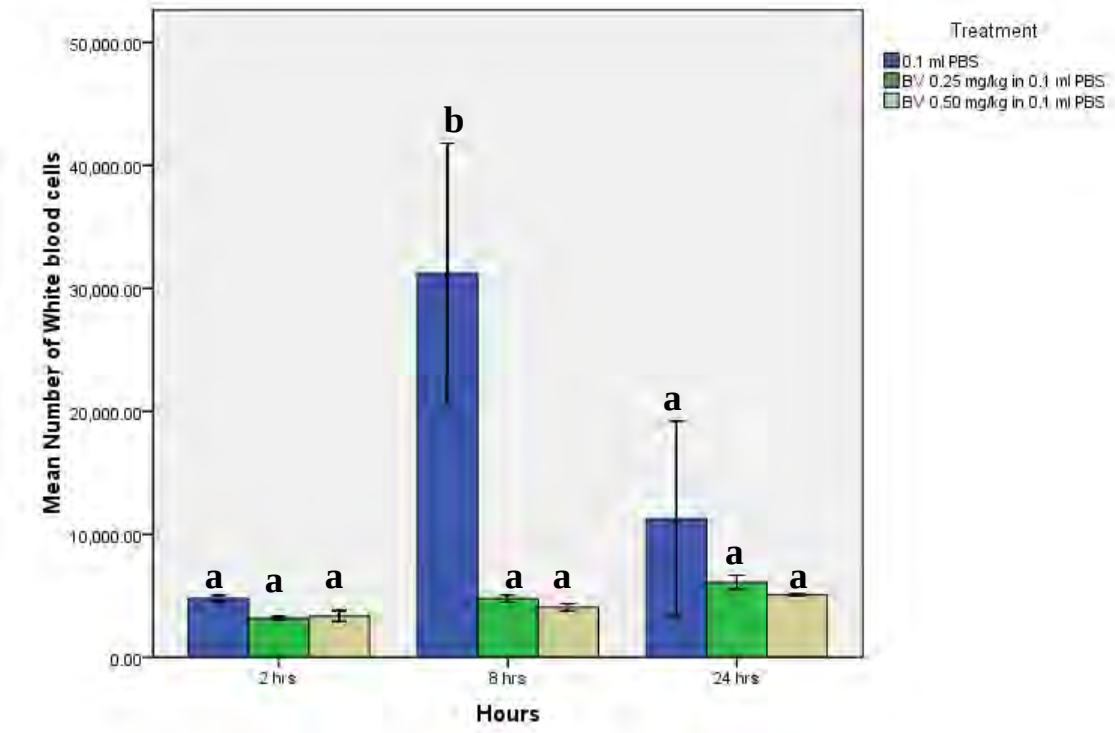

Figure 4.21. Amount of white blood cells at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

Neutrophil

The treatment groups showed difference significant in amount of neutrophil between low dose and high dose at $p<0.05$. The rats treated with 0.5 mg BV in 0.1 ml PBS at 24 hours had the highest amount of neutrophil. On the other hand the control group had lowest amount of neutrophil at 24 hours. However, There was not difference significant between treated group of 0.25 mg BV in 0.1 ml PBS and control group at 2 hours. In creasing of neutrophil showed system inflammatory response (Netto *et al*., 2004).

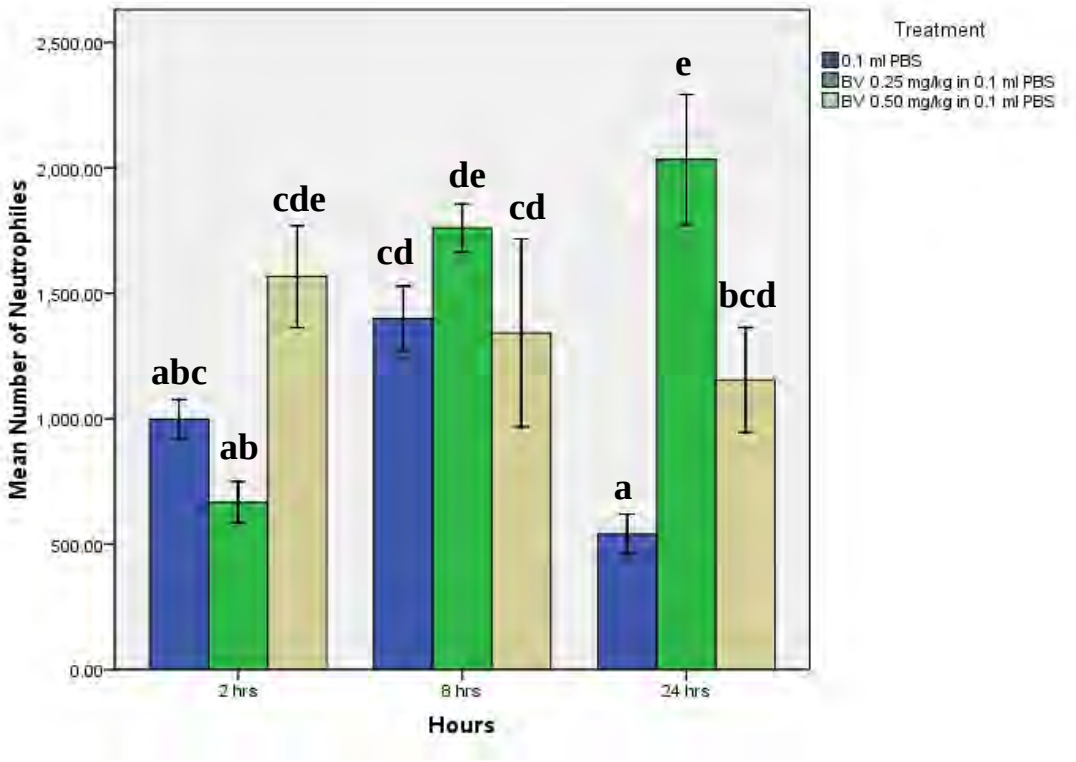

Figure 4.22. Amount of neutrophil at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

Lymphocyte

The results showed that there were difference significant in amount of lymphocyte between the treatment group at $p<0.05$. The highest amount of lymphocyte was found in control group but there was not significant difference from treatment groups of 0.25 mg BV in 0.1 ml PBS at 2 hours and 0.5 mg BV in 0.1 ml PBS at 24 hours. On the other hand, there was not significant difference in amount of lymphocyte between the rats treated with 0.5 mg BV in 0.1 ml PBS at 2 hours and 0.25 mg BV in 0.1 ml PBS at 8 hours. Lymphocyte showed cytotoxicity function to destroy protein allergen. The decrease in number of lymphocyte mainly caused by the generalized pain (Nogueira *et al.*, 2007).

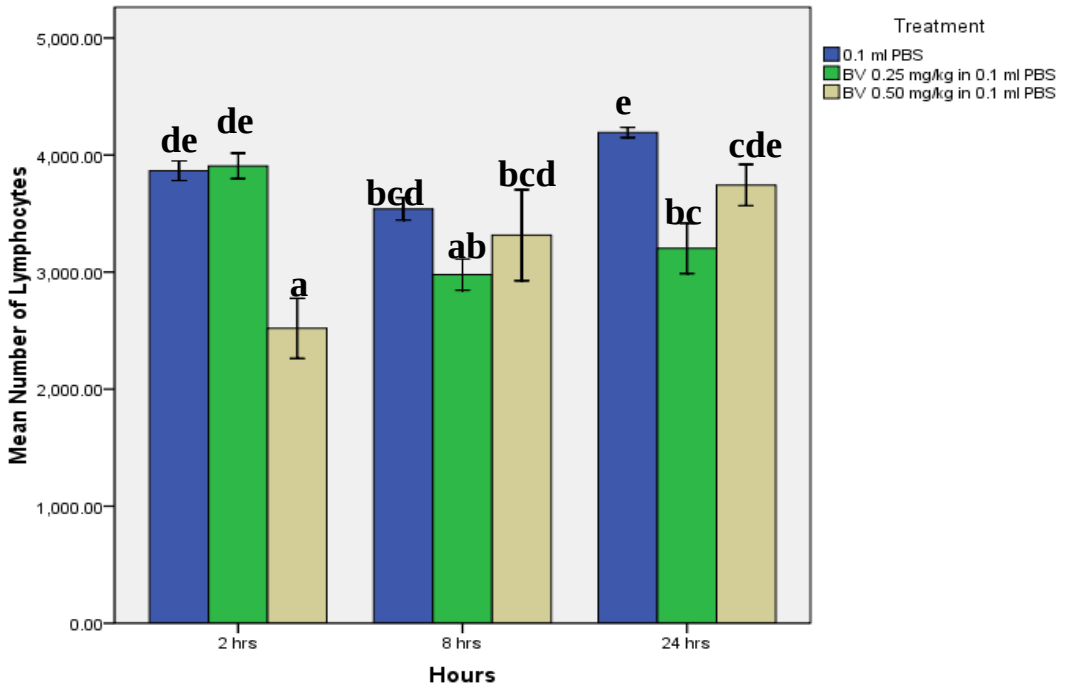

Figure 4.23. Amount of lymphocyte at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

Eosinophil

There was not difference significant in amount of eosinophil between control and both treatment groups at 2, 8 and 24 hours.

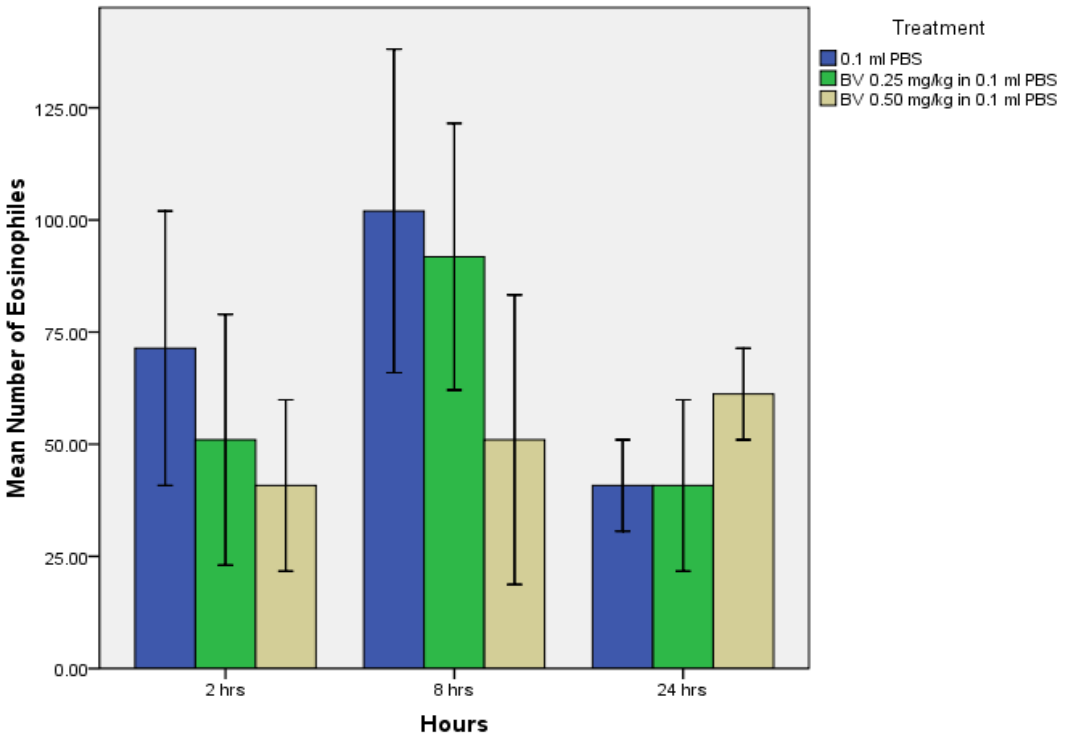

Figure 4.24. Amount of eosinophil at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

Monocyte

There was not difference significant in amount of monocyte between control and both treatment groups at 2, 8 and 24 hours.

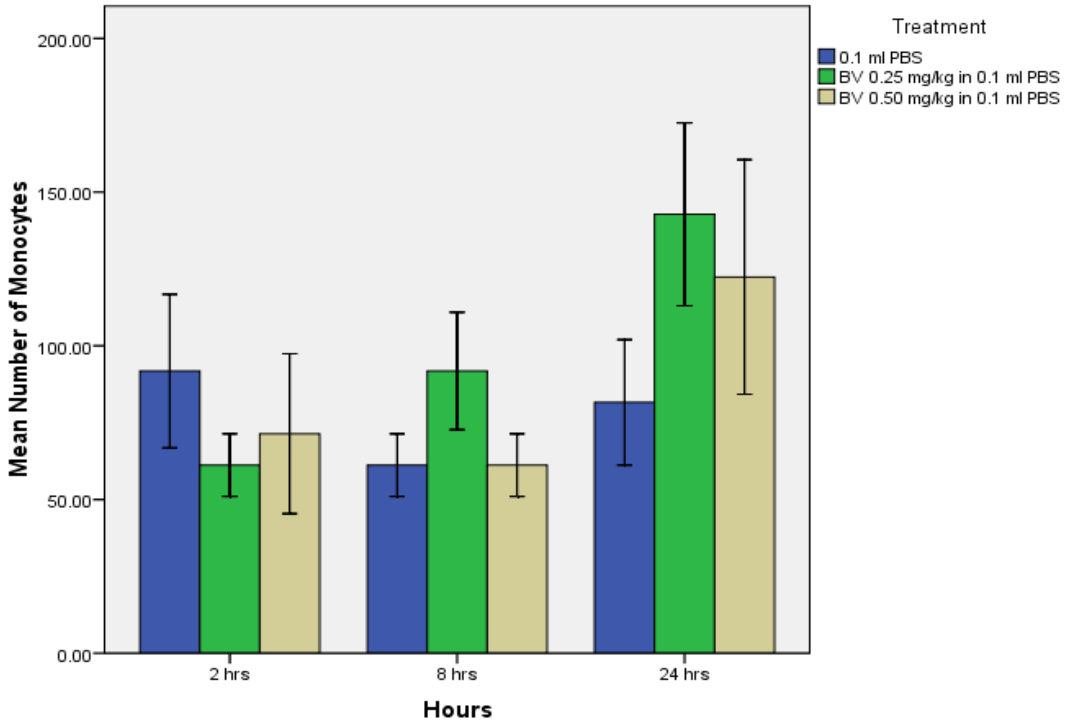

Figure 4.25. Amount of monocyte at 2h, 8h, and 24h after injected with 0.1 ml PBS (blue), BV 0.25 mg (Green) and 0.5 mg (yellow) in 0.1 ml PBS

Eosinophil and monocyte from this study were not significant difference from control group. These may cause from the concentration iof *A. dorsata* venom which had a toxin protein less than to function excited of these white blood cells.

CHAPTER V
CONCLUSION

In this research, amount of venom, components and the effect of *A. dorsata* venom were carried out. High performance liquid chromatography (HPLC) method was used for separation and purification. Mass spectrometry method was used for analysis. The final part was to study the effect of *A. dorsata* venom of liver, kidney and blood tissues in rats by using histological techniques.

The amount of dried weight venom of *A. dorsata* is about 0.170 ± 0.016 mg per bee. *A. dorsata* venom is higher than other *Apis* due to the larger size.

Three of BV component (apamine, phospholipase A2, melittin) are common found in *Apis* spp. whereas the enzyme glycosyltransferease found in BV component. Glycosyltransferase is an enzyme involve in transfer sugar from sugar donor to acceptor such as protein or lipid also called glycosylation. Occurrence of this enzyme in BV component suggested that some of BV component require glycocylation.

There were three major responses to BV including blood congestion sinusoidal space dilation and lipid droplets accumulation on hepatocyte. The virulence of responding is higher when the exposed time is longer and concentration of BV is higher.

This experiment indicated that BV is a cause of local inflammation of liver cell. The result supported by the increasing in amount of Kupffer cells in sinusoid space, the cell that involve in innate immunity.

The major responses found in the kidney including proximal tubular degeneration and haemorrhage in renal cortex and collecting duct

degeneration, haemorrhage and white blood cells infiltration in renal medulla. The virulence of responding is higher when the exposed time is longer. It was found that the amount of BV did not involve in the virulence of responding.

Effect of BV on blood tissue Haemoglobin, platletes, eosinophil, monocyte were found not significant difference but red blood cells, haematocrit, lymphocyte, white blood cells, neutrophil were found significant difference. Suggest that BV has responding to immune of mammalian.

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APPENDICES

APPENDIX A

Chemical reagents

1. 10% Buffered formalin

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

2. Phosphate buffer saline Ph 7.4

Anhydrous disodium phosphate (Na_2HPO_4)	28.39	g
Sodium Chloride (NaCl)	4.38	g
ddH_2O	1000	ml

3. Eosin

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

4. Haematoxylin

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

5. Giemsa stain	1	g
Giemsa powder	66	ml
Absolute methanal	66	ml
Glycerol		
6. Phosphate buffer saline Ph 7.4		
Solution A		
Potassium Hydrogen phthalate (KH_2PO_4)	9.1	g
ddH_2O	1000	ml
Solution B		
Anhydrous disodium phosphate (Na_2HPO_4)	9.5	g
ddH_2O	1000	ml

Routine paraffin thehnique

70% ethanol (several days)

⇩

90% Ethanol (60 min.)

⇩

95% Ethanol (60 min.)

⇩

95% Ethanol (30 min.)

⇩

Ethanol absolute (60 min.)

⇩

Ethanol absolute (180 min.)

⇩

Ethanol absolute (180 min.)

⇩

Ethanol absolute (180 min.)

⇩

Xylene (30 min.)

⇩

Xylene (30 min.)

⇩

Xylene in paraplast 1:1 (60 min.)

⇩

Paraplast (60 min.)

⇩

Paraplast (60 min.)

⇩

Embed in paraplast

Routine H&E staining

Deparaffinize sections in xylene (2 times; 10 mins each)

⇩

Re-hydrate in absolute alcohol (2 time; 5 mins each)

⇩

95% alcohol for 2 minutes

⇩

70% alcohol for 2 miuntes

⇩

Briefly wash in distilled water

⇩

Stain in Mayer’s hematoxylin solution for 8 mins

⇩

Wash in running tap water for 5 mins

⇩

Differentiate in 1% acid alcohol for 30 seconds

⇩

Wash running tap water for 1 min

⇩

Dips in 95% alcohol

⇩

Stain in eosin Y solution for 40 seconds

⇩

Dehydrate through 95% alcohol

⇩

Absolute alcohol (2 times; 5 mins each)

Routine H&E staining (cont.)

Clear in xylene (2 times; 5 mins each)

⇩

Mounting slide

APPENDIX B

Descriptive of histopathological in live tissue

Treats			Blood congestion	Fatty degeneration	Sinusoidal dilation
Control	2 hrs	Median	0	0	0
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level	mild lesion level
	8 hrs	Median	0	0	0
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level	mild lesion level
	24 hrs	Median	0	0	0
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level	mild lesion level
0.25 mg BV in 0.1 ml PBS	2 hrs	Median	1	1	1
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	moderate lesion level	moderate lesion level	sever lesion level
	8 hrs	Median	1	1	2
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	moderate lesion level	moderate lesion level	sever lesion level
	24 hrs	Median	2	2	2
		Minimum	mild lesion level	no observable lesion	mild lesion level
		Maximum	sever lesion level	sever lesion level	sever lesion level
0.50 mg BV in 0.1 ml PBS	2 hrs	Median	2	1	2
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	moderate lesion level	moderate lesion level	moderate lesion level
	8 hrs	Median	2	2	3
		Minimum	mild lesion level	no observable lesion	mild lesion level
		Maximum	moderate lesion level	moderate lesion level	sever lesion level
	24 hrs	Median	3	3	3
		Minimum	mild lesion level	moderate lesion level	mild lesion level
		Maximum	sever lesion level	sever lesion level	sever lesion level

Kruskal-wallis test of liver tissue in control group at 2-24 hrs

	Blood congestion	Fatty degeneration	Sinusoidal dilation
Chi-Square	10.633	4.139	5.305
df	2	2	2
Asymp. Sig.	.005	.126	.070

Kruskal-wallis test of liver tissue treated with 0.25 mg/kg BV in 0.1 ml PBS 2-24 hrs

	Blood congestion	Fatty degeneration	Sinusoidal dilation
Chi-Square	52.265	56.103	10.828
df	2	2	2
Asymp. Sig.	.000	.000	.004

Kruskal-wallis test in liver tissue treated with 0.50 mg/kg BV in 0.1 ml PBS at 2-24 hrs

	Blood congestion	Fatty degeneration	Sinusoidal dilation
Chi-Square	69.538	110.075	45.191
df	2	2	2
Asymp. Sig.	.000	.000	.000

Kruskal-wallis test in liver tissue at 2 hrs in control group and both treatments

	Blood congestion	Fatty degeneration	Sinusoidal dilation
Chi-Square	71.952	24.749	83.636
df	2	2	2
Asymp. Sig.	.000	.000	.000

Kruskal-wallis test in liver tissue at 8 hrs in control group and both treatments

	Blood congestion	Fatty degeneration	Sinusoidal dilation
Chi-Square	82.670	81.691	103.144
df	2	2	2
Asymp. Sig.	.000	.000	.000

Kruskal-wallis test in liver tissue at 24 hrs in control group and both treatments

	Blood congestion	Fatty degeneration	Sinusoidal dilation
Chi-Square	104.141	107.916	105.207
df	2	2	2
Asymp. Sig.	.000	.000	.000

Number of Kupffer cell of control group treated with 0.1 ml PBS

Treats			No. of Kupffer cell
Control	2 hours	Mean	21.0000
		S.E.	.89443
		Minimum	19.00
		Maximum	24.00
	8 hours	Mean	17.8000
		S.E.	1.24097
		Minimum	14.00
		Maximum	21.00
	24 hours	Mean	18.6000
		S.E.	1.36382
		Minimum	14.00
		Maximum	21.00

Number of Kupffer cell treated with 0.25 mg/kg BV in 0.1 ml PBS

Treats			#No. of Kupffer cell
0.25 mg BV in 0.1 ml PBS	2 hours	Mean	26.2000
		S.E.	1.15758
		Minimum	24.00
		Maximum	29.00
	8 hours	Mean	28.8000
		S.E.	.80000
		Minimum	27.00
		Maximum	31.00
	24 hours	Mean	30.4000
		S.E.	3.05941
		Minimum	22.00
		Maximum	39.00

Number of Kupffer cell in treated with 0.50 mg/kg BV in 0.1 ml PBS

Treats			#No. of Kupffer cell
0.50 mg BV in 0.1 ml PBS	2 hours	Mean	36.8000
		S.E.	.20000
		Minimum	36.00
		Maximum	37.00
	8 hours	Mean	38.0000
		S.E.	.31623
		Minimum	37.00
		Maximum	39.00
	24 hours	Mean	39.4000
		S.E.	.24495
		Minimum	39.00
		Maximum	40.00

Test of Homogeneity of Variances

	Levene Statistic	df1	df2	Sig.
Number of Kupffer Cell	8.066	8	36	.000

Oneway ANOVA of Kupffer cells

Parameter		Sum of Squares	df	Mean Square	F	Sig.
Number of Kupffer Cell	Between Groups	2778.311	8	347.289	39.715	.000
	Within Groups	314.800	36	8.744		
	Total	3093.111	44			

Multiple Comparisons
Post Hoc Tests by Duncan test

Number of Kupffer Cell

		Subset for alpha = 0.05			
Treats	N	1	2	3	4
Control 8 hrs.	5	17.8000			
Control 24 hrs.	5	18.6000			
Control 2 hrs.	5	21.0000			
Low 2 hrs.	5		26.2000		
Low 8 hrs.	5		28.8000	28.8000	
Low 24 hrs.	5			30.4000	
High 2 hrs.	5				36.8000
High 8 hrs.	5				38.0000
High 24 hrs.	5				39.4000
Sig.		.114	.173	.398	.198

Descriptive of histopathological in renal cortex of kidney tissue

Treats			Proximal tubular degeneration	Haemorrhage in renal cortex
Control	2 hrs	Median	0	0
		Minimum	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level
	8 hrs	Median	0	0
		Minimum	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level
	24 hrs	Median	0	0
		Minimum	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level
0.25 mg BV in 0.1 ml PBS	2 hrs	Median	1	1
		Minimum	no observable lesion	no observable lesion
		Maximum	moderate lesion level	moderate lesion level
	8 hrs	Median	2	1
		Minimum	no observable lesion	no observable lesion
		Maximum	sever lesion level	sever lesion level
	24 hrs	Median	2	2
		Minimum	mild lesion level	mild lesion level
		Maximum	sever lesion level	sever lesion level
0.50 mg BV in 0.1 ml PBS	2 hrs	Median	2	1
		Minimum	mild lesion level	no observable lesion
		Maximum	sever lesion level	moderate lesion level
	8 hrs	Median	2	2
		Minimum	mild lesion level	no observable lesion
		Maximum	sever lesion level	moderate lesion level
	24 hrs	Median	2	2
		Minimum	mild lesion level	mild lesion level
		Maximum	sever lesion level	sever lesion level

Descriptive of histopathological in renal medulla of kidney tissue

Treats			Heamorragh in renal medulla	White blood cell in renal medulla	Collecting duct degeneration in renal medulla
Control	2 hrs	Median	0	0	0
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level	mild lesion level
	8 hrs	Median	0	0	0
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level	mild lesion level
	24 hrs	Median	0	0	0
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	mild lesion level	mild lesion level	mild lesion level
0.25 mg BV in 0.1 ml PBS	2 hrs	Median	0	1	1
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	moderate lesion level	moderate lesion level	moderate lesion level
	8 hrs	Median	2	1.5	2
		Minimum	mild lesion level	mild lesion level	mild lesion level
		Maximum	sever lesion level	moderate lesion level	sever lesion level
	24 hrs	Median	2	2	2
		Minimum	mild lesion level	mild lesion level	mild lesion level
		Maximum	sever lesion level	sever lesion level	sever lesion level
0.50 mg BV in 0.1 ml PBS	2 hrs	Median	1	1	1
		Minimum	no observable lesion	no observable lesion	no observable lesion
		Maximum	moderate lesion level	sever lesion level	sever lesion level
	8 hrs	Median	2	2	2
		Minimum	mild lesion level	mild lesion level	moderate lesion level
		Maximum	sever lesion level	sever lesion level	sever lesion level
	24 hrs	Median	2	2	2
		Minimum	mild lesion level	mild lesion level	moderate lesion level
		Maximum	sever lesion level	sever lesion level	sever lesion level

Kruskal-wallis test in kidney tissue at 2 hrs in control group treated with 0.1 ml PBS

	Proximal tubular degeneration	Haemorrhage in renal cortex	Collecting duct degeneration in renal medulla	Heamorragh in renal medulla	White blood cell in renal medulla
Chi-Square	6.458	.499	.780	.489	.436
df	2	2	2	2	2
Asymp. Sig.	.040	.779	.677	.783	.804

Kruskal-wallis test in kidney tissue treated with 0.25 mg/kg BV in 0.1 ml PBS at 2-24 hrs

	Proximal tubular degeneration	Haemorrhage in renal cortex	Collecting duct degeneration in renal medulla	Heamorragh in renal medulla	White blood cell in renal medulla
Chi-Square	56.751	69.598	38.743	72.573	93.214
df	2	2	2	2	2
Asymp. Sig.	.000	.000	.000	.000	.000

Kruskal-wallis test in liver tissue treated with 0.50 mg/kg BV in 0.1 ml PBS at 2-24 hrs

	Proximal tubular degeneration	Haemorrhage in renal cortex	Collecting duct degeneration in renal medulla	Heamorragh in renal medulla	White blood cell in renal medulla
Chi-Square	7.171	65.111	70.145	46.621	56.971
df	2	2	2	2	2
Asymp. Sig.	.028	.000	.000	.000	.000

Kruskal-wallis test in liver tissue at 2 hrs in control group and both treatments

	Proximal tubular degeneration	Haemorrhage in renal cortex	Collecting duct degeneration in renal medulla	Heamorragh in renal medulla	White blood cell in renal medulla
Chi-Square	99.253	54.151	51.962	43.367	46.642
df	2	2	2	2	2
Asymp. Sig.	.000	.000	.000	.000	.000

Kruskal-wallis test in liver tissue at 8 hrs in control group and both treatments

	Proximal tubular degeneration	Haemorrhage in renal cortex	Collecting duct degeneration in renal medulla	Heamorragh in renal medulla	White blood cell in renal medulla
Chi-Square	106.870	83.625	114.335	80.565	118.507
df	2	2	2	2	2
Asymp. Sig.	.000	.000	.000	.000	.000

Kruskal-wallis test in liver tissue at 24 hrs in control group and both treatments

	Proximal tubular degeneration	Haemorrhage in renal cortex	Collecting duct degeneration in renal medulla	Heamorragh in renal medulla	White blood cell in renal medulla
Chi-Square	106.592	115.285	116.378	102.880	111.272
df	2	2	2	2	2
Asymp. Sig.	.000	.000	.000	.000	.000

Descriptive of blood tissue

Treats			No. of Red blood cell	No. of Haemoglobin	No. of Haematocrit
Control	2 hrs	Mean	5.3600	13.4000	37.4000
		S.E.	.11662	.24495	.87178
		Minimum	5.20	13.00	35.00
		Maximum	5.80	14.00	40.00
	8 hrs	Mean	4.9000	12.2000	34.4000
		S.E.	.06325	.20000	.24495
		Minimum	4.70	12.00	34.00
		Maximum	5.10	13.00	35.00
	24 hrs	Mean	5.0880	12.8000	35.6000
		S.E.	.08405	.20000	.60000
		Minimum	4.80	12.00	34.00
		Maximum	5.30	13.00	37.00
0.25 mg BV in 0.1 ml PBS	2 hrs	Mean	4.9200	12.6000	36.2000
		S.E.	.14629	.60000	1.39284
		Minimum	4.60	11.00	33.00
		Maximum	5.40	14.00	40.00
	8 hrs	Mean	5.2500	12.4000	36.2000
		S.E.	.31219	.74833	2.00998
		Minimum	4.40	10.00	30.00
		Maximum	6.04	14.00	41.00
	24 hrs	Mean	5.1660	12.4000	35.4000
		S.E.	.05564	.24495	.50990
		Minimum	5.04	12.00	34.00
		Maximum	5.30	13.00	37.00

0.50 mg BV in 0.1 ml PBS	2 hrs	Mean	5.5800	13.8000	39.0000
		S.E.	.13928	.20000	.44721
		Minimum	5.20	13.00	38.00
		Maximum	5.90	14.00	40.00
	8 hrs	Mean	4.9860	12.2000	34.6000
		S.E.	.08658	.20000	.50990
		Minimum	4.70	12.00	33.00
		Maximum	5.20	13.00	36.00
	24 hrs	Mean	5.0380	12.6000	35.4000
		S.E.	.08686	.24495	.40000
		Minimum	4.70	12.00	34.00
		Maximum	5.20	13.00	36.00

Descriptive of blood tissue (Con.1)

Treats			No. of Platelets	No. of White blood cell	No. of Neutrophil
Control	2 hrs	Mean	523600.0000	4780.0000	998.2000
		S.E.	17304.91260	247.79023	78.39732
		Minimum	487000.00	3800.00	735.00
		Maximum	576000.00	5100.00	1173.00
	8 hrs	Mean	508200.0000	31220.0000	1399.6000
		S.E.	37225.52887	10576.88045	129.71415
		Minimum	411000.00	5200.00	1056.00
		Maximum	631000.00	53000.00	1716.00
	24 hrs	Mean	459000.0000	11260.0000	540.4000
		S.E.	23287.33561	7939.81108	79.71236
		Minimum	403000.00	2500.00	372.00
		Maximum	539000.00	43000.00	817.00
Low dose	2 hrs	Mean	456800.0000	3180.0000	667.4000
		S.E.	22009.99773	124.09674	82.10457
		Minimum	376000.00	2800.00	462.00
		Maximum	509000.00	3500.00	957.00
	8 hrs	Mean	548600.0000	4760.0000	1760.8000
		S.E.	29763.40034	292.57478	95.63389
		Minimum	435000.00	4000.00	1386.00
		Maximum	603000.00	5600.00	1920.00
	24 hrs	Mean	503600.0000	6100.0000	2033.8000
		S.E.	20068.88138	564.80085	258.74976
		Minimum	443000.00	4800.00	1519.00
		Maximum	551000.00	7600.00	2964.00

High dose	2 hrs	Mean	573400.0000	3340.0000	1566.4000
		S.E.	27518.35751	436.57760	203.29353
		Minimum	496000.00	2000.00	860.00
		Maximum	652000.00	4600.00	2028.00
	8 hrs	Mean	429800.0000	4060.0000	1341.8000
		S.E.	98961.30557	290.86079	374.93325
		Minimum	44000.00	2900.00	559.00
		Maximum	580000.00	4400.00	2508.00
	24 hrs	Mean	512200.0000	5060.0000	1155.4000
		S.E.	34229.22728	87.17798	209.07358
		Minimum	414000.00	4800.00	800.00
		Maximum	602000.00	5300.00	1850.00

Descriptive of blood tissue (Con.2)

Treats			No. of Eosinophiles	No. of Basophiles	No. of Lymphocytes	No. of Monocytes
Control	2 hrs	Mean	71.4000	.0000	3865.8000	91.8000
		S.E.	30.60000	.00000	84.42002	24.98480
		Minimum	.00	.00	3723.00	51.00
		Maximum	153.00	.00	4182.00	153.00
	8 hors	Mean	102.0000	.0000	3539.4000	61.2000
		S.E.	36.06245	.00000	94.86548	10.20000
		Minimum	.00	.00	3264.00	51.00
		Maximum	204.00	.00	3774.00	102.00
	24 hours	Mean	40.8000	.0000	4192.2000	81.6000
		S.E.	10.20000	.00000	43.87186	20.40000
		Minimum	.00	.00	4029.00	51.00
		Maximum	51.00	.00	4284.00	153.00
Low dose	2 hrs	Mean	51.0000	.0000	3906.6000	61.2000
		S.E.	27.93385	.00000	108.90620	10.20000
		Minimum	.00	.00	3519.00	51.00
		Maximum	153.00	.00	4182.00	102.00
	8 hrs	Mean	91.8000	.0000	2978.4000	91.8000
		S.E.	29.73785	.00000	133.57717	19.08245
		Minimum	.00	.00	2448.00	51.00
		Maximum	153.00	.00	3162.00	153.00
	24 hrs	Mean	40.8000	.0000	3202.8000	142.8000
		S.E.	19.08245	.00000	214.80628	29.73785
		Minimum	.00	.00	2550.00	51.00
		Maximum	102.00	.00	3621.00	204.00

High dose	2 hrs	Mean	40.8000	.0000	2519.4000	71.4000
		S.E.	19.08245	.00000	256.82944	26.00500
		Minimum	.00	.00	1836.00	.00
		Maximum	102.00	.00	3315.00	153.00
	8 hrs	Mean	51.0000	.0000	3315.0000	61.2000
		S.E.	32.25523	.00000	389.07351	10.20000
		Minimum	.00	.00	2142.00	51.00
		Maximum	153.00	.00	4284.00	102.00
	24 hrs	Mean	61.2000	.0000	3743.4000	122.4000
		S.E.	10.20000	.00000	175.63559	38.16491
		Minimum	51.00	.00	3111.00	51.00
		Maximum	102.00	.00	4080.00	255.00

Test of Homogeneity of Variances

	Levene Statistic	**df1**	**df2**	**Sig.**
Number of Red blood cell	5.656	8	36	.000
Number of Haemoglobin	4.648	8	36	.001
Number of Haematocrit	5.252	8	36	.000
Number of Platelets	2.334	8	36	.039
Number of White blood cell	16.668	8	36	.000
Number of Neutrophiles	4.221	8	36	.001
Number of Band	7.615	8	36	.000
Number of Eosinophiles	2.065	8	36	.066
Number of Basophiles	.	8	.	.
Number of Lymphocytes	5.292	8	36	.000
Number of Monocytes	2.723	8	36	.019

Oneway ANOVA of Blood tissue

Parameter		Sum of Squares	df	Mean Square	F	Sig.
Number of Red blood cell	Between Groups	1.988	8	.248	2.470	.030
	Within Groups	3.621	36	.101		
	Total	5.609	44			
Number of Haemoglobin	Between Groups	12.044	8	1.506	2.151	.056
	Within Groups	25.200	36	.700		
	Total	37.244	44			
Number of Haematocrit	Between Groups	82.178	8	10.272	2.300	.042
	Within Groups	160.800	36	4.467		
	Total	242.978	44			
Number of Platelets	Between Groups	8.493E10	8	1.062E10	1.215	.318
	Within Groups	3.145E11	36	8.735E9		
	Total	3.994E11	44			
Number of White blood cell	Between Groups	3.215E9	8	4.019E8	4.118	.001
	Within Groups	3.514E9	36	9.760E7		
	Total	6.729E9	44			
Number of Neutrophiles	Between Groups	9582019.111	8	1197752.389	6.383	.000
	Within Groups	6755587.200	36	187655.200		
	Total	1.634E7	44			
Number of Eosinophiles	Between Groups	20808.000	8	2601.000	.796	.609
	Within Groups	117565.200	36	3265.700		
	Total	138373.200	44			
Number of Basophiles	Between Groups	.000	8	.000	.	.
	Within Groups	.000	36	.000		
	Total	.000	44			
Number of Lymphocytes	Between Groups	1.095E7	8	1368140.450	7.199	.000
	Within Groups	6841670.400	36	190046.400		
	Total	1.779E7	44			
Number of Monocytes	Between Groups	33408.400	8	4176.050	1.588	.163
	Within Groups	94676.400	36	2629.900		
	Total	128084.800	44			

Multiple Comparisons
Post Hoc Tests by Duncan test

Number of Red blood cell

Treats	N	Subset for alpha = 0.05	
		1	2
Control 8 hrs.	5	4.9000	
Low 2 hrs.	5	4.9200	
High 8 hrs.	5	4.9860	
High 24 hrs.	5	5.0380	
Control 24 hrs.	5	5.0880	
Low 24 hrs.	5	5.1660	5.1660
Low 8 hrs.	5	5.2500	5.2500
Control 2 hrs.	5	5.3600	5.3600
High 2 hrs.	5		5.5800
Sig.		.055	.066

Number of Haematocrit

Treats	N	Subset for alpha = 0.05	
		1	2
Control 8 hrs.	5	34.4000	
High 8 hrs.	5	34.6000	
Low 24 hrs.	5	35.4000	
High 24 hrs.	5	35.4000	
Control 24 hrs.	5	35.6000	
Low 2 hrs.	5	36.2000	36.2000
Low 8 hrs.	5	36.2000	36.2000
Control 2 hrs.	5	37.4000	37.4000
High 2 hrs.	5		39.0000
Sig.		.060	.062

Number of White blood cell

Treats	N	Subset for alpha = 0.05	
		1	2
Low 2 hrs.	5	3.1800E3	
High 2 hrs.	5	3.3400E3	
High 8 hrs.	5	4.0600E3	
Low 8 hrs.	5	4.7600E3	
Control 2 hrs.	5	4.7800E3	
High 24 hrs.	5	5.0600E3	
Low 24 hrs.	5	6.1000E3	
Control 24 hrs.	5	1.1260E4	
Control 8 hrs.	5		3.1220E4
Sig.		.276	1.000

Number of Neutrophil

Treats	N	Subset for alpha = 0.05				
		1	2	3	4	5
Control 24 hrs.	5	5.4040E2				
Low 2 hrs.	5	6.6740E2	6.6740E2			
Control 2 hrs.	5	9.9820E2	9.9820E2	9.9820E2		
High 24 hrs.	5		1.1554E3	1.1554E3	1.1554E3	
High 8 hrs.	5			1.3418E3	1.3418E3	
Control 8 hrs.	5			1.3996E3	1.3996E3	
High 2 hrs.	5			1.5664E3	1.5664E3	1.5664E3
Low 8 hrs.	5				1.7608E3	1.7608E3
Low 24 hrs.	5					2.0338E3
Sig.		.123	.100	.070	.054	.115

Number of Lymphocytes

Treats	N	Subset for alpha = 0.05				
		1	2	3	4	5
High 2 hrs.	5	2.5194E3				
Low 8 hrs.	5	2.9784E3	2.9784E3			
Low 24 hrs.	5		3.2028E3	3.2028E3		
High 8 hrs.	5		3.3150E3	3.3150E3	3.3150E3	
Control 8 hrs.	5		3.5394E3	3.5394E3	3.5394E3	
High 24 hrs.	5			3.7434E3	3.7434E3	3.7434E3
Control 2 hrs.	5				3.8658E3	3.8658E3
Low 2 hrs.	5				3.9066E3	3.9066E3
Control 24 hrs.	5					4.1922E3
Sig.		.105	.070	.080	.061	.146

VITA

Miss Thanyalak Thakodee was born on January 3, 1983 in Nakhornprathom province, Thailand. She obtained a Bachelor Degree of Science, Department of Biology, Faculty of Science, Prince of Songkhla University, Thailand in 2004. After that continued her studying in Master's Degree of science, Program in Biotechnology at Chulalongkorn University and will graduate in 2009.