EFFECT OF TEMPERATURE ON BIOLOGICAL ACTIVITIES OF BEE VENOM FROM 3 DIFFERENT SPECIES OF HONEY BEE IN THAILAND

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MASTER OF SCIENCE
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Thesis Title	Effect of Temperature on Biological Activities of Bee Venom from 3 Different Species of Honey Bee in Thailand
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ABSTRACT

Bee venom (BV) is one of well-known honeybee products, used as traditional alternative medicine. Due to its protein components such as melittin, BV has been reported to possess pharmaceutical properties including anticancer and antimicrobial activities. The activities of BV would be varied due to different species and temperature. In this study, BV from 3 species of honeybee (*Apis mellifera*, *A. dorsata* and *A. florea*) were investigated for its antibacterial activities against five skin pathogens and anticancer activity against B16F10 melanoma cell. All BV samples were prepared using 10 kDa molecular weight cut-off column to separate complex components according to molecular weight. Prior to determine the effects of temperature on MIC, BV samples were incubated at 40°C, 60°C, 80°C for 5 minutes and unincubated (control). The results indicated that *A. mellifera* BV showed the lowest MIC (6.25-12.5 µg/ml) at 40°C against *Streptococcus pyogenes* and *Staphylococcus aureus* while *A. dorsata* BV showed the lowest MIC (12.5-25 µg/ml) at control and 40°C against *S. aureus*. Meanwhile, BV from *A. florea* was indicated the MIC over 100 µg/ml among temperature levels against all bacteria species.

Further to determine the anticancer activities of BV, BV peptide focused on melittin was generated the interaction simulation with anti-apoptotic protein of cancer cell by computational *in silico* study. We found that melittin have potential to block the activity of anti-apoptotic protein by referring to hydrogen bond and hydrophobic interaction with anti-apoptotic protein together with docking energy score. Additionally, BV samples were tested for inhibitory concentration (IC) against melanoma cells and AO/PI fluorescence staining. The result indicated that BV from each species were presented different inhibitory concentration causing 20 percent of death cell (IC_{20}) and different stages of cell apoptosis could not be observed but secondary necrosis which cells completely dead could be observed for all BV samples. Moreover, samples at IC_{20} were BV incubated at 40°C, 60°C, 80°C for 5 minutes and unincubated (control) and tested for cell cytotoxicity. BV from all species had an activity to decrease cell viability of melanoma compared to control after incubated at 40 to 80°C. BV incubated at 60°C from *A. mellifera*, at 40 and 60°C from *A. dorsata* and 40°C to 80°C from *A. florea* showed the lowest cell viability. In conclusion, each species demonstrated different activities on gram-positive bacteria skin pathogens and melanoma cells at different temperatures. Therefore, BV have shown potential biological activities which could be used as alternative component aiming for anticancer and antimicrobial properties.

Keywords: Bee Venom Activity, Cell Viability, Minimum Inhibitory Concentration

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CHAPTER 1

INTRODUCTION

1.1 Background and Importance of the Research

Honeybee (HB) products have been used in ancient Egypt, Greece and China since thousands of years ago. Bee venom (BV), one of the interesting HB products, has been applied for various health applications known as apitherapy (An et al., 2014).

BV, a scentless and colorless liquid with pH 4.5 to 5.5, is produced by female worker bees to defend their colonies against intruders (An et al., 2014; Wehbe et al., 2019). BV mainly consists of water and dry weight of BV consists of peptides, enzymes, amino acids and volatile compounds. The main bioactive compounds in BV are consist of melittin (40-60%), phospholipase A_2 (12-15%) and hyaluronidase (2-4%) (Chen et al., 2016). Due to its corresponding bioactive compounds, BV have been applied for many pharmaceutical properties such as anticancer (Oršolić, 2012) with various types for example lung cancer (Zhang & Chen, 2017), breast cancer (Duffy et al., 2020) and also skin cancer (Han et al., 2022). Moreover, researchers are exploring the potential of BV in cancer treatment. Computational methods, like *in silico* molecular docking, simulate the interaction between BV and proteins in cancer cells, providing insights into its potential effects on the cancer cell cycle. Laboratory experiments, such as the MTT assay, further evaluate BV's impact by assessing its effect on cell viability.

The previous researches have been reported about anti-inflammatory (Lee et al., 2014) and antioxidant (Somwongin et al., 2018). It is also reported to have many antimicrobial activities for example anti multi-drug resistance bacteria (MDRB) (Fadl, 2018). Beside that, BV is reported as anti-acne (Lee et al., 2014) and skin infection (Sangboonruang et al., 2021).

Therefore, cosmetic industries and dermatologist are interested in BV because of their activity against skin diseases for example atopic dermatitis, acne, vitiligo and facial wrinkles (An et al., 2018; Han et al., 2013, 2015; Jeon et al., 2007).

The biological activities of BV are quite varied due to many factors such as biological and physical factors which affect peptide structure and biological activity of BV. The biological factor was related to the various species of honeybee. It has been reported that different species of honeybee have a different sequence of protein in their venom especially melittin which resulted in different of biological properties for example antibacterial activities against gram-positive and gram-negative bacteria (Maitip et al., 2021) and antioxidant activities (Somwongin et al., 2018). In Thailand, there are 5 species of honeybee genus *Apis* including *Apis mellifera*, *A. dorsata*, *A. florea*, *A. cerana* and *A. andreniformis* (Buawangpong & Burgett, 2019). *A. mellifera* and *A. cerana* are cavity nesting bee which harbor its colony inside of the cavity while *A. dorsata*, *A. florea* and *A. andreniformis* are open-air nesting which its colony expose to the environment.

Moreover, the physical factors such as temperature have been known for changing in the secondary structure of protein components due to protein degradation. It is interesting to note that temperature could alter melittin secondary structure peptide folding and affect to protein-lipid interaction (Klocek et al., 2009). Thus, the antimicrobial activity could be affected by these changes of protein structure.

In this study, BV from 3 species of honeybee (*A. mellifera*, *A. dorsata* and *A. florea*) will be used to examine the effects of honeybee species and temperature on antibacterial and anticancer activities.

1.2 Research Objectives

1.2.1 To examine the effects of BV from 3 species of honeybee in Thailand on the antibacterial and anticancer activities.

1.2.2 To examine the effects of temperature on the antibacterial and anticancer activities of BV from 3 species of honeybee in Thailand.

1.3 Scope of Research

BV (An et al., 2014) which obtained from honeybee genus *Apis* including *Apis mellifera*, *A. dorsata* and *A. florea*. BV were collected by electricity collector for cavity nest species, *A. mellifera* and reservoir dissection species, *A. dorsata* and *A. florea*. Due to adverse effect of BV which is allergic reaction (Möbs et al., 2019), The crude BV were separated according to molecular weight to remove allergens by using 10 kDa protein concentrator then upper part (>10 kDa) and lower part (LMW, <10 kDa) were obtained. The crude BV and LMW part were measured total protein concentration and performed SDS-PAGE to determine the protein concentration and component of LMW part compared to control. The LMW part was examined the effect temperature on antibacterial activities by incubated under different temperature conditions (40°C-80°C) and control (unincubated). After incubation, LMW BV samples were tested for antibacterial activities using resazurin-based assay. Moreover, the anticancer activities of BV were predicted using computational *in silico* molecular docking against anti-apoptotic protein in cancer cell. After that, all LMW BV samples were determined the inhibitory concentration (IC) to obtained the appropriate concentration to perform Acridine orange (AO)/ Propidium iodide (PI) fluorescence staining for observing cell morphology after treat with BV samples. Additionally, the effect of temperature on anticancer activities by incubated under the same conditions as mentioned in antibacterial activities part. Finally, the statistical analysis was performed by using SPSS software. The significant different of cytotoxicity of bee venom at different temperature was evaluated in this study.

CHAPTER 2

LITERATURE REVIEW

2.1 Honeybee

Honeybees are the insect in phylum Arthropoda, order Hymenoptera, family Apidae, genus *Apis*. Honeybees in genus *Apis* are considered as one of economic insect due to their role as pollinators for agricultural crops and economic plants to fight with global food security from global warming and climate changes (Khalifa et al., 2021). Moreover, honeybees are economic resources producing variety of products including honey, pollen, propolis and BV.

2.1.1 Honeybee Species

According to nest habits, there are 2 types of honeybees, cavity and open-air nest (Figure 2.1), the cavity nest is a nest which has only one small entrance. *A. mellifera* and *A. cerana* are the species with cavity nest while *A. dorsata*, *A. florea* and *A. andreniformis* are the species with open-air nest (Baracchi & Tragust, 2017).

A)

Figure 2.1 Bee hives in nature

B)  

Note (A) Cavity nest (B) Open-air nest

Source Taulman (2017), Kellam (2023), Marshall, (2013) and Heaf (2021)

Figure 2.1 (continued)

Each honeybee species has their unique characteristics (Figure 2.2 and Table 2.1) including (Al-Ghamdi et al., 2013; Guerin, 2020).

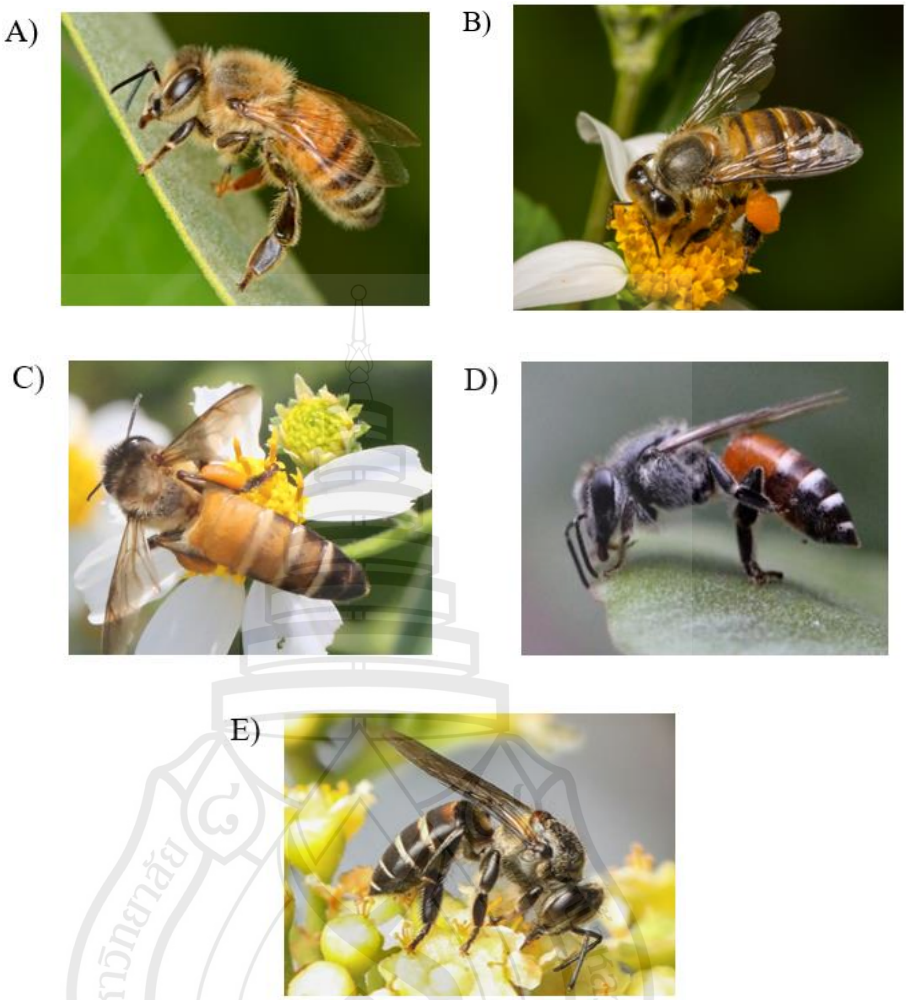
2.1.1.1 *A. mellifera* or European honeybee, the most popular honeybee species which can be found in Europe then spread to other regions.

2.1.1.2 *A. cerana*, the greatest distribution range among Asian honeybee. Their bodies are same size as or smaller than *A. mellifera*. The colonies are form as multiple combs in cavities with small entrance.

2.1.1.3 *A. dorsata* or giant honeybee, the bee bodies are quite large compared to honeybee from other species. They build large single open-air colonies and hang from trees or buildings.

2.1.1.4 *A. florea* or red dwarf honeybee, the small size honeybee with red-brown color compared to other species. They build small open-air colonies on branches and the special behavior is they migrate less than 200 meters from old colonies.

2.1.1.5 *A. andreniformis* or black dwarf honeybee, the characteristic of this species are similar to *A. florea* except black color of back part of bee bodies.



Note (A) *A. mellifera* (B) *A. cerana* (C) *A. dorsata* (D) *A. florea* (E) *A. andreniformis*

Source Rojas-Sandoval (2022), ผึ้งโพรง (*Apis cerana*) (2024), Chen (2016), Habib (n.d.) and Singapore Nature Photography (2021)

Figure 2.2 The honeybee species found in Southeast Asia and Thailand

Table 2.1 The type of nest and species distribution of Asian honeybee species in Thailand

Types of nest	Species	Distribution in Thailand
Cavity nest	*A. mellifera*	All over Thailand
	A. cerana	All over Thailand especially Southern
	A. dorsata	All over Thailand
Open-air nest	*A. florea*	All over Thailand except Southern
	A. andreniformis	Northern and Chanthaburi

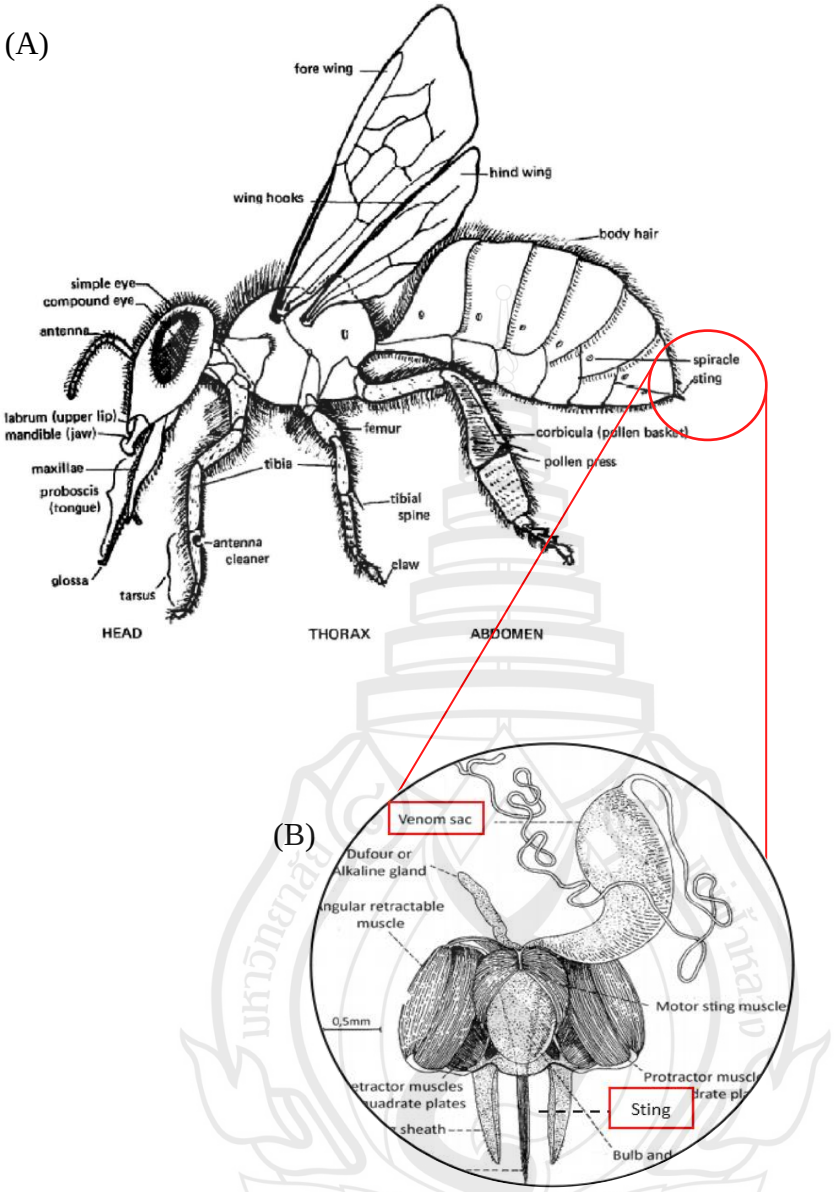
2.1.2 Honeybee Anatomy (Wongsiri et al., 2012)

Honeybee is the invertebrate insect which have the same general characteristic as other common insects. The adult honeybees are separated into 3 parts including head, thorax and abdomen (Figure 2.3A).

2.1.2.1 Head is composed of compound eyes, ocelli, antenna and mouth. The mouth parts are at the bottom of head and the honeybee's mouth part are chewing lapping and sucking. Worker bee can use mouth for bite pollen, pick bee wax to build their nest and have the mandibular gland to produce foraging pheromone.

2.1.2.2 Thorax is separated into 4 segments and composed of legs and wings. The hind legs are the biggest pair and can be collected pollen called pollen basket.

2.1.1.3 Abdomen is the location of wax gland, scent gland or Nasanov's gland and sting. There is the sharp spine at the tip of bee's abdomen, called barb or barb sting, connect with venom sac which used for sting and release venom to predators or intruders (Figure 2.3B).



Note (A) External morphology of honeybee (B) Sting and venom sac

Source Ramachandra et al. (2012) and Ferreira Jr et al. (2012)

Figure 2.3 The morphological characteristic of honeybee

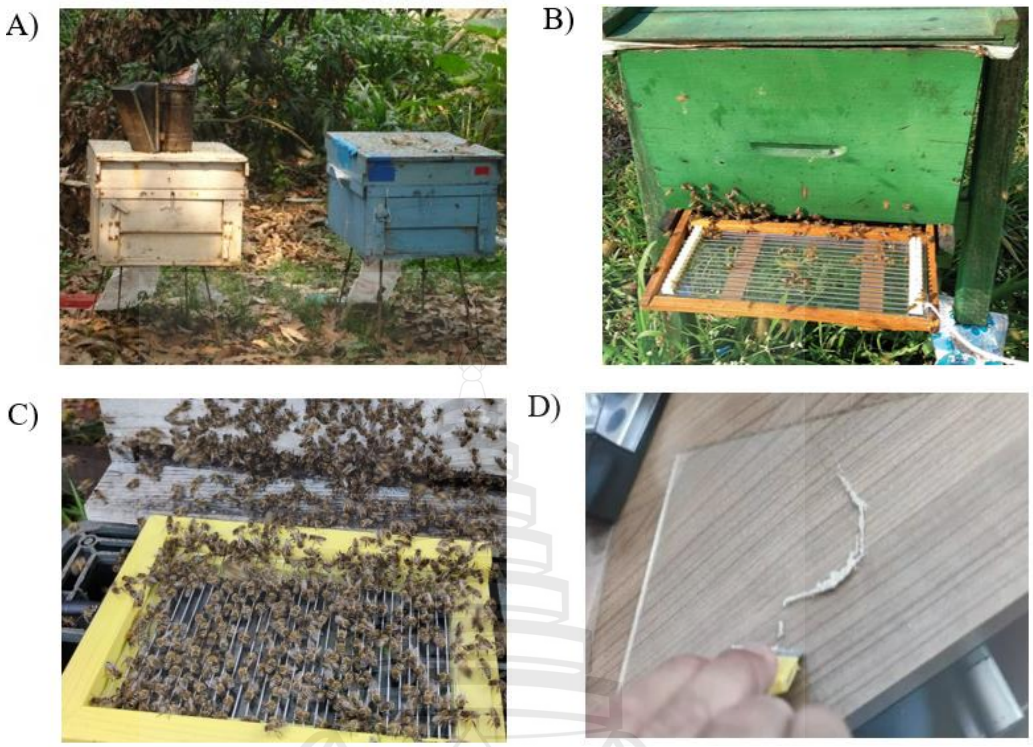
2.2 Bee Venom

BV is produced in venom gland of honeybee which located in the abdomen and reserve in venom sac (Maitip et al., 2021). BV can be obtained from *A. mellifera*, *A. dorsata*, *A. flore*a, *A. cerana* and *A. andreniformis* which can be found in Southeast Asia and Thailand (Guerin, 2020).

2.2.1 Bee Venom Collection

There are two main methods to collect BV from honeybee including electric stimulation (Figure 2.4) for *A. mellifera*. BV from *A. dorsata* and *A. florea* were collected by reservoir dissection.

For electric stimulation, the stimulator was designed to collect venom from cavity bees by not lose their sting and stay alive. The stimulators are better to design the suitable distance between conductor and glass slide which not allow bees to be trapped between them. Moreover, power of electric stimulator and work period should be controlled during the collection to avoid invariable honeybee health degeneration. This method was divided into two ways including external and internal, based on device location. The internal is when the device locate inside the hive, the whole colonies can become stressed and the temperature inside will be increased. However, the external was more suggested way to avoid negative effect on honeybee.



Note (A) Bee boxes (B) External electric stimulation (C) Bees sting on stimulator device (D) BV powder

Source Oliveira Orsi et al. (2024) and SMART-BEE (n.d.)

Figure 2.4 Bee hives and BV collection for bee keeper in Thailand

Normally, bee hives or bee boxes have the area in front of the entrance to put the external electric stimulator. The cavity nest has small entrance that makes it easier to control BV collection by electric stimulator (Figure 2.4B, 2.4C).

The other collection method is reservoir dissection. This method is designed for laboratory scale which suitable for all honeybee species. In order to collect venom, venom sac will be dissected under the dissection microscope then the venom sac will be pulled by hand using two fine-tipped tweezers from abdomen of honeybee. This is one of the methods to reduce interferences from deposition of venom on glass plate during electric stimulation process.

2.2.2 BV Storage

After collection, all of crude BV can be stored at -20°C with or without lyophilization (Lee et al., 2015). Mahfouz, H. M. et. al. has been reported about antimicrobial activity of BV which store under different condition. The result was indicated that the minimum inhibitory concentration is different depend on species of tested bacteria strain and could be the same or different in different storage condition. The storage condition has less effect on antimicrobial activity than the concentration of BV sample (Mahfouz et al., 2020).

The other research has been studied about the melittin content in BV with different storage condition, room temperature and freezer, in short term storage (1, 2, 3 and 6 months). After storage period, the melittin content was analyzed using RP-HPLC-PDA and compare between each storage condition and time period. The result has shown that for short term storage not more than 6 months in both conditions have no negative effect on melittin content (Flanjak et al., 2021).

2.2.3 Bee Venom Characteristics

2.2.3.1 Components

BV generally consists of water (Wenhua et al., 2006) and the others (12%) which contain complex components. The components are peptide, enzyme and low molecular weight compound. Among the complex component, peptide and enzyme are the majority including melittin (40-60%), phospholipase A_2 (12-15%) and hyaluronidase (2-4%) while the minor components have been reported as apamin, mast cell degranulating (MCD), mineral, amino acid and bioactive compounds (Bellik, 2015) (Table 2.2).

Table 2.2 The major and minor components in crude BV

Class of molecules	**Component**	**Molecular weight (kDa)**	**Amino acid residues**	**Contents (%in dry weight)**
Peptides	Melittin	3	26	40-60
	Apamin	2	18	2-3
	Mast cell degranulating (MCD) peptide	2.5	22	2-3
	Adolapin	11	103	1
Enzymes	Phospholipase A_2	19	128	10-12
	Hyaluronidase	38	350	1.5-2
Amine	Histamine	0.3	-	1.5
	Dopamine	0.18	-	0.13-1
Other molecules		-	-	10-16

2.2.3.2 The biological properties of major components in bee venom

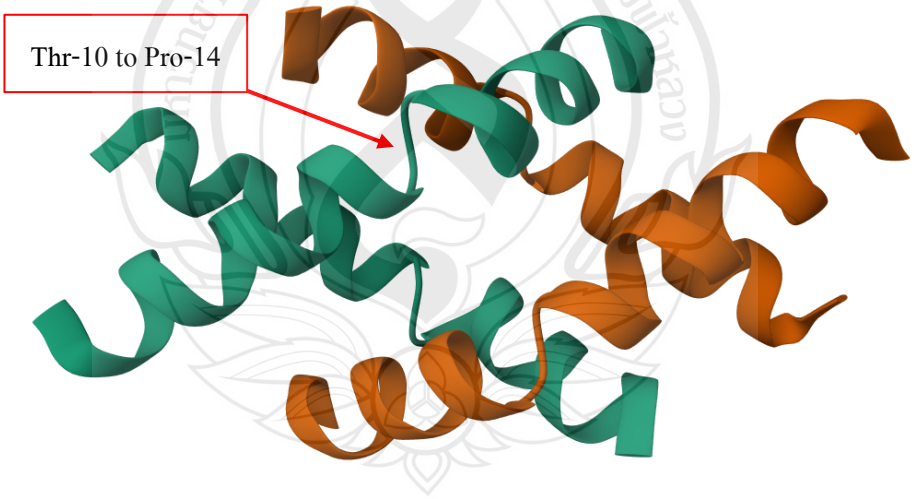
1. Melittin

In BV, melittin is the main peptide component which consist of short peptide composed of 26 amino acid residues. Its molecular weight is about 3 kDa. The amino acid sequences of melittin are different in each species of honeybee (Table 2.3).

Table 2.3 The amino acid sequences of each honeybee species with highlight different amino acid among four species

Habitats	Species	Amino acid sequences
		5 10 14 15 22 25 26
Cavity nest	*A. mellifera*	G I G A V L K V L T T G L P A L I S W I K R K R Q Q
	A. cerana	G I G A V L K V L T T G L P A L I S W I K R K R Q Q
Open-air nest	*A. dorsata*	G I G A I L K V L S T G L P A L I S W I K R K R Q E
	A. florea	G I G A I L K V L A T G L P T L I S W I K N K R K Q

Melittin could be formed as monomeric and tetrameric which both could be soluble in water. The carboxyl-terminal is hydrophilic and has lytic activity, while the amino-terminal is hydrophobic with no lytic activity (Raghuraman & Chattopadhyay, 2007).



Note Arrow refers to the position of threonine and proline

Source Eisenberg et al. (1990)

Figure 2.5 Tetramer structure of melittin in aqueous solution

The three-dimension secondary structure of melittin is two segments of bent rod α-helix according to the presence of proline-14. Proline is a cyclic amino acid which introduces the rigidity and constraints in peptide chain by disruption of the amino acid residues 10-14 resulting in a kink and bend in melittin structure. The bend is important for tetrameric formation by allows closer contact between melittin chains and promote the stabilization of tetramer (Figure 2.5). The tetramer structures of melittin are formed by hydrophobic interaction of non-polar amino acid for example valine, leucine, isoleucine and tryptophan. The stabilization of four α-helix chains of melittin is happened from balancing the inside hydrophobic interaction and electrostatic repulsion of positive charge amino acid in closely perfect symmetry (Terwilliger, 1982).

The tetramer structure of melittin plays a crucial role in its functions including induced their solubility in aqueous solution and enhanced membrane disruption property or lytic activity. The lytic activity or pore formation of melittin is non-specific activity and can happen in both prokaryotic and eukaryotic cells and responsible in anti-microbial, antifungal, antitumor and hemolytic activity (Pino-Angeles & Lazaridis, 2018; Rady et al., 2017; Xu et al., 2020).

2. Phospholipase A_2 (PLA_2)

PLA_2 is the main enzyme of BV in single peptide form with 128 amino acids and molecular weight about 19 kDa. PLA_2 is not harmful when it is pure but, in combination with melittin it could be allergen as inducer of immunoglobulin E (IgE)-mediated anaphylaxis. It works as major allergen in BV and it is an enzyme which can hydrolyzes phospholipid. PLA_2 can be found in mammalian pancreas, reptile venom and insect venom. PLA_2 from BV is the secretory and Ca^{2+} -dependent type (Darwish et al., 2021).

3. Hyaluronidase (HYA)

Hyaluronidase is the enzyme with single polypeptide chain with 350 amino acid residues which acts as spreading factor by damage hyaluronic acid, polysaccharide in connective tissue and extracellular fluid to small oligosaccharides, which can cause changes of the integrity of local tissue and extracellular fluid (Bala et al., 2018). Hyaluronidase will act as spreading factor to separate tissue cell and pave the way for other venom constituents to penetrate into the body. For this reason,

hyaluronidase was used as the medicine to cure wound scar and skin burning (Topchiyeva & Mammadova, 2016).

2.2.4 Allergic Reaction

The BV allergy is one of the members of Hymenoptera venom allergy which cause by insects in order Hymenoptera for example honeybee, wasp and ant. In general, BV components can trigger immune system and lead to allergic reaction (Żabicka & Gomułka, n.d.). There are five classes of Hymenoptera allergy include normal local reaction, large local reaction, systemic anaphylactic reaction, systemic toxic reaction and unusual reaction (Komi et al., 2018). The allergic reaction symptoms can be different in each person and range from mild reaction such as itchy, red burning skin and swelling to severe reaction such as swollen tongue, difficult breathing and loss of consciousness.

The all of symptoms are cause by allergen components in BV such as PLA_2, HA, melittin and apamin (Sobotka et al., 1976). The previous research has been reported that PLA_2 and HA are the major allergens in BV by promote IgE-mediated hypersensitivity in human (Padavattan et al., 2007). Moreover, the re-exposure to BV allergen will activate the production and release bio-mediators in mast cells and basophils to take response for type-1 hypersensitivity allergic reaction (an allergic reaction stimulated by re-exposure to specific allergen).

2.2.5 Effects of Environment Factors on the Stability of Protein

There are many factors which affect to protein structure and also biological activity such as physical and biological factors. There are many previous researches which have been reported about the change of protein structure affect to their biological property for example

Effect of temperature

Temperature is one of the denaturants which affect to protein structure and their biological activities. There are many researches which supports the effect of temperature on protein structure for example Akazawa et. al. has been reported about heat denaturation of each type of antibodies such as IgE and IgG. The result revealed that each type of antibodies was denatured in different temperature for example IgG was denatured at higher than 65°C and lost their antigen-binding activity at 90°C. For

other classes of antibodies, IgA and IgM, were denatured more than 75% at 72°C due to the different domain structure in each antibody (Akazawa-Ogawa et al., 2018).

The other research has been done on enzyme, catalase, horseradish peroxidase and α-chymotrypsin, as a model protein. These three enzymes were heated in solid and aqueous state at 70°C then FTIR spectroscopy, size-exclusion chromatography and biological activity were performed. The result revealed that solid state of protein model after thermal exposure showed no significant different compared to control. In the other hand, aqueous state or protein solution found to have significant different compared to control. From the experiment, can be concluded that the changes of secondary structure were affected to loss of biological activities of protein (Zeeshan et al., 2019).

As the effect of temperature which can make change in protein structure and biological activity also make change on melittin. The previous research has been reported about the effect of temperature on structure of melittin at 30-80°C by NOEs and NMR spectrum together with chemical shifts. From this research, at temperature 30-70°C, tetramer still intact but monomer which assembly into dimer and tetramer have been shown separation about 2 Å and this is the real structural change which confirmed by NMR data (Iwadate et al., 1998). The other research has been reported about binding enthalpy and aggregation of BV peptide on lipid membrane which affect to pore formation and biological activity such as antimicrobial activity (Klocek et al., 2009).

2.2.6 Bee Venom Screening

Apart from pH and temperature, the other way to make change in protein component of BV is protein purification. The crude BV have many components mixed together for example peptide, enzyme, amine and other molecules. To purified crude BV, there are many methods such as molecular weight cut-off ultrafiltration, chromatography column, gel filtration and immunosorbent.

The molecular weight cut-off ultrafiltration has varied pore size depend on which peptide is wanted to remove. The previous research has been reported about PLA_2-free BV by stirred ultrafiltration with 10 kDa molecular weight cut-off membrane to remove allergen in this case is PLA_2 which can cause adverse effect to severe anaphylactic shock in some case. The result has shown that PLA_2-free BV (PBV) can

inhibit factor which led to the production of pro-inflammatory mediator. However, PBV still maintain cell proliferation and UV-protection in human dermal fibroblast cell. The PBV have the efficiency to use as cosmetic ingredient and improve therapeutic profile of purified BV (Lee et al., 2015).

The other novel method to purified melittin from crude BV by cation-exchange chromatography column. The previous research has been studied about influence of pH, types of buffers, salt concentration and types of cation-exchange column. After purification, SDS-PAGE, RP-HPLC, PLA_2 enzyme assay and HYA enzyme assay were performed to verified recovery yield of melittin and removal of PLA_2 and HYA. The result has shown that strong cation-exchange resin with sodium phosphate buffer pH 6.0 was made a recovery yield of melittin up to 93% and removed PLA_2 and HYA around 99% and 96% respectively (Teoh et al., 2017).

The other research has mentioned about hyaluronidase (HYA) purification. HYA was found to be a minor allergen in BV. HYA has been purified from BV, *A. mellifera*, by separated from PLA_2 and other components. The method was proved to be complicated because of many steps to separate HYA from PLA_2. The HYA purification by using gel filtration, ion-exchange chromatography, affinity chromatography and remove PLA_2 by rabbit anti-PLA_2 immunosorbent column. After purification, the product was analyzed by SDS-PAGE to confirm purity of final product. The result has shown that there are less than 0.1% PLA_2 and less than 1.5% acid phosphatase in final product. Purified HYA was found to be sticky when highly pure and low concentration. However, HYA have slightly different molecular weight in different purification method (KEMENY et al., 1984).

2.3 Biological Activities of Bee Venom

BV have been used as traditional medicine or folk medicine for a long time from the past. BV is a complex mixture of active component such as enzyme, peptide and volatile compound which causes BV to have pharmaceutical properties. BV have many pharmaceutical properties for example anticancer, anti-inflammatory, antioxidant and antimicrobial.

2.3.1 Anticancer Activity

For anticancer activity, BV have an efficiency to inhibit many kinds of cancer for example prostate cancer, lung cancer, breast cancer and melanoma by the induction of apoptosis.

BV have anticancer activity against lung cancer and breast cancer, the most common cancer type in the world (Jung et al., 2018), through the activation of death receptor (DRs) and the inhibition of nuclear factor kappa B (NF-κB). The DRs activation was performed by the interaction between DR and thier ligand. After that, caspasses and Bax (pro-apoptotic protein) will be stimulated while the reduction of Bcl-2 (anti-apoptotic protein) which lead to apoptosis of cancer cell. The activation of DRs can induce cancer cell apoptosis, especially chemoresistant cancer cell (K. E. Choi et al., 2014).

The other research has been mentioned about BV on the expression of Bax, p53 and Bcl_2. The result has shown that the synergistic activity between melittin and PLA_2 have the positive result on gene expression of Bax and p53, also decrease Bcl_2 expression in breast and liver cancer cell (MCF7 and HepG2) (El Sharkawi et al., 2015).

One of the interesting anticancer of BV is the activity against human melanoma A2058 cells. The result of previous research has been mentioned that there are 4 main pathways including ROS generation, AIF and EndoG translocation, interference of MAPKs kinases family and calcium -dependent apoptosis in human melanoma cells. The ROS has involved in BV apoptotic process. BV treatment was increased the activities of caspase-2 and -3 with AIF and EndoG translocated from mitochondria to cytosol responsible for caspase-independent apoptosis pathway. BV can be interfered with MAPKs kinases family such as JNK and ERK. For examine effect of JNK and ERK in BV apoptisis process by pretreat A2058 human melanoma with JNK and ERK inhibitor. The result has shown that cell death retio reduce to 40% compared to control with BV only which cell death ratio equal to 60%. However, when pretreat with ERK inhibitor cell death ratio was accelerated after treat with BV. The result inferred that JNK might cooperate with BV-induced cell apoptosis while the activation of ERK will protect cell from BV. The other pathway is BV induce calcium cytosolic in A2058 cell. From the result indicated that BV-induced A2058 cell apoptosis by a calcium-dependent pathway (Tu et al., 2008).

2.3.2 Antimicrobial Activity

The antimicrobial activity is one of the interesting biological activities of BV. Nowadays, there are 700,000 people who death from drug-resistance pathogens and will be increased to 10 million within 2050. Therefore, modern medicine was more interested in organic extract from plant and animal which have antimicrobial activity instead of antibiotics drug. The previous researches have been reported about antimicrobial activity through 3 main process including conformational changes, inter-peptide cooperation and membrane disturbance. The conformational changes of melittin structure from random coil to α-helix (Therrien et al., 2016) and form amphiphilic structure to facilitate the membrane insertion (Illya & Deserno, 2008). The inter-peptide cooperation is about the cooperation of peptide and embedded peptide to reduce free-energy barrier and the insertion would be easier. The other process of how melittin inserts into cell membrane is membrane disturbance which related to Peptide/Lipid ratio (P/L ratio) and lipid types. The difference of lipid types was effected to area per lipid (APL) and membrane thickness (MT) that affect to melittin action. Under the effects of melittin, the membrane stress is changed significantly and lead to membrane deformation was found to increase with increase of P/L ratio. Furthermore, the interesting actions, lipid extraction is a demonstration of melittin-membrane interaction and occur as peptide penetration into membrane resulting in membrane deformation. Melittin was inserted into membrane by pore formation and this is the key step of bacterial killing by melittin (Hong et al., 2019).

The previous research has been reported that BV have antimicrobial activity against both gram-positive and gram-negative bacteria. The result has indicated that MIC of apitoxin on gram-negative bacteria was higher than gram-positive bacteria (Hegazi et al., 2014). Therefore, gram-negative was found to have more resistant than gram-negative because the outer membrane of gram-negative compose of lipopolysaccharide which interrupt the insertion of peptides and reduce diffusion rate of outer macromolecules through cell membrane (Fennell et al., 1968; Mahfouz et al., 2020; Nader et al., 2021; Picoli et al., 2017).

There are many researches that have been reported about antimicrobial activities of BV in various bacteria species such as foodborne pathogen (Lamas et al., 2020) and also pathogenic fungus (Isidorov et al., 2023). The previous researches have been

mentioned about antimicrobial activities of BV against Methicillin-resistant *Staphylococcus aureus* (MRSA), the main problem in world's health care and community infection, both *in vitro* and *in vivo*. For *in vitro*, BV was used to test antimicrobial activity on 5 *Streptococcus* sp. and 3 *Staphylococcus aureus* (MRSA) strains. The MIC result was reported range between 1.56 and 12.5 µg/ml for *Streptococcus* sp. but for MRSA strains were more susceptible to BV by MIC range from 0.78-3.13 µg/ml. For *in vivo*, the experiment was done on MRSA-infected mice by inject 20 mg/kg BV. There are no significant different in control group and BV treatment group (Choi et al., 2015). The other research has indicated that BV have antibacterial activity against MRSA for *in vitro* study. For *in vivo* study, BV was treated in pneumonia-induced mice model and the result has shown that no abscesses were presented, lung edema and inflammatory were reduced with BV treatment (Kong et al., 2020).

The other interesting bacteria species are skin pathogen and acne-inducing bacteria. The previous research has been demonstrated about antimicrobial activity of BV against acne-inducing bacteria. In this experiment, the different 4 strains of skin pathogen including *P. acnes*, clindamycin-resistance *P. acnes*, *S. epidermidis* and *S. pyogenes* were tested for MIC. The result has shown that MIC values were between 0.086-0.121 µg/ml. Therefore, BV have a potential to inhibited skin pathogen and also inhibited multiple drug resistance strains from acne patients after long-term acne treatment (Han et al., 2010).

BV has become the interesting natural product since their pharmaceutical properties was found. However, most researches have been reported about the properties of BV from European honeybee or *A. mellifera* but the other species in genus *Apis* are not well-known. So, the research trend about the apitoxin is gradually increasing (Han et al., 2015; Şenel, 2019).

2.3.3 Bio-assay/Activity Measurement

2.3.3.1 The colorimetric assay for cell viability and cell cytotoxicity (Aslantürk, 2017).

1. MTT assay is one of the popular assays to detect viability and cytotoxicity of cells by measure the activity of mitochondrial enzyme which reduce MTT to purple water-insoluble formazan precipitate.

2. XTT assay is the colorimetric assay based on the activity of cell to reduce tetrazolium salt XTT to water-soluble orange formazan precipitate. This method is suitable for cell proliferation due to ability to response to growth factors.

3. The other assays for example WST-1, WST-8, LDH and SRB.

2.3.3.2 The common antimicrobial activity assay (Balouiri et al., 2016)

1. Agar disk diffusion is the method for susceptibility testing. The accepted standard values were reported by Clinical and Laboratory Standard Institute (CLSI) for microbe testing. This method was used the disc-contained test compound and put on microbes inoculated agar plate and observed the inhibition zone.

2. Broth dilution is one of the most common methods to examine the minimum inhibitory concentration of tested compound against microorganisms. The experiment was performed on 96 well plate as the tested compounds is diluted in serial dilution and tested on 0.5 McFarland microbial inoculum. Apart from turbidity, there are some colorimetric methods to measure the MIC value for example resazurin and tetrazolium.

3. Flow cytometry is the method which use the fluorescence dye to stain nucleic acid of microbe cells. The flow cytometry can report the viable, injure and dead cells in short period.

2.3.4 Cell Apoptosis Stages (Häcker, 2000; Kamalidehghan et al., 2018)

There are many researches have been studied about fluorescence staining. The aims of performing fluorescence staining assay were observed the cell morphology and separated characteristics of cell live and cell dead. The common staining methods for example Acridine orange (AO)/Propidium iodide (PI), Acridine orange/Ethidium bromide (EB), DAPI (4’, 6-diamido- 2-phenylindole). The different stages of cell apoptosis were mentioned below.

1. Early apoptosis is the early stage of the programmed cell death. The chromatin is condensed and membrane blebs is formed. Bright green with orange nucleus when observed under fluorescence microscope.

2. Blebbing of cell membrane occurs during apoptosis. This stage is involved the formation of membrane bulges known as blebs.

3. Late apoptosis or secondary necrosis is the advanced stage in programmed cell death. The red death cells were observed under fluorescence microscope.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bee Venom Collection

BV was collected from 3 species of honeybee including *A. mellifera* (AM), *A. dorsata* (AD) and *A. florea* (AF) during January in 2019 at Mae Fah Luang University, Thailand. BV from *A. mellifera* (AMBV) was collected by using bee venom collector or electric stimulator while BV from *A. dorsata* (ADBV) and *A. florea* (AFBV) were collected by reservoir dissection (de Graaf et al., 2021). Briefly, BV sac attached to a stinger from a worker's abdomen was dissected then transferred and mashed on a petri dish which placed on ice bath. All crude BV was scraped off and pooled into 1.5 ml microcentrifuge tube. BV solution was prepared by dissolving 100 mg BV with 1 ml sterile distilled water and was filtered through a 0.22 µm and BV solution was kept at -20°C until use.

3.2 Bee Venom Preparation

3.2.1 Molecular Weight Cut-off

AMBV, ADBV and AFBV solutions (100 mg/ml) were separated complex components according to molecular weight by using 10 kDa protein concentrator (Amicon Ultra-0.5 mL, Millipore®, Merck Ltd.) and centrifuged at 13000 rpm for 30 minutes. Then, the elute (low molecular weight part, LMW) part was collected and filtered by a 0.22 µm syringe. LMW part of AMBV, ADBV and AFBV were kept at -20°C.

3.2.2 Total Protein Measurement

Total protein of crude and LMW part of AMBV, ADBV and AFBV (<10 kDa) were measured using Bradford assay kit (Bio-Rad). A mixture of samples which consist of 5 µl of crude and LMW BV solutions and 250 µl 1X dye reagent was added into 96-well plates then incubated at room temperature for 5 minutes. After that, the absorbance at 595 nm wavelength was measured. The concentration of protein was calculated by using a standard curve of BSA standard concentration range from 0.125-1.5 mg/ml.

3.2.3 Bee Venom Protein Component Analysis

After molecular weight cut-off, BV samples which compose of crude AMBV and LMW part from 3 species were analyzed protein composition and size by using SDS-PAGE. For briefly method, the gel was casted by using 16% polyacrylamide (30% Acrylamide/Bis-acrylamide solution 19:1 Bio-rad) and the 20 µl of sample was mixed with 4 µl loading dye then heated at 95°C for 10 minutes and spin down. The volume 10 µl of mixture and protein ladder (Precision Plus Protein™ Dual Xtra, Bio-rad) were loaded into SDS-PAGE well and run by using condition 150V for 40 minutes. Inside the tank was used tris-tricine as buffer and outside the tank was used tris-HCl as a running buffer. After that, the gel was stained with 0.1% Coomassie blue R250 for 30 minutes then washed by de-stain solution for 5-6 times or until all stained background was removed then the gel was observed under gel documentation.

3.3 Determination of Antibacterial Activities of BV

Effect of Temperature on Minimum Inhibitory Concentration (MIC) of Bee Venom Using Resazurin-based Assay

The skin pathogens (*Staphylococcus aureus* TISTR 746, *S. epidermidis* TISTR 518, *Streptococcus pyogenes* DMST 17020 and *Propionibacterium acnes* DMST 14916) and gram-negative *Pseudomonas aeruginosa* (TISTR 1287) were used for the determination of the minimum inhibitory concentration (MIC). Briefly (Elshikh et al., 2016), A 100 µl of culture media was added in every well of 96-well plates then 100 µl of BV (incubated at different temperature and unincubated) at different concentrations (0-100 µg/ml) was added and diluted in 2-fold dilution. Besides, the stock culture of

each bacteria species was cultured and adjusted by the 0.5 McFarland standard ($1.5x10^8$ CFU/ml). Each 5 µl of bacteria culture was added into 96-well plates. After 24 hours, 30 µl of 0.015% resazurin was added and observed the color of resazurin (blue, dead) and resorufin (pink, live) in each tested concentration (Jia et al., 2020). The lowest concentration of BV that inhibited microbial growth was the MIC.

3.4 Determination of Anticancer Activities of BV

3.4.1 *In silico* Study of Inhibition of Bee Venom Peptide Against Anti-Apoptotic Protein in Cancer Cell

3.4.1.1 Materials

A peptide sequences of melittin from AMBV, ADBV and AFBV were studied.

3.4.1.2 Molecular docking analyses by HPEPDOCK

The structure of anti-apoptotic protein (AAPs) in Bcl-2 family members including Mcl-1 (2PQK), Bcl-2 (6GL8) and Bfl-1 (5UUP) as receptor in complex with ligand was obtained from Protein Data Bank (PDB). The chain A (ligand) and chain B (receptor) was prepared using BIOVIA Discovery Studio Visualizer (BIOVIA, Dassault Systemes, Discovery Studio 2021 Client), removing water and heteroatoms. After localize bond, 2D structure was performed to use as reference structure or positive control.

After that, the complex structure was trimmed and saved in PDB format then use for analysis in HPEPDOCK server (http://huanglab.phys.hust.edu.cn/hpepdock/). The trimmed chain B was uploaded as receptor into the server and chain A was added as ligand then the binding site of receptor was defined. The result was shown the top 10 model of receptor-ligand interaction which predicted by server and docking score of top 10 model. The docked model was checked for interaction, particularly, hydrogen bond and hydrophobic interaction by using BIOVIA Discovery Studio Visualizer.

For BV sample, the docking process was performed as same as the reference peptide. However, the receptor was used AAPs in Bcl-2 family. The ligands were used

melittin sequences from AMBV, ADBV and AFBV. After that, the result was compared with reference peptide.

3.4.2 Determination of Inhibitory Concentration Using MTT Assay

B16F10 melanoma cell lines were cultured in DMEM media (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C with 5% CO_2. Cell lines were collected by centrifugation at 1500 rpm, 5 minutes, 25°C then pellets were resuspended by DMEM media and cell density (cells/ml) was measured by using a counting chamber. Cell lines ($2x10^3$ cells/well) were seeded on 96-well plates and incubated at 37°C with 5% CO_2. After 24 hours, LMW BV from AM, AD and AF were added with different concentrations (0-30 µg/ml) by 2-fold dilution. (Lim et al., 2019) After 72 hours, BV treatments were removed and the 50 µL of MTT solution (2 mg/mL; EKEAR Bio@Tech) was added to each well, and the cells were incubated for 3 hours and DMSO solubilizing solution was added then incubated for 15 minutes under dark condition. The absorbance of each well was measured at 570 nm wavelength by the Microplate Spectrophotometer (Thermo ScientificTM MultiskanTM GO, Finland). The cell viability was calculated by using following equation.(Kamiloglu et al., 2020) and plotted with log [BV] to find the inhibitory concentration that leads to a 20 percent decrease in cell viability (IC_{20}) as a reference point.

Percentage cell viability = ((Absorbance of sample)/ (Absorbance of control)) x100

3.4.3 Acridine Orange (AO)/Propidium Iodide (PI) Fluorescence Staining

Confirmation of dead cell, B16F10 melanoma cell line was examined apoptosis cells using AO/PI staining and observed under fluorescence microscopy. Briefly, B16F10 ($2.1x10^3$ cells/well) was seeded in 12-well plates and incubated for 24 hours. BV from all species at IC_{20} was added into each well. After 72 hours, cell lines were detached and centrifuged at 200 xg for 5 minutes and cell pellet was resuspended by PBS. The cell solution was stained with AO (100 µg/ml) and PI (100 µg/ml) mixture at a dilution of 1:1. The cell morphology was observed immediately at 100x magnification under fluorescence microscope (Nikon ECLIPSE Ti series) at blue (ex 475 nm/ em 535 nm) and green (ex 525 nm/ em 660 nm) excitation channel.

3.4.4 Effect of Temperature on Cell Cytotoxicity of Bee Venom Using MTT Assay

For the effects of temperature, BV from 3 species at IC_{20} was incubated individually at 40°C, 60°C and 80°C for 5 minutes. After incubation, the BV and unincubated BV (control) were tested for cell cytoxicity on B16F10 melanoma cell line to monitor the effects of temperature on anticancer activity of BV.

3.5 Statistical Analysis

All data on the effects of different temperatures of BV from three species on cell cytotoxicity was performed using IBM SPSS Statistic (version 26) analysis software and data were expressed as the mean ± standard deviation. All data were normal distributed, then were analyzed using two-way analysis of variance (ANOVA) for significant differences. Any significant differences were found, differences among treatments were analyzed using multiple comparisons of means with Tukey's HSD

CHAPTER 4

RESULTS AND DISCUSSION

In this study, BV from 3 species of honeybee which were AMBV, ADBV and AFBV were examined for biological activities. These three BV samples were separated LMW from high molecular weight components by using 10 kDa protein concentrator.

4.1 Bee Venom Samples Protein Components

4.1.1 Total Protein Concentration of Bee Venom Samples

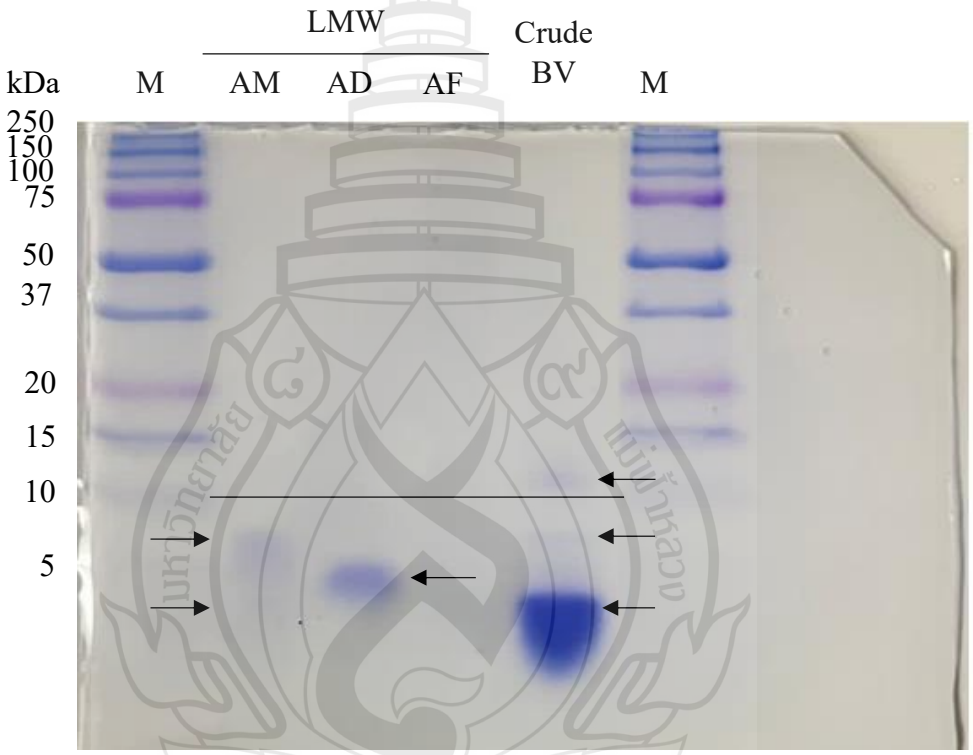
Before any experiments were performed, BV samples were measured the quantity of protein components by using Bradford assay to examine the total protein concentration. The total protein concentration of crude and LMW part of BV from 3 species was shown in Table 4.1.

Table 4.1 The amount of protein in crude and LMW part of BV from 3 species

Species	Protein amount (mg/g sample)	
	Crude	**LMW (<10 kDa)**
A. mellifera	89.0±9.02	4.42±0.044
A. dorsata	104.4±4.64	4.60±0.047
A. florea	89.8±0.66	2.68±0.055

4.1.2 Protein Molecular Weight Analysis

After cut-off, the high molecular weight protein components were excluded around 95-96% when compared to total protein of crude BV before cut-off. Furthermore, protein molecular weight analysis was performed for LMW part of BV (<10 kDa) and crude BV by using SDS-PAGE to analyze the peptide components in BV samples. The result has shown in Figure 4.1.



Note M: Marker, LMW part of BV from AM: *A. mellifera*, AD: *A. dorsata*, AF: *A. florea* and Crude BV: crude BV from *A. mellifera*. Black arrow refers to band of protein component in BV

Figure 4.1 16% SDS-PAGE-Tricine of LMW fraction and crude BV

From Figure 4.1, LMW part and crude BV were analyzed protein components by using 16% polyacrylamide gels which is suitable for separated protein components MW range 1-70 kDa (Schägger & Von Jagow, 1987).

Crude BV from AM was shown three bands on polyacrylamide gel. These bands were expected to be phospholipase A_2 (~19 kDa), Adolapin (~11 kDa) and the most intense band was related to small peptide such as melittin (~3 kDa) which is the main component in BV (40-60%). After molecular weight cut-off, the noticeable target is protein band with size less than 10 kDa.

From SDS-PAGE profile, AMBV revealed two bands in 5-10 kDa molecular weight. In contrast, ADBV displayed a single intense band with a molecular weight below 10 kDa (Figure 4.3). The small size protein from these two samples were expected to be small peptide in BV such as melittin, apamin and mass cell degranulating (MCD). According to the result, the molecular weight cut-off was a preliminary method used for separate the high molecular weight components such as phospholipase A_2. Following fractionation through a 10 kDa molecular weight cut-off column, BV samples may exhibit multiple bands on SDS-PAGE because BV mixtures could contain various components with molecular weights below 10 kDa, allowing them to pass through the column membrane.

4.2 Determination of Antibacterial Activities of BV

Effects of Temperature on Minimum Inhibitory Concentration

From the result, AMBV and ADBV have shown the various MIC values range from 6.25 to 100 µg/ml. For *S. aureus*, the MIC values at 40-80°C equal to 12.5-50 µg/ml among AMBV and ADBV. In *S. epidermidis*, the MIC were increasing from 12.5 to 100 µg/ml followed the increasing temperature for both AMBV and ADBV. In case of *S. pyogenes*, the MIC values were increasing from 6.25 to 25 µg/ml followed by increasing temperature for AMBV. For gram-positive *P. acnes*, the activity of AM was increased in the same direction as temperature increases which is 25 to over 100 µg/ml.

Table 4.2 Minimum inhibitory concentrations (MIC) of BV from three species against four gram-positives of skin pathogen

Microorganisms	Honeybee species	MIC (µg/ml)			
		Control	**40°C**	**60°C**	**80°C**
Staphylococcus aureus	AM	12.5-25	6.25-12.5	12.5-25	25-50
	AD	12.5-25	12.5-25	25-50	25-50
	AF	>100	>100	>100	>100
Staphylococcus epidermidis	AM	12.5-25	12.5-25	12.5-25	25-50
	AD	25-50	25-50	50-100	50-100
	AF	>100	>100	>100	>100
Streptococcus pyogenes	AM	50-100	6.25-12.5	50-100	>100
	AD	>100	>100	>100	>100
	AF	>100	>100	>100	>100
Propionibacterium acnes	AM	50-100	25-50	50-100	>100
	AD	50-100	50-100	>100	>100
	AF	>100	>100	>100	>100
Pseudomonas aeruginosa	AM	>100	>100	>100	>100
	AD	>100	>100	>100	>100
	AF	>100	>100	>100	>100

The MIC values of AMBV were less than ADBV means AMBV has better antibacterial activities than ADBV because the interaction between cationic peptide and anionic bacteria cell membrane needs electrostatic attraction by increasing peptide net charge resulting in stronger peptide binding which could be increased antimicrobial and hemolytic activities (Jiang et al., 2008). Therefore, melittin from AM which carry +5 net charge because of glutamine at position 26th could have better antibacterial activities than melittin from AD with +4 net charge due to negative charge glutamic acid at position 26th.

The MIC values from AF were over 100 μg/ml against all pathogens and temperature levels. The reason could be peptide self-association which prevent peptide penetration to bacterial cell membrane. The optimum hydrophobicity range could be obtained the maximum antimicrobial activity but beyond this optimum point the increasing of hydrophobicity lead to decrease antimicrobial potential of the peptide. The less antimicrobial activity of peptide because more hydrophobicity could affect to more strong dimerization of peptide that would be the obstacle when peptide insert into cell membrane. The self-association of peptide had no effect on hemolytic activity or the accession of peptide to eukaryotic membrane (Chen et al., 2007).

For the gram-negative bacteria, *P. aeruginosa*, the MIC values were found to be over 100 μg/ml among different temperatures and honeybee species. Gram-negative bacteria possess a unique cell envelope structure. The outer membrane, composed primarily of lipopolysaccharides, acts as a selective barrier overlying the peptidoglycan layer. The lipopolysaccharides mimic a lipid bilayer and significantly influences the rate of melittin insertion and folding. The different lipid composition also affected to the kinetics of melittin insertion, the formation of α-helix structure of melittin and melittin-lipid interaction (Constantinescu & Lafleur, 2004). Therefore, gram-negative bacteria have less sensitivity to melittin compared to gram-positive bacteria.

4.3 Determination of Anticancer Activities of BV

In this study, BV samples were prepared by separating LMW from high molecular weight components by using 10 kDa protein concentrator. Additionally, LMW part was tested the anticancer activities before temperature treatment and the effects of temperature on anticancer activities were performed by treating BV samples at different temperature.

4.3.1 *In silico* Study of Inhibition of Bee Venom Peptide Against Anti-Apoptotic Protein in Cancer Cell

Cancer cells have a one common characteristic which is the evasion from cell dead pathway or apoptosis by express high level of AAP and this characteristic is very important to cancer therapeutics because the overexpression of AAPs can cause drug

resistance in patients. Therefore, bioactive compounds able to target AAPs by interact and block the function were interesting for cancer therapy (Shahar & Larisch, 2020).

The *in silico* study is the computational method to predict the interaction or blocking between receptor which is AAP and ligand which is the bioactive compounds. In this study, the Bcl-2 family including Bcl-2, Mcl-1 and Bfl-1 (Qian et al., 2022) were focused because of the responsibility in regulation of the release of mitochondrial apoptotic factors such as cytochrome c (Tsujimoto, 1998). These AAPs were docked with melittin from AM, AD, AF and compared with positive control (reference peptide).

HPEPDOCK web server is the server for predict the protein-peptide binding and generate docking energy scores. The docking energy scores are the relative ranking score for different binding model and can be referred to binding affinity. A lower docking energy score was indicated stronger interaction between protein and peptide which directly proportional to binding affinity (Biswas et al., 2022).

After docking between receptor and ligand, the lower-scoring peptides compared to references peptide (see Table 4.3) were selected including melittin from AM with Mcl-1 and Bcl-2, melittin from AD with all three receptor and melittin from AF with Bcl-2. The docking energy score was related to weak/strong of the interaction. Therefore, these models were paired together to generate the 2D diagram and consider the interaction between ligand and binding site of receptor.

Table 4.3 Docking energy score of melittin and anti-apoptotic protein of cancer cell interaction generated by HPEPDOCK server

References peptide/ Melittin peptide	**Sequences**	**ID**	**Docking energy score**		
			Mcl-1	**Bcl-2**	**Bfl-1**
Selective Bfl-1 peptide	GVREIAYGLRRAADDV NAQVER	5UUP	-251.56	-215.42	-256.75
Bim BH_3	GRPEIWIAQELRRIGDE FNAYYA	2PQK	-249.87	-214.42	-211.92
A. mellifera	GIGAVLKVLTTGLPALI SWIKRKRQQ	P01501	-253.99	-224.06	-203.64
A. dorsata	GIGAILKVLSTGLPALIS WIKRKRQE	P01502	-251.77	-218.99	-229.36
A. florea	GIGAILKVLATGLPTLIS WIKNKRKQ	P01504	-220.90	-233.32	-204.42

There are hydrogen bonds and hydrophobic interaction found in the interaction between AAP (receptor) and melittin from AM, AD and AF (ligand). The hydrogen bonds are divided into two types, conventional and carbon hydrogen bond, in these receptor and ligand interaction. The conventional hydrogen bonds have many types of interaction including N-H-O, O-H-O, S-H-O, S-H-N and the bonds were separated by distance around 3.0 Å. Meanwhile, the carbon hydrogen bonds (C-H-O) were defined as weak hydrogen bond which is weaker than conventional hydrogen bond because the distance of the bonds was approximately 3.4 Å and longer than conventional hydrogen bond around 0.4 Å. Apart from hydrogen bonds, others important interaction was hydrophobic interaction. The hydrophobic interaction was important for interaction, stability, folding of protein and main driving force of receptor-ligand interaction.

Hydrophobic interaction was more influenced to receptor-ligand binding than hydrogen bond (de Freitas & Schapira, 2017).

The interaction between AAP and selected peptides were shown in Figure 4.2 and Table 4.4. Melittin from AM was formed hydrophobic interaction with two key binding sites, Val220 and Met321, of AAP of Mcl-1 while melittin from AD was formed one hydrophobic interaction and one conventional hydrogen bond with that two key binding sites. As mentioned before about the relation of interaction strength and docking score, this can be referred to docking energy of interaction (Table 4.3) between melittin from AM and Mcl-1 (-253.99) which is slightly lower than melittin from AD (-251.77) means the interaction of melittin from AM with Mcl-1 was stronger than melittin from AD. Whereas, melittin from AF was showed the hydrogen bond and hydrophobic interaction with Bcl-2 in both key binding site and non-key binding site. The docking energy score was obviously lower than melittin from AM and AD.

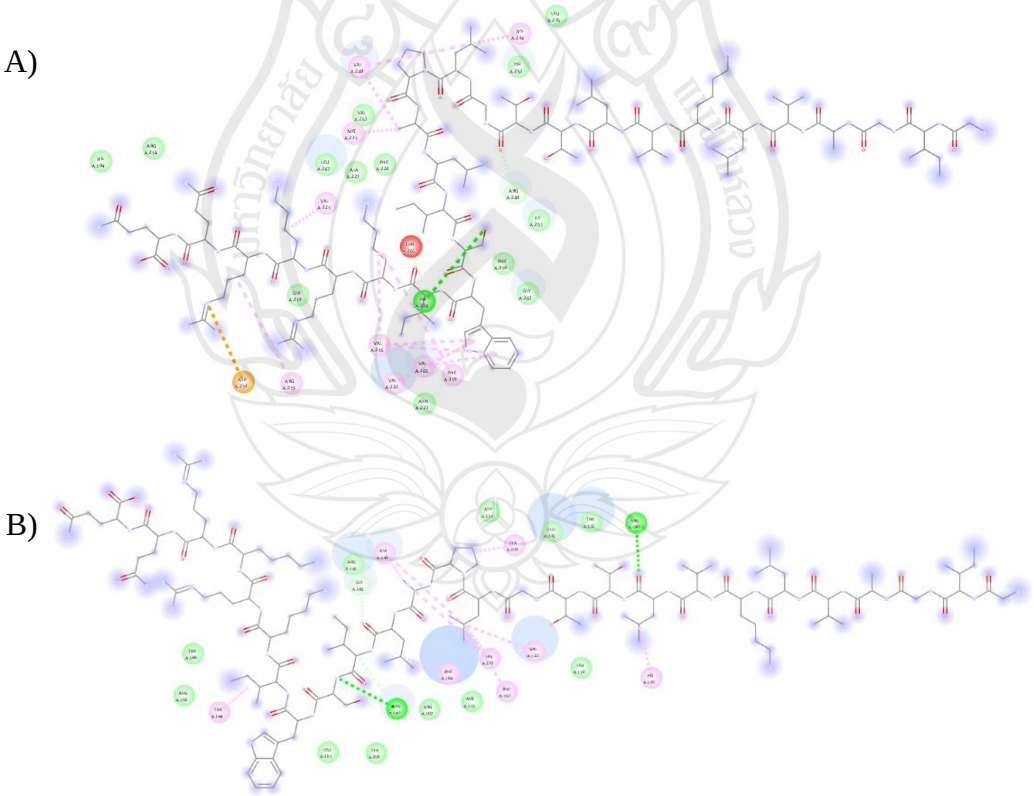
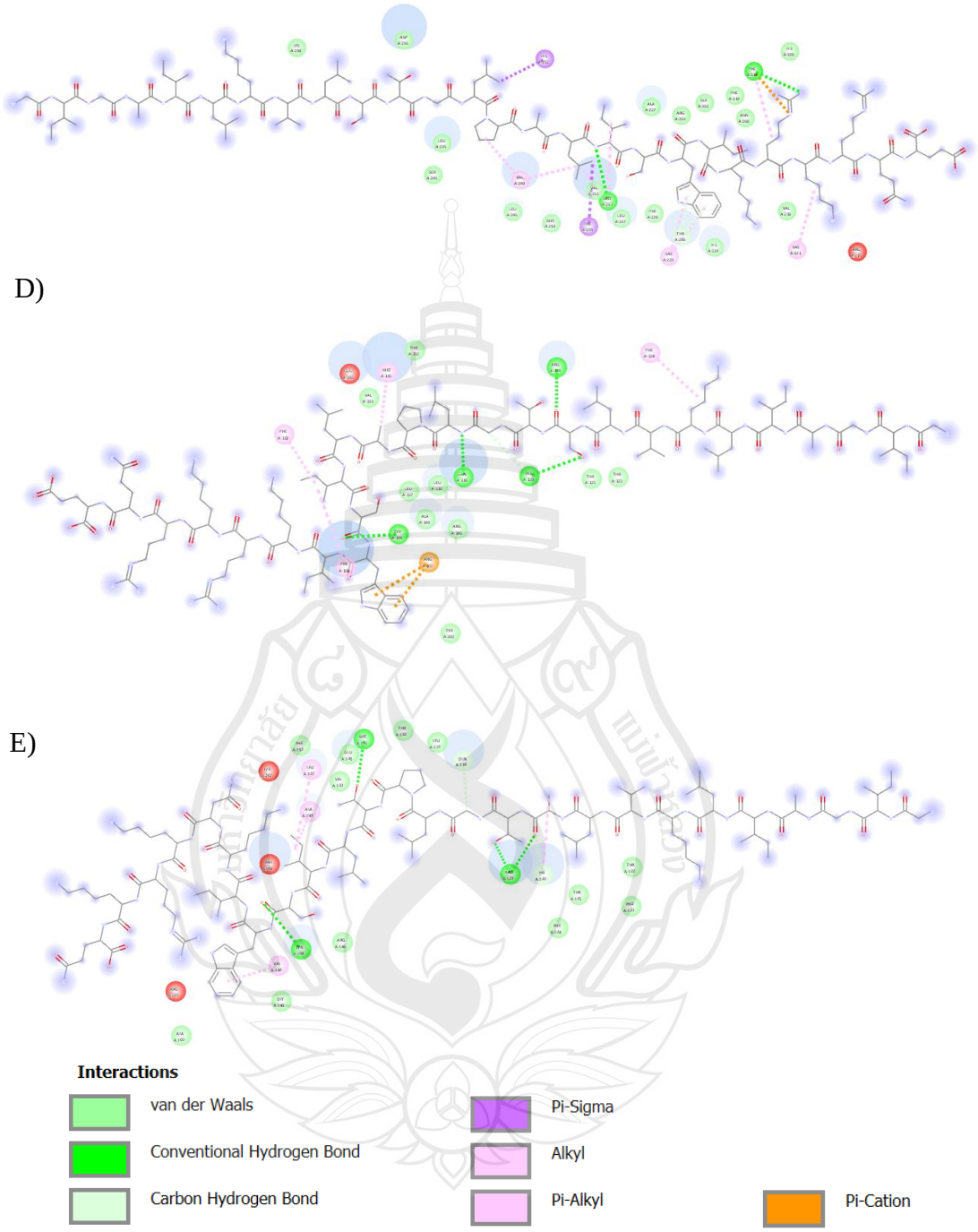


Figure 4.2 2D diagrams of melittin from 3 species of honeybee interact with AAP active site generated by Discovery Studio Visualizer

C)

D)

E)



Note (A) AM with Mcl-1 (B) AM with Bcl-2 (C) AD with Mcl-1 (D) AD with Bcl-2 (E) AF with Bcl-2

Figure 4.2 (continued)

Table 4.4 Interaction of melittin from 3 species of honeybee with anti-apoptotic protein

Anti-apoptotic protein	Amino acid of anti-apoptotic protein	Type of interaction		
		AM	**AD**	**AF**
Mcl-1	Val220*	Hydrophobic	Hydrophobic	-
	Met231*	Hydrophobic	S-H-N	-
	Val321	Hydrophobic	Hydrophobic	-
Total no. of interaction	**Hydrophobic**	**3**	**2**	**-**
	H-bond	**-**	**1**	**-**
Bcl-2	Phe104*	Hydrophobic	Hydrophobic	-
	Tyr108*	Hydrophobic	O-H-O	O-H-O
	Met115*	-	-	S-H-O
	Gln118*	-	N-H-O	C-H-O
	His120	Hydrophobic	O-H-O	C-H-O
	Arg129	N-H-O	N-H-O	N-H-O
	Val148	-	-	Hydrophobic
Total no. of interaction	**Hydrophobic**	**3**	**1**	**1**
	H-bond	**1**	**4**	**5**

Note *amino acid residues are the key binding site of AAP.

4.3.2 Determination of Inhibitory Concentration using MTT assay

The inhibitory concentration was at concentrations of 1.828, 2.128 and 31.623 µg/ml, the cell viability of melanoma cell line reached 80 percent from AMBV, ADBV and AFBV, respectively (Figure 4.4). These concentrations were considered as the concentration caused 20 percent of death cell (IC_{20}). However, our BV concentration could not be reached 50 percent (IC_{50}) due to low the limitation amount of BV samples. Therefore, the inhibitory concentration (IC_{20}) was used as the reference point for AO/PI fluorescence staining and cell cytotoxicity which has been used in the study from Da-Costa-Rocha and Prieto (2021).

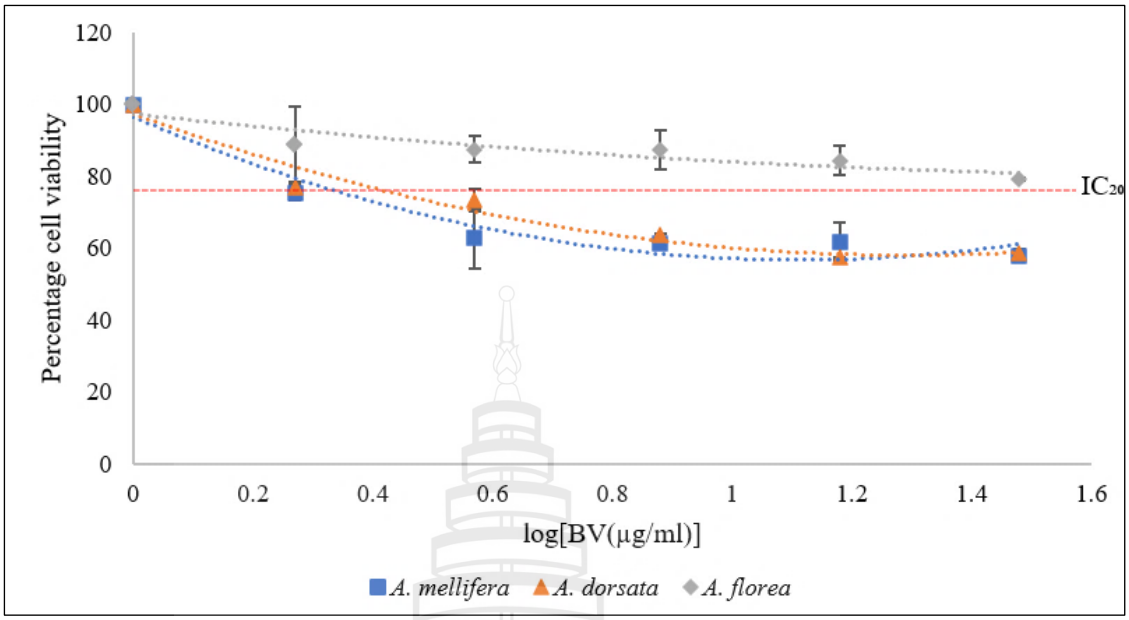
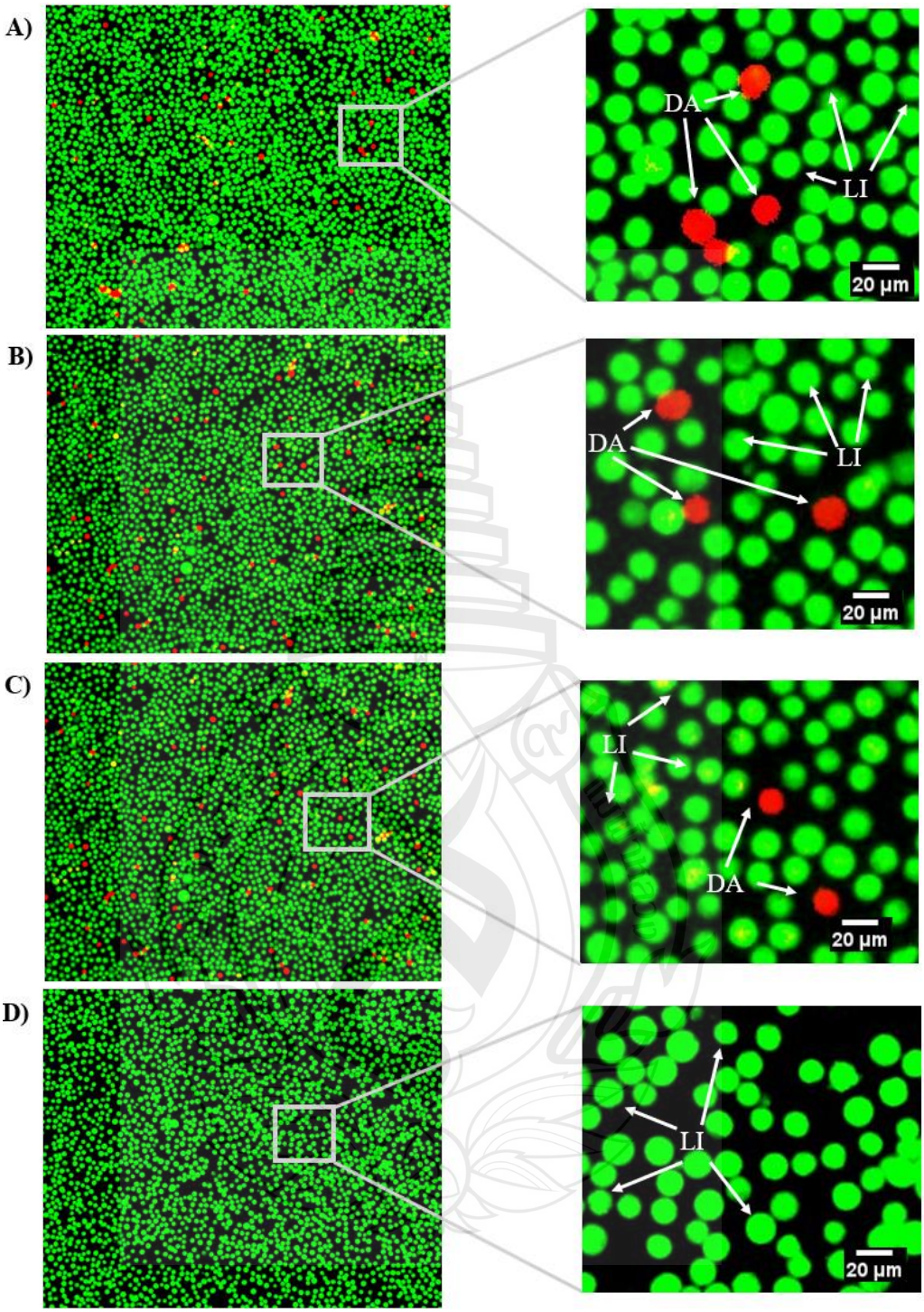


Figure 4.3 Percentage of cell viability (mean ± SD.) of bee venom (BV)-treated B16F10 melanoma cell line by AMBV, ADBV and AFBV

4.3.3 Acridine Orange (AO)/Propidium Iodide (PI) Fluorescence Staining

AMBV, ADBV and AFBV at IC_{20} were used to perform AO/PI fluorescence staining on B16F10 melanoma cell line. AO/PI fluorescence staining was used to confirm the effects of BV on cell viability and apoptotic pathway of melanoma cell line. At 72 hours after incubation, melanoma cell lines were found to have late apoptosis or secondary necrosis cell around 20 percent together with healthy live cell compared to control (untreated) which have only live cell (Figure 4.4).



Note (A) AMBV (B) ADBV (C) AFBV (D) Control (Untreated) at 72 hours. LI: indicated live cell in green color, DA: indicated dead cell in red color.

Figure 4.4 Cell morphological of B16F10 melanoma cell line under fluorescence microscope at 100X magnification after treated with IC_{20} concentrations

A study about the insertion of melittin into cell membrane was reviewed that melittin change the structure from random-coiled to α-helix due to hydrophobic and hydrophilic amino acid organization. After membrane attachment of melittin, lipid extraction was happened and then melittin was formed U-shape which purpose for penetrated into lipid bilayer of cell membrane. Melittin can be formed transmembrane pore and passed through cell membrane in around 2100 nanosecond (Hong et al., 2019). Therefore, the different stages of cell apoptosis for example membrane blebbing or early apoptosis could not observe under fluorescence microscope after 72 hours of incubation period. Our result was differed from previous research that study about the apoptosis of TSGH-8301 bladder cancer cell induced by BV after incubated for 48 hours. The result was shown nuclear condensation and apoptotic bodies formation in TSGH-8301 bladder cancer cell (Ip et al., 2012). Thus, it can be confirmed the activity of BV on apoptotic pathway of cancer cell.

4.3.4 Effect of Temperature on Cell Cytotoxicity of Bee Venom

BV from 3 species at IC_{20} were incubated at different temperature range from 40 to 80°C and tested for cell cytotoxicity using MTT assay.

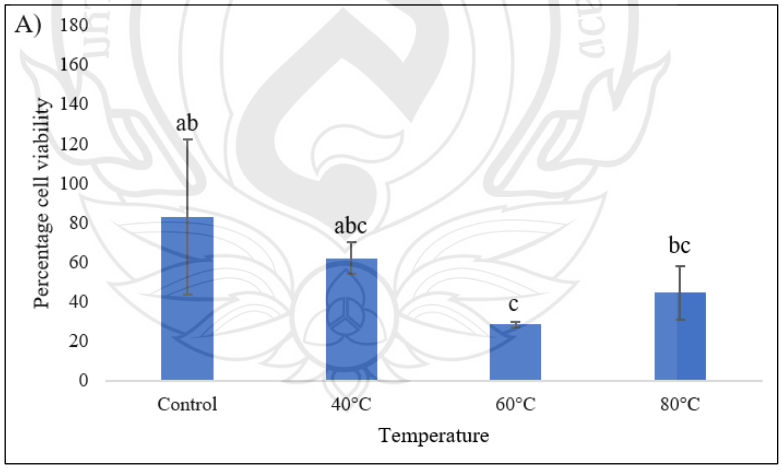
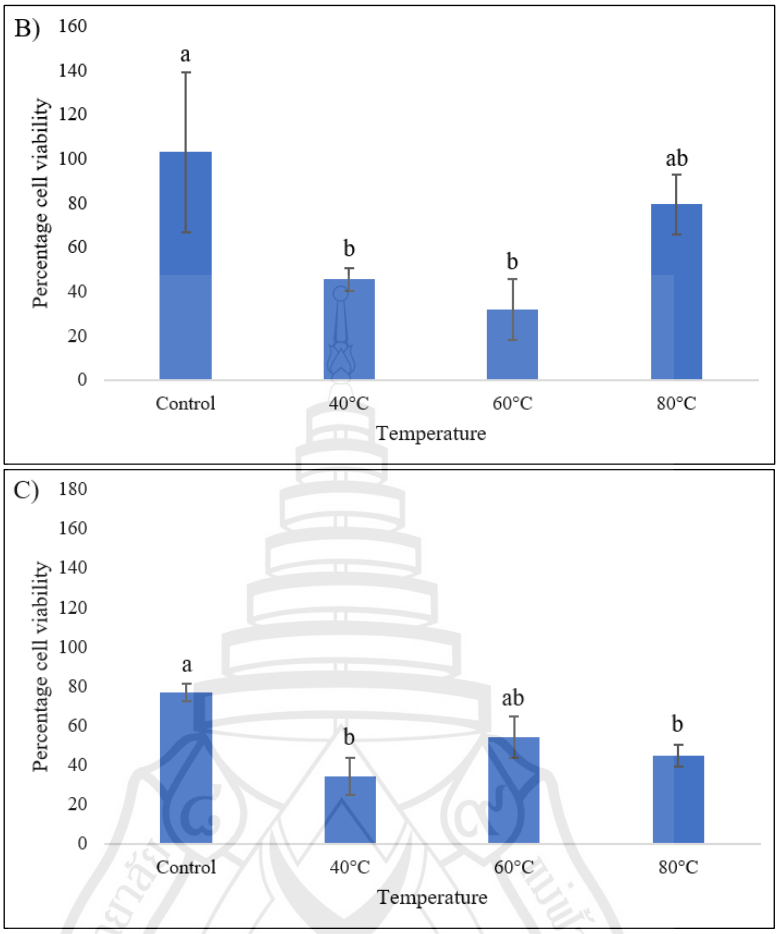


Figure 4.5 Percentage of cell viability (mean ± S.D.) at 72 hours after treated with BV incubated among different temperature on B16F10 melanoma cell line



Note Effect of temperature on (A) AMBV (B) ADBV (C) AFBV. Bars with different letters are significantly different at $p < 0.05$.

Figure 4.5 (continued)

For the effects of temperature, there was no interaction among temperature and honeybee species ($p = 0.081$) which means the effects of temperature and honeybee species should be considering separately. AMBV exhibited the lowest cell viability at 60°C, significantly different from 40°C and 80°C ($p = 0.149$) (Figure 4.5A). This suggests an optimal temperature range for AMBV activity. In contrast, ADBV showed the lowest cell viability at both 40°C and 60°C (Figure 4.5B), indicating a negative effect on viability at these specific temperatures.

The reduction of cell viability at 40°C and 60°C could be occurred due to a better activity of BV than at 80°C according to the study of Wilcox and Eisenberg (1992) which has been mentioned that the tetramer structure of melittin from AMBV

had a stability at 35.5 to 43°C. The mechanism of melittin action was happened in its tetrameric form which essential for its biological activities for example enhancing membrane disruption, exerting enzymatic activity and cell cytotoxicity (Terwilliger, 1982). Tetramer structure also could be promoted the ability to disrupt membrane by the aggregation of melittin chain into tetramer which could increase both peptide concentration at membrane interface and the interaction between peptide to support the activities of each peptide chain (Hong et al., 2019). However, the stability of tetrameric melittin was decrease at higher temperature due to the decrease of enthalpy change (ΔH) and entropy change (ΔS) resulted in weaker interaction such as hydophobic interaction that contribute to the stability and dynamic of tetramerization process. From our result, it is an interesting note that when AMBV and ADBV samples were incubated at 80°C, the percentage of cell viability was increased because of monomer structure starts to separate at temperature above 70°C (Iwadate et al., 1998).

Melittin from AM has threonine at position 10th has an impact on stability of α-helix structure through hydrogen bond forming with backbone carbonyl groups resulting in proper peptide folding in monomer unit and the stability of hydrophobic core in tetrameric formation (Terwilliger, 1982). Moreover, threonine in melittin chain is the polar amino acid with hydroxyl side chain (Figure 4.6). The hydroxyl side chain could form hydrogen bond with other molecules which enhance solubility of melittin in aqueous solution and the higher helical propensity of α-helix structure of melittin lead to higher cytotoxicity and antibacterial activities (Ma, 2015).

										10				14							22				
AM	G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	Q
AD	G	I	G	A	I	L	K	V	L	S	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	E
AF	G	I	G	A	I	L	K	V	L	A	T	G	L	P	T	L	I	S	W	I	K	N	K	R	K	Q

Figure 4.6 Melittin sequences from AMBV, ADBV and AFBV

However, AMBV and ADBV when incubate at different higher temperature was made more stable percentage of cell viability compared to AFBV. The possible reason is amino acid arginine at position 22nd in melittin sequences of AM and AD were different from asparagine from AF melittin in the same position (Figure 4.6). The

positively charge arginine was exhibited the stable charge interaction with negative phosphate group in lipid bilayer and this interaction is crucial for peptide penetration through cell membrane by overcome the energy barrier of hydrophobic core of lipid bilayer. Previous studies have shown that altering a peptide sequence through single amino acid substitutions can significantly enhance its biological functions. Replacing amino acids with one that is more hydrophobic could increase the peptide's interaction with lipid bilayers (Tan et al., 2014) while substituting with more aromatic amino acids could promote cyclic peptide formation, thereby improving the peptide's ability to penetrate cell membranes (Wessolowski et al., 2004).

Moreover, arginine still could be interacted with lipid phosphate at higher temperature because of arginine side chain can form a unique high-temperature peptide strand which is independent of temperature or membrane composition, resulting in stable interaction even in high temperature condition (Su et al., 2009).

Furthermore, melittin from BV among all species were stated the biological activities due to bend rod of melittin chain. The amino acid residue, proline, at position 14^{th} (Figure 4.6) which carried cyclic side chain connect with backbone twice and cause the kink in melittin chain between residues 10^{th} to 14^{th}. This kink was necessary for closer packing and stability of tetramer forming lead to enhancing of membrane disruption (Terwilliger, 1982).

In conclusion, there are many critical factors which could be affected to biological activities of BV including amino acid sequence, charge, structure, hydrophobicity and external environment such as temperature.

CHAPTER 5

CONCLUSION

This study was examined the effects of different temperature on the antibacterial and anticancer activities of BV from AM, AD and AF. The experiment starts from BV collection and preparation, crude BV solution was separated the complex components using 10 kDa molecular weight cut-off column. The amount of LMW part equal to 4.42±0.044, 4.60±0.047 and 2.68±0.055 mg per g sample for AMBV, ADBV and AFBV, respectively.

The LMW part of BV samples were incubated at different temperature (40-80°C) for 5 minutes and tested for the effects of temperature on antibacterial activities compared to control or unincubated. The results indicated that AMBV showed the lowest MIC (6.25-12.5 µg/ml) at 40°C against *Streptococcus pyogenes* and *Staphylococcus aureus* while ADBV showed the lowest MIC (12.5-25 µg/ml) at control and 40°C against *S. aureus*. Meanwhile, AFBV was indicated the MIC over 100 µg/ml among temperature levels against all bacteria species. From the result, the MIC values were different due to different temperature, BV from different honeybee species and pathogens.

Before the effects of temperature on anticancer activities, the computational method was performed for observing the interaction between bee venom peptide, specifically melittin from different species of honeybees, and anti-apoptotic proteins involved in cancer cells, particularly the Bcl-2 family proteins (Bcl-2, Mcl-1, Bfl-1). The docking energy score together with interaction at key amino acid were shown a strong interaction between melittin especially melittin from AMBV and the anti-apoptotic proteins. The LMW part of BV samples were examined the anticancer activities by determining the inhibitory concentration (IC) and AO/PI fluorescence staining to confirm the influence of BV samples on cell live/dead.

BV from 3 species were found to have the effects on B16F10 melanoma cell line calculated as 20 percent of cell death (IC_{20}) and each BV samples were shown the different IC_{20}. The IC_{20} was used for AO/PI fluorescence staining

After 72 hours incubation period, we could not observe the different stages of cell apoptosis. Even though, this staining could be confirmed the effect of BV on B16F19 cell viability. For the effects of temperature on anticancer activities, the LMW part of BV samples were incubated at different temperature (40-80°C) for 5 minutes and tested for cell viability compared to control or unincubated. The results was reviewed that the the percentage of cell viability was decrease at 40°C and 60°C then slightly increase at 80°C compared to control which mean the activity of BV was increase when incubated at 40°C and 60°C then decrease at 80°C.

In conclusion, the biological activities of BV could be varied, depending on the amino acid sequences, tetramer formation and stability of their peptide structure at different temperatures. This study was provided evidence for the potential of pharmacological or biological activities including anticancer and antimicrobial activities, relevant to skin conditions. BV demonstrates promise for the development of alternative therapeutics for illness caused by cancer and pathogenic bacteria especially skin diseases.

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APPENDICES

APPENDIX A

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

A.1 Buffer and solution

1. 1.5M Tris pH 8.8 (Separating gel buffer)

Tris	9.0855 g
Distilled water	30 ml

Adjust pH to 8.8 with HCl

Adjust volume to 50 ml, store at 4°C

2. 0.5M Tris pH 6.8 (Stacking gel buffer)

Tris	3.0285 g
Distilled water	30 ml

Adjust pH to 6.8 with HCl

Adjust volume to 50 ml, store at 4°C

3. 1M Tris, 1M Tricine, 1%SDS pH 8.25 (10X Cathode buffer)

Tris	60.57 g
Tricine	89.585 g
SDS	5 g
Distilled water	500 ml

Do not adjust pH

Dilute to 1X by distilled water before use

4. 2M Tris-HCl pH 8.9 (10X Running buffer)

Tris	121.14 g
Distilled water	400 ml

Adjust pH to 8.9 with HCl

Adjust volume to 500 ml

5. 0.2M EDTA

EDTA	2.92 g
Distilled water	50 ml
Adjust pH to 8 and heat at 56°C until dissolved	

6. 3.3% Stacking gel

Acrylamide-Bis	2.5 ml
Stacking gel buffer	1.9 ml
Distilled water	10.3 ml
0.2M EDTA	150 µl
10% APS	150 µl
TEMED	7.5 µl

7. 16% Separating gel

Acrylamide-Bis	10 ml
Separating/spacer gel buffer	10 ml
Distilled water	6.7 ml
Glycerol	3.2 ml
10% APS	100 µl
TEMED	10 µl

A.2 SDS-PAGE gel casting and running

1. Assemble the glass plate with casting frame and casting stand follow the instructions and test the leakage by pouring distilled water.

2. Prepare 16% separating gel (Appendix B.1, 7.) and pour into glass plate (3-4 cm) then overlaid top surface with distilled water. Leave at room temperature until set (around 30 minutes).

3. Discard distilled water on top surface and replace with 3.3% stacking gel (Appendix B.1, 6.) pour into glass plate (1-2 cm) and leave at room temperature until set.

4. Assemble the glass plate with electrode chamber and pour tris-tricine (Appendix B.1, 3.) into electrode chamber and tested the leakage.5. Put electrode chamber into gel tank (Mini-PROTEAN Tetra Cell) and pour tris-HCl (Appendix B.1, 4.) into tank.

6. Load 10 µl protein ladder and samples into each well. Close lid and connect with power supply and run 150V for 40 minutes.

APPENDIX B

PERFORMANCE STANDARDS FOR ANTIMICROBIAL SUSCEPTIBILITY TESTING

Table B1 Susceptibility testing of tested bacteria strains compared with CLSI (Clinical and Laboratory Standards Institute) 27th edition

Antibiotics (Positive control)	**Bacteria strain**	**MIC from experiment (µg/ml)**	**MIC from standard (µg/ml)**
Gentamicin	*Staphylococcus aureus*	≤ 2	≤ 4
	Staphylococcus epidermidis	≤ 0.125	
	Pseudomonas aeruginosa	2-4	
Vancomycin	*Streptococcus pyogenes*	1-2	≤ 1
	Propionibacterium acnes	4-8	≥ 4

CURRICULUM VITAE

CURRICULUM VITAE

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