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Effects of temperature on the biological activities of bee venom from three honeybee species in Thailand

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ABSTRACT: Bee venom (BV) samples collected from three honeybee species (*Apis mellifera*, *Apis dorsata*, and *Apis florea*) were investigated for its anticancer activity against B16F10 melanoma cells and antimicrobial activity against five skin pathogens. Crude BV samples were prepared, and the low molecular weight BV were obtained by removing proteins with molecular weight larger than 10 kDa using the cut-off column. The BV samples were then evaluated for inhibitory concentration (IC) and acridine orange/propidium iodide fluorescence staining against melanoma cells. At IC_{20}, only the secondary necrosis stage of cancer cell apoptosis was observed. BV samples were incubated at 40, 60, and 80 °C for 5 min, and then the anticancer and antimicrobial activities were determined. The lowest cell viability was observed from *A. mellifera* BV incubated at 60 °C, *A. dorsata* BV incubated at 40 and 60 °C, and *A. florea* BV incubated at 40 and 80 °C. The minimum inhibitory concentration (MIC) results showed that BV from *A. mellifera* incubated at 40 °C demonstrated the lowest MIC (6.25–12.5 µg/ml) against *Streptococcus pyogenes* and *Staphylococcus aureus* while BV from *A. dorsata* incubated at 40 °C and control exhibited the lowest MIC (12.5–25 µg/ml) against *S. aureus*. Meanwhile, BV collected from *A. florea* possessed the MIC over 100 µg/ml among the treatments. In conclusion, incubation of the BV from each honeybee species at different temperatures led to different activities on melanoma cell lines and gram-positive bacteria, causing skin infection.

KEYWORDS: bee venom activity, cell viability, minimum inhibitory concentration

INTRODUCTION

Honeybee products have been used in ancient Egypt, Greece, and China for thousands of years. BV is an interesting honeybee product used in traditional medicine, also known as apitherapy [1].

BV, a scentless and colorless liquid with a pH of 4.5–5.5 [1], is produced by female worker bees to defend their colonies against intruders [2]. BV mainly consists of water, peptides, enzymes, amino acids, and volatile compounds, with its main bioactive compounds being melittin (40–60%), phospholipase A_2 (12–15%), and hyaluronidase (2–4%) [3]. Due to its bioactive compounds, BV has been applied in many pharmaceutical applications, not only because of its anticancer properties [4] against lung cancer [5], breast cancer [6], and skin cancer [7] but also for its anti-inflammatory [8] and antioxidant properties [9]. It is also reported to have many antimicrobial activities, including anti-multi-drug-resistant bacteria (MDRB) [10] and anti-acne [8] and to treat skin infections [11]. Therefore, the cosmetic industry and dermatologists are interested in BV because of its activity against skin diseases such as atopic dermatitis, acne, vitiligo, and facial wrinkles [12–15].

The biological activities of BV are affected by the biological and physical factors that affect the peptide structure and biological activity of BV [16]. Thus, the biological factors vary according to the different species of honeybees [17]. It has been reported that various species of honeybees have different sequences of peptides in their venom, especially melittin, which results in diverse antibacterial activities against gram-positive and gram-negative bacteria [17] as well as antioxidant activities [9]. In Thailand, there are at least 5 *Apis* honeybee species, including *A. mellifera*, *A. dorsata*, *A. cerana*, *A. florea*, and *A. andreniformis* [18]. *A. mellifera* and *A. cerana* are cavity-nesting bees that harbor their colonies inside the cavity, while *A. dorsata*, *A. florea*, and *A. andreniformis* are open-air nesting bees with colonies exposed to the environment.

Moreover, physical factors such as temperature have been known to change the secondary structure of protein components due to protein degradation. The temperature could alter melittin secondary structure peptide folding and affect the protein-lipid interaction [16]. Thus, antimicrobial activity could be affected by such changes in protein structure. Furthermore, the allozymes of cytosolic malate dehydrogenase (cMHD) across a range of *Apis* demonstrate differing degrees of thermostability [19]. More than 90% of Thai *A. mellifera* were identified as either homozygous for the slow or heterozygous for the fast allele, which exhibits more resistance to heat denaturation and enhances enzymatic activity at high temperatures [20]. In this study, the BV collected from 3 species of honeybees (*A. mellifera*, *A. dorsata*, and *A. florea*) was used to examine the effects of honeybee species at different

temperatures on the cell viability of melanoma cell lines and the MIC against gram-positive bacteria causing skin infection and gram-negative bacteria.

MATERIALS AND METHODS

Bee venom collection

BV was collected from 3 species of honeybees: *A. mellifera*, *A. dorsata*, and *A. florea* (in January 2019 at Mae Fah Luang University, Thailand) using 2 methods: an electric stimulator and reservoir dissection. The method of BV collection depended upon honeybee habitats. BV from the cavity nest honeybee, *A. mellifera*, was collected by a BV collector which stimulated honeybees stinging on a glass plate using electric shocks. The BV was then secreted and dried on a glass plate. Moreover, BV from open-air nest honeybees, *A. dorsata* and *A. florea*, was collected by reservoir dissection [21]. Briefly, adult bees were anesthetized at −20 °C overnight and kept in a petri dish on ice. The sting apparatus was then removed from its abdomen using fine-tipped tweezers, and the venom sac was then separated from the sting apparatus and dried on a petri dish. After that, 100 mg of all crude BV powder scraped from the glass plate and petri dish were dissolved in 1 ml of sterile distilled water. The crude BV solution was filtered through a 0.22 µm filter and kept at −20 °C until use.

Bee venom preparation

BV complex components with a molecular weight of over 10 kDa were removed using a 10-kDa molecular weight cut-off column (Amicon Ultra-0.5 ml, Millipore®, Merck Ltd., Germany), and 500 µl of crude BV solution (100 mg/ml) was then added to the column and centrifuged at 13,000 rpm for 30 min. Low molecular weight of less than 10 kDa (LMW) BV was eluted and collected in a 1.5 ml microcentrifuge tube and filtered through a 0.22 µm syringe filter in order to keep it at −20 °C.

The total protein of crude BV and LMW BV from 3 species (*A. mellifera*, *A. dorsata*, and *A. florea*) was measured using Bradford assay (BSA assay kit, Bio-Rad, USA). Briefly, a mixture of samples consisting of 150 µl of BV solution and 150 µl of 1X dye reagent were loaded into 96-well plates and incubated at room temperature for 5 min. After that, all samples were measured for absorbance at 595 nm in 3 replicates, and the protein concentration of crude BV and LMW BV was calculated using a standard curve of BSA at concentrations ranging from 1.25–25 µg/ml.

Determination of inhibitory concentration (IC) using cytotoxicity assay

The B16F10 melanoma cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) and incubated at 37 °C with 5% CO_2. Approximately 2×10^3 cells/well were seeded on 96-well plates and incubated at 37 °C with 5% CO_2. After 24 h, the cell lines were treated with various concentrations (0–30 µg/ml) of BV in 3 replicates for 72 h [22]. After that, 50 µl of MTT solution (2 mg/ml; EKEAR Bio@Tech, China) was added to each well, and the cells were incubated for 3 h; DMSO solubilizing solution was added and then incubated for 15 min in dark conditions. The absorbance of each well was measured at 570 nm by the Microplate Spectrophotometer (Thermo Scientific™ Multiskan™ GO, Finland). The cell viability was calculated following the equation below [23] and plotted with log [BV] to find the inhibitory concentration that leads to a 20% decrease in cell viability (IC_{20}) as a reference point.

$$\text{Percentage cell viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Detection of apoptosis by acridine orange/propidium iodide (AO/PI) staining

Briefly, B16F10 (2.1×10^3 cells/well) was seeded in 12-well plates and incubated for 24 h. All BV samples at IC_{20} were added to each well. After 72 h, treated and untreated cells were collected and centrifuged at 200×*g* for 5 min, and the cell pellet was resuspended by PBS. The cell suspension 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 10 × magnification under a fluorescent microscope at the blue (ex 475 nm/em 535 nm) and green (ex 525 nm/em 660 nm) excitation channels.

Determination of cell cytotoxicity from BV incubated at different temperatures

To evaluate the effect of temperature, BV collected from 3 species at IC_{20} was incubated individually at 40, 60, and 80 °C for 5 min. After incubation, the incubated and unincubated BV (control) were tested for cell cytotoxicity on the B16F10 melanoma cell line in 3 replicates to monitor the effect of temperature on the anticancer activity of BV.

Determination of minimum inhibitory concentration (MIC)

The skin pathogens (*S. aureus* TISTR 746, *S. epidermidis* TISTR 518, *S. pyogenes* DMST 17020, and *Propionibacterium acnes* DMST 14916) and gram-negative *Pseudomonas aeruginosa* TISTR 1287 were used for the MIC determination. Briefly [24], the stock culture of each bacteria species was cultured and adjusted by the 0.5 McFarland standard (1.5×10^8 CFU/ml). Each 5 µl of bacteria culture was added to 96-well plates. Then, 100 µl of BV (incubated at different temperatures) at different concentrations (0–100 µg/ml) was added and mixed into the 96-well plates for 3 replicates. After 24 h, 30 µl of 0.015% resazurin was added, and the color changes were observed: resazurin (blue, dead)

and resorufin (pink, alive) at each tested concentration [25]. The lowest concentration of BV inhibiting microbial growth was the MIC.

Statistical analysis

All data concerning the effects of different temperatures on BV collected from 3 species on cell cytotoxicity were analyzed using IBM SPSS Statistic (version 26) software and expressed as the mean ± standard deviation. All data were normally distributed and then analyzed using a two-way analysis of variance (ANOVA) to identify significant differences. Any significant differences among treatments were analyzed using multiple comparisons of means with Tukey's Honestly Significant Difference (HSD).

RESULTS AND DISCUSSION

The BV showed biological activities for anticancer against various types of cancer [26, 27], according to the action of their protein components. In this study, BV samples from 3 honeybee species were removed to obtain the protein component with a molecular weight of over 10 kDa. The total protein component of LMW BV decreased by around 95–97% compared to that of crude BV (Table 1).

Table 1 The total protein concentration of crude and LMW BV collected from 3 species.

Honeybee species	Protein concentration (mg/ml)	
	Crude BV	LMW BV (< 10 kDa)
A. mellifera	8.90 ± 0.902	0.442 ± 0.004
A. dorsata	10.44 ± 0.464	0.460 ± 0.005
A. florea	8.98 ± 0.066	0.268 ± 0.006

To study the anticancer effect of LMW BV collected from 3 different species, IC_{20} was examined [28, 29]. The results revealed that at concentrations of 1.828, 2.128, and 31.623 µg/ml of LMW BV, the cell viability of melanoma cell lines reached 80% after being treated with BV collected from *A. mellifera*, *A. dorsata*, and *A. florea*, respectively (Fig. 1). Thus, these concentrations were used as BV at IC_{20}.

After that, BV at IC_{20} was used to perform AO/PI fluorescence staining to identify dead cells after treatment with BV. After incubation, dead cells were found to be in the secondary necrosis stage, stained by orange-red fluorescent PI. All cells treated with BV collected from 3 species were stained in red except for the control (Fig. 2). These scenarios could be explained by the quick insertion of BV peptide into the cell membrane for only a nanosecond [30]. After 72 h of incubation, cell morphology showed no difference in the apoptosis stage for membrane blebbing, early apoptosis, or late apoptosis.

Apart from the different species and sequences of BV peptide, temperature may also influence BV peptide

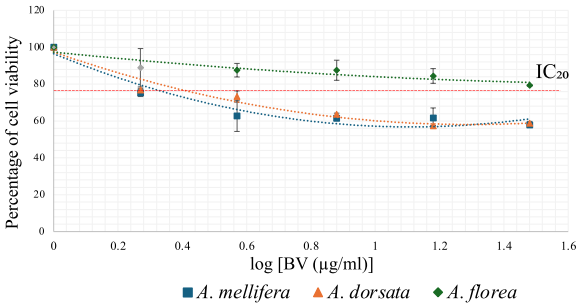

Fig. 1 Percentage of cell viability (mean ± SD) in the B16F10 melanoma cell line after being treated with LMW BV collected from *A. mellifera*, *A. dorsata*, and *A. florea*.

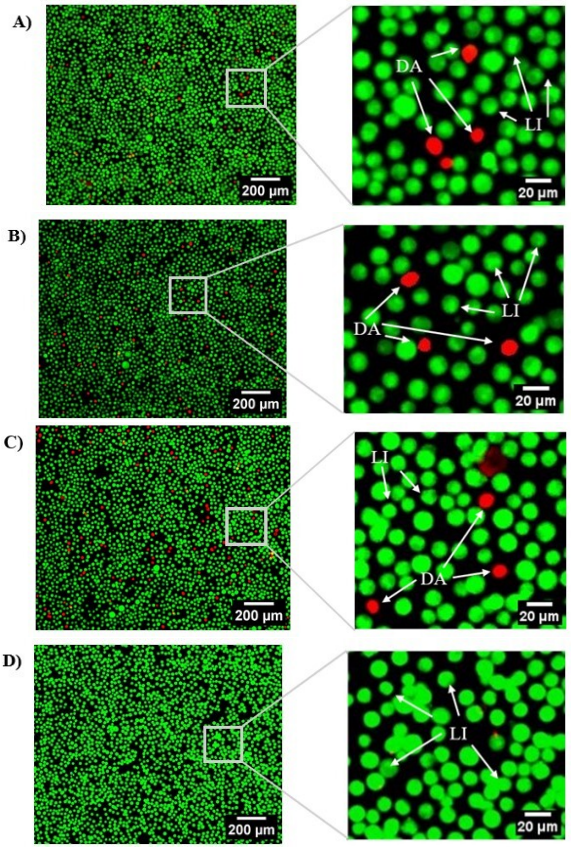

Fig. 2 Cell morphology analysis using AO/PI staining of the B16F10 melanoma cell line at 72 h after being treated with IC_{20} concentrations of BV collected from (A) *A. mellifera*, (B) *A. dorsata*, (C) *A. florea*, and (D) Control (Untreated). LI: live cell indicated in green and DA:dead cell indicated in red.

structure. BV collected from 3 species at IC_{20} and incubated at different temperatures (40–80 °C) were tested for cell cytotoxicity.

There was no interaction between temperature

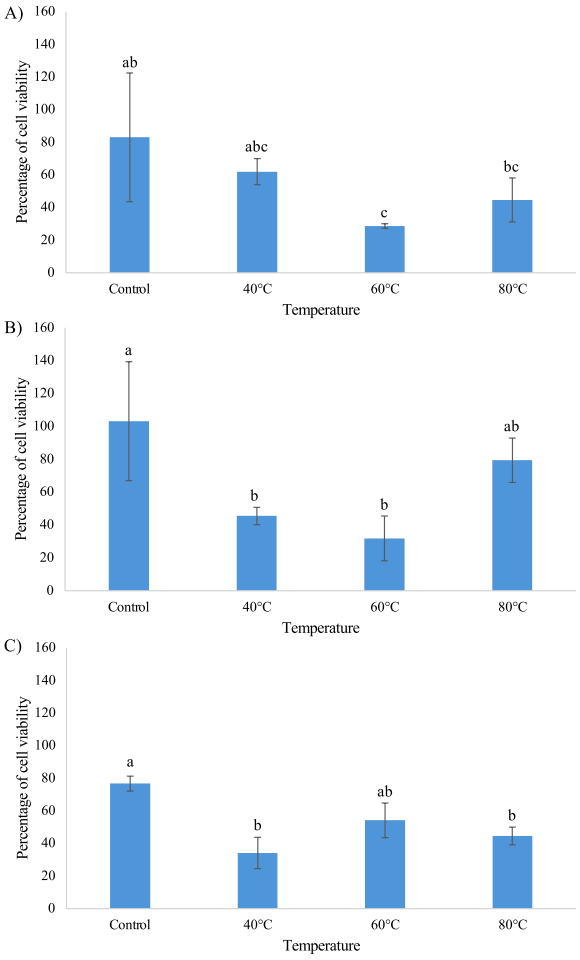

Fig. 3 Percentage of cell viability (mean ± SD) in the B16F10 melanoma cell line at 72 h after being treated with BV incubated at different temperatures. Bars with different letters are significantly different at $p < 0.05$. BV collected from (A) *A. mellifera*, (B) *A. dorsata*, and (C) *A. florea*.

and honeybee species ($p = 0.081$), meaning that the effects of temperature and honeybee species could be considered separately. Meanwhile, BV collected from *A. mellifera* at 60 °C exhibited the lowest percentage of cell viability, which, although significantly lower than that of the control, was not significantly different from BV at 40 and 80 °C (Fig. 3A). BV incubated at 60 °C could be an optimal level for the anticancer activity of BV collected from *A. mellifera*, whereas BV collected from *A. dorsta* at 40 and 60 °C exhibited the lowest percentage of cell viability, which was significantly lower than that of the control (Fig. 3B). The cell viability of BV collected from *A. mellifera* and *A. dorsata* was observed to decrease at temperatures from 40–60 °C and slightly increased in number at 80 °C due to the better activity of BV incubated at 40 and 60 °C than BV incubated at 80 °C. These results aligned with the study by Wilcox et al [31], who revealed that melittin from *A. mellifera* could form a tetramer and exhibited maximum stability at 35.5–43 °C and then decreased in stability at higher temperatures due to the unfolding of melittin. The tetramer structure plays an essential role in enhancing peptide aggregation by increasing the peptide concentration and structural support between each peptide chain at the membrane surface. Interestingly, the percentage of cell viability increased when BV was incubated at 80 °C due to the monomer unit in the tetramer structure beginning to separate above 70 °C. Therefore, the activity of BV decreased at 80 °C [32, 33].

However, the percentage of cell viability after being treated with BV collected from *A. florea* at 40, 60, and 80 °C showed no significant difference (Fig. 3C). The percentage of cell viability from all incubated BV collected from *A. florea* at 40 and 80 °C was significantly lower than that of the control which showed a susceptibility to temperature changes. It is possible that amino acid sequences of melittin at the 22nd position of BV collected from *A. florea* (asparagine) were different from *A. mellifera* (arginine) and *A. dorsata* (arginine) [17]. Since a positive charge of arginine is involved in the formation of ionic bonds with the lipid bilayer, it could result in stronger lipid-protein interaction with a negatively charged phosphate group involving cell membranes of *A. mellifera* and *A. dorsata* than that of *A. florea* [34].

The antimicrobial activity of 3 BV samples was tested by determining the MIC against 5 pathogenic bacteria (Table 2). According to the results, BV collected from *A. mellifera* and *A. dorsata* exhibited various MIC values from 6.25–100 µg/ml. For *S. aureus*, the MIC values at 40–80 °C were equal to 12.5–50 µg/ml among BV collected from *A. mellifera* and *A. dorsata*. In *S. epidermidis*, the MIC increased from 12.5–100 µg/ml, following the increase in temperature for both BV collected from *A. mellifera* and *A. dorsata*. In the case of *S. pyogenes*, the MIC values increased from 6.25–25 µg/ml, followed by an increase in temperature for BV collected from *A. mellifera*. For gram-positive *P. acnes*, the activity of *A. mellifera* increased in the same direction as the temperature increased from 25 to over 100 µg/ml. Moreover, the findings revealed that BV collected from *A. florea* had MIC values of more than 100 µg/ml against gram-positive bacteria.

The MIC values of BV collected from *A. mellifera* against *S. aureus* and *S. epidermidis* were 12.5–25 µg/ml, lower than those revealed in the previous study by Maitip et al [17]. This was because the BV samples in the present study were prepared using a molecular weight cut-off protein concentrator to separate complex components and concentrate protein components before performing cell cytotoxicity and MIC.

Additionally, the different MIC values among treat-

ments were affected by BV being collected using different incubated temperatures, honeybee species, and pathogens. In *A. mellifera*, the MIC values decreased at 40 and 60 °C while slightly increased at 80 °C compared to the control against all pathogens. However, the MIC values of *A. dorsata* BV at 40 °C were the lowest against *S. aureus*.

Table 2 Minimum inhibitory concentrations (MIC) of bee venom (BV) from 3 species against 4 gram-positive skin pathogens.

Microorganism	Treatment	MIC (µg/ml)		
		A. mellifera	*A. dorsata*	*A. florea*
S. aureus	Control	12.5–25	12.5–25	>100
	40 °C	6.25–12.5	12.5–25	>100
	60 °C	12.5–25	25–50	>100
	80 °C	25–50	25–50	>100
S. epidermidis	Control	12.5–25	25–50	>100
	40 °C	12.5–25	25–50	>100
	60 °C	12.5–25	50–100	>100
	80 °C	25–50	50–100	>100
S. pyogenes	Control	50–100	>100	>100
	40 °C	6.25–12.5	>100	>100
	60 °C	25–50	>100	>100
	80 °C	>100	>100	>100
P. acnes	Control	50–100	50–100	>100
	40 °C	25–50	50–100	>100
	60 °C	>100	>100	>100
	80 °C	50–100	>100	>100

The MIC values from *A. florea* were over 100 µg/ml against 5 pathogens at all temperatures, which could be due to the self-association of hydrophobic amino acid in the peptide sequence. Hydrophobicity in the optimum range could increase antimicrobial activity. However, when a peptide creates higher hydrophobicity over the optimum range, the antimicrobial activity of the peptide could decrease due to its self-association preventing it from penetrating through the bacterial cell membrane [35].

The MIC values for the gram-negative bacteria *P. aeruginosa* were found to be over 100 µg/ml among different temperatures and honeybee species because lipopolysaccharide, an important outer membrane component of gram-negative bacteria, acts as the lipid bilayer cover in the peptidoglycan structure. According to Vaiwala et al [36], the slow diffusion of melittin through lipid on the cell membrane [37] and the lipid components on the cell membrane were found to affect the melittin folding rate.

The results reveal that the temperature could enhance antimicrobial activity and cell cytotoxicity. The activities of BV increased when incubated at 40–80 °C compared to the control. Moreover, BV incubated at 40–60 °C exhibited better activities than that at 80 °C due to the tetramer structure of melittin having the greatest stability at 30–70 °C [32, 33], enabling it to act as a lytic agent to pass through the cell membrane [38].

CONCLUSION

This study examined the effect of BV collected from different honeybee species and at various temperatures on cytotoxicity and antimicrobial activity. The findings revealed that BV collected from 3 honeybee species exhibited different IC_{20} on the B16F10 melanoma cell line. Moreover, BV incubated at various temperatures showed different cytotoxicity and antimicrobial activity. The percentage of cell viability in BV collected from *A. mellifera* and *A. dorsata* decreased at 40 and 60 °C and slightly increased at 80 °C when compared to control. Moreover, the percentage of cell viability in BV collected from *A. florea* decreased at 40 and 80 °C when compared to the control. The alteration in temperature could be due to the MIC values being based on BV collected from different honeybee species and pathogens. This study highlights the potential of pharmacological (anticancer and antimicrobial) activities of BV samples in applications to address skin-related problems.

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