SOME MORPHOLOGICAL ASPECTS OF ASIAN HONEY BEE (*Apis cerana*) AND ISOLATION OF ITS MELITTIN CONTENT

Mumu N. N., M. Sarker[1], M. R. Islam[1], S. Akand and M. A. Bashar

Environmental Biology and Biodiversity Laboratory (EBBL), Department of Zoology, University of Dhaka, Dhaka-1000, Bangladesh; [1]Department of Biochemistry and Molecular Biology, University of Dhaka, Dhaka-1000, Bangladesh

Abstract

Melittin is a major component of honey bee venom that has many biological effects including anticancer properties. The melittin content of Asian honey bee (*Apis cerana* F.) venom was isolated and quantify by reversed phase-high performance liquid chromatography (RP-HLPC) in Bangladesh. Melittin content was found to present in 59.3% of total venom content in Asian honey bee venom. Venom compounds were investigated at 254 nm and the retention time of venom-melittin was compared with an external standard (Sigma-Aldrich). This finding was compared with previously published findings. By comparing the present data with the earlier studied findings, a projection has been made on the quantification of melittin compound.

Key words: Melittin, *Apis cerana*, RP-HPLC, Venom sac.

INTRODUCTION

The principal form of defense in honey bee is venom (Owen *et al.* 1977). The main reservoir of venom in honey bees is venom sac. In the honey bee, a single venom gland secretes a mixture of at least 50 identified components. And many of these components have significant toxic effects on other invertebrate and vertebrate species (Bridges and Owen 1984, Kokot and Matysiak 2009, Vetter and Visscher 1998). Approximate estimation of venom carried by an individual worker bee is 1 to 2 mg of liquid with 0.1 mg of dried material per sting (O'Connor *et al.* 1967).

Melittin, a toxic peptide, is the dominant and main lethal component of honey bee venom (Schmidt *et al.* 1986). Its main effect include- membrane- activeness, diminishing of surface tension of membranes and stabilizing them, serving as anti-inflammatory in a very small doses, stimulating smooth muscles, activating the hypophysis and adrenal glands, increasing of capillary permeability, increasing of blood circulation, lowering the blood pressure and blood coagulation, influencing the central nervous system, acting as immunostimulatory and immunosuppressive, radiation protective, antibacterial, antifungal, antiviral, anti-atherosclerosis and endosomolytic (Benton and Mulfinger1989, Jeong *et al.* 2011, Lee and Bae 2016). Study suggests that melittin has anticancer properties and it is capable of killing tumor cells (Mahmoodzadeh *et al.* 2013, 2015). Researchers are also considering bee venom as a cure for AIDS as bee venom destroys HIV virus particle (Hood *et al.* 2013).

Several venom fractions were isolated and main components were identified mainly by liquid chromatography and capillary electrophoresis by various authors (Benton *et al.* 1963, Habermann and Reitz 1965). More researches were done on the detailed separation, identification, structures, mode of action, biological activity of venom (Choi and Kang 2001, Han *et al.* 2007). The quantification of melittin compound was also done by RP/HPLC, but it was only in *Apis mellifera* species (Chmielewska and Szczesna 2004, Zhou *et. al.* 2010, Pacakova *et al.* 1995, De Graaf *et al.* 2009).

The objective of the present study is to separate, identify and quantify melittin in bee venom of the Asian honey bee (*Apis cerana*) by RP-HPLC. Before going to attempt the small task of melittin separation, some morphological aspects were examined in detail for proper identification of the honey bee (*A. cerana*).

MATERIAL AND METHODS

Sample collection and identification

The four species of honeybees viz. *Apis dorsata*, *A. cerana*, *A. mellifera*, *A. florea* are available in Bangladesh. They were collected from Curzon Hall campus, University of Dhaka and from a local apiary in Sonargaon, Bangladesh. Along with colouration and wing venation, body size and stripes on abdomen were used to identify the bee species. In addition to the body size and colouration of four different species, their activities on foraging behaviour were also recorded (Fig. 1). For the final identification the wings of the species were temporarily mounted using glycerin on a slide following the methodology of Niem and Trung (1999), Engel (1999), Abrol (2013), Ruttner (1988).

a b c d

Fig. 1. Four different species of honey bees (a. *Apis cerana,*b. *A. dorsata,* c. *A. mellifera,* d. *A. florea*) available in Bangladesh are seen on foraging activities. Their morphological differences and colour variations are very distinct to differentiate the species.

Sample preparation

After collection of the honey bees with a sweeping net, they were anesthetized by chloroform. The last segment of abdomen was taken out with a forcep; sometimes only sting was disssected out carefully. The muscles were cleaned with a needle. The venom sac's length (VSL) was measured from base to apex of the sac with a micrometer in 4x magnification under a stereomicroscope (Fig. 2). Euromex-Holland microscope attached with a camera and image analysis software were used for capturing pictures. The body sizes of the honey bee species were measured with a measuring scale. Here, the body sizes of honey bees are taken as area of their body. This study was conducted following the methodology of Zhao *et al.* (2015), Perveen *et al.* (2012).

All dissecting procedures were performed under the stereomicroscope (model SQF-E) at EBBL (Environmental Biology and Biodiversity Laboratory, Department of Zoology, University of Dhaka). For chemical analysis, the venom was manually collected. After collecting and anesthetizing, the sting with venom sac and gland was drawn out with a fine forcep, sometimes the last segment was also drawn out and then the venom sac was cut to make free from the sting.

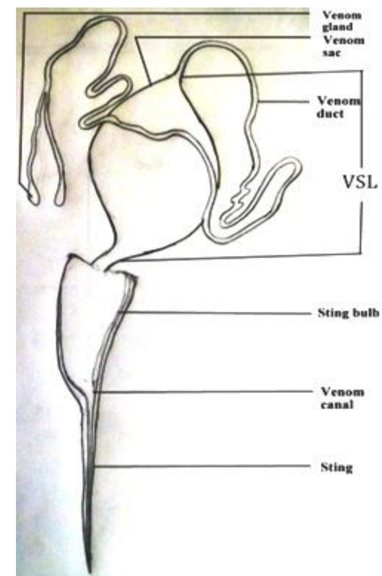

Fig. 2. VSL measurement from a typical honey bee-sting apparatus.

The content of all venom sacs was squeezed out and rinsed with 120 µL nano pure water in a 1.5 mL centrifuge tube and was centrifuged for 10 minutes at 14,000 rpm at 4ºC. After centrifugation the supernatant was collected by micropipette and stored at -20ºC. The chemical analysis was performed according to the methodology followed by Dong *et al.* (2015), Chmielewska and Szczesna (2004) and Mahmoodadeh *et al.* (2015).

Chemicals for RP-HPLC

Standard melittin (CAS no. 20449-79-0), solvents for HPLC like- acetonitrile (AcN) (CAS no. 75-05-8) and trifluoroacetic acid (TFA) (CAS no. 76-05-1) were purchased from Sigma–Aldrich, Fisher and Roth & Co, respectively. The working solution of melittin was prepared by 2 fold dilution of the stock. The working solution was stored at -20ºC. The standard melittin concentration used for HPLC was 5µg/µL.

Chromatographic condition for RP-HPLC

In order to facilitate venom profiling and melittin isolation RP-HPLC were carried out using U HPLC Dionex Ultimate 3000, Thermo Scientific, USA and it consisted of the following instruments:

- Chromatographic columns with C18 packing,
- Detector: Diode-array detector (DAD) and UV
- Auto injector: injection volume 10 µL
- Conditions for gradient method: 35% - 55% AcN/0.1%TFA for 15 minutes
- Flow rate of mobile phase: 1mL/min
- Separation temperature: 30ºC
- The analysis was done at 4 wavelengths-214nm, 220nm, 235nm, 254 nm

Venom compounds were identified at 254 nm. The retention time of the isolated melittin was compared with an external standard (Sigma- Aldrich). The purity of the isolated melittin was evaluated with the same HPLC condition. The procedure was repeated three times to make a reproducible result.

Data analysis

To assess whether the venom sac's length (VSL) differs interspecifically or not, a one way ANOVA test was performed among the VSL of four species of honey bees. Beforehand, the assumptions were checked with the Shapiro-Wilk test for the normality and the Levene's test for the homogeneity of variances. A Tukey's honestly significant difference (HSD) post hoc test was conducted to show where difference lies. A Pearson product-moment correlation was performed between venom sac size and body size. The statistical analyses were performed using R-3.3.2 for Windows 10.

RESULTS AND DISCUSSION

Some morphological aspects of four different honey bees have been considered in the present investigation giving emphasis both on the primary data (*Apis cerana*) and the secondary data (*A. dorsata, A. mellifera* and *A. florea*). *A. dorsata,* the giant honeybee and *A. florea*, the dwarf honeybee were identified mainly by their size. Among them *A. dorsata* was found the largest in size and *A. florea* the smallest (Table 1). In addition to size *A. florea* were very much prominent in body colour, as their first scleritic of abdomen was red in colour. Furthermore, the white hairs on the thorax, abdomen and also on the hind tibiae were adequate to identify this little species.

Table 1. Body size of four species of honey bees (worker) in Bangladesh.

Honeybee species	No. of bees examined (n)	Body area (Length x Breadth)		
		Min (mm)	Max (mm)	Average±SD
Apis dorsata	11	132	210	174.63±24.52
Apis mellifera	10	96	102.3	98.82±2.82
Apis cerana	11	45.6	75	60.64±10.32
Apis florea	7	33	44	40.00±4.77

Apis cerana and *A. mellifera* are medium sized honeybee and among them *A. mellifera* was larger and more robust, its body was found more hairy with uneven black stripes and three abdominal stripes. On the other hand, *A. cerana* had four prominent and consistent abdominal stripes with even black band across the entire abdomen. However, for more accurate identification, wing venations were analyzed. The general shape and number of veins were very similar, but *A. cerana* had the extended radial vein, which was never more than a tiny spur in *A. mellifera* (Fig. 3).

Apis cerana *Apis mellifera*

Fig. 3. Hind wing of *A. cerana* and *A. mellifera;* the radial vein indicated by an arrow.

VSL measurement

The data for the venom sac length is normally distributed for all of the species (Shapiro test: *p-value* = 0.178 (*A. dorsata*), 0.970 (*A. mellifera*), 0.991 (*A. cerana*), 0.553 (*A. florea*)). The variances are statistically equal among the sample (Levene's test: *p-value* = 0.738). The one way ANOVA test indicates that VSL is significantly different among the species studied (Table 2).

Table 2. Venom sac length of four species of honey bees.

Honey bee species	No. of bees examined (n)	Venom sac length			F value	p value
		Min (μm)	Max (μm)	Average±SD		
Apis dorsata	11	16	25	21.125±2.232		
Apis mellifera	10	13	18	15.667±1.966	111.5	3.17e-14
Apis cerana	11	10	14	11.625±1.302		
Apis florea	7	5	5.5	4.967±0.712		

The output of Tukey's honestly significant difference (HSD) post hoc test gives the differences in mean, confidence level and the adjusted p-values for all possible pairs. The confidence levels (Fig. 4) and p- values indicate that there is significant difference of the length of venom sac among all possible pair of four species (Table 3).

Table 3. Tukey's (HSD) post hoc test of the four species of honey bees.

Honey bee species	Difference	lwr	upr	Adjusted p value
A.dorsata-A.cerana	9.500000	7.167781	11.832219	0.0000089
A.florea-A.cerana	-6.658333	-9.177418	-4.139249	0.0000009
A.mellifera-A.cerana	4.041667	1.522582	6.560751	0.0009634
A.florea-A.dorsata	-16.158333	-18.677418	-13.63924	0.0000008
A.mellifera-A.dorsata	-5.458333	-7.977418	-2.939249	0.0000203
A.mellifera-A.florea	10.700000	8.006986	13.393014	0.0000010

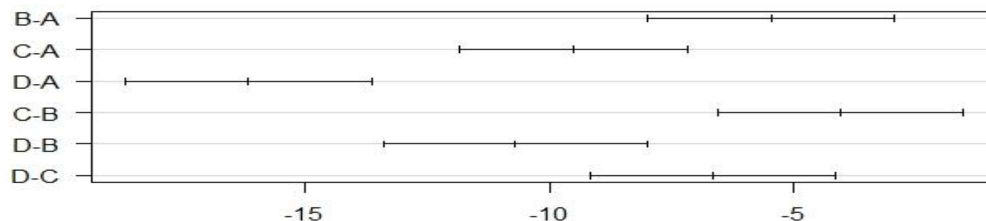

Fig. 4. Difference in mean levels (95% family-wise confidence level) of honey bee species (A= *Apis dorsata*, B= *A. mellifera,* C= *A. cerana*, D= *A. florea).*

Fig. 5 shows that *A. dorsata* have the largest venom sac while *A. florea* have the smallest one among the four species. Therefore, it stands that in case of VSL characteristic the sequence of honey bees is- *A. dorsata* > *A. mellifera* > *A. cerana* > *A. florea*.

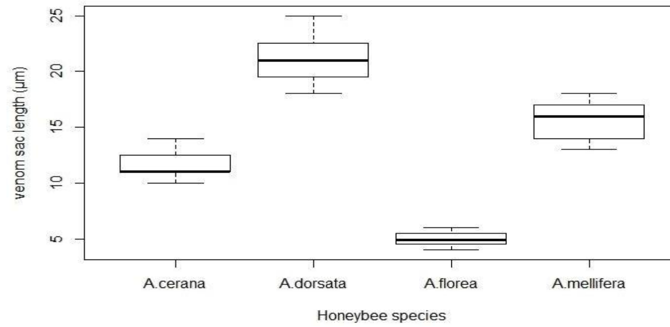

Fig. 5. VSL of four different species of honey bees.

The result of Pearson product-moment correlation between venom sac and body size of honey bees showed a positive relationship, where, p-value is less than 0.01, therefore this relationship is statistically significant (r= 0.8979779, p value= 9.208e-11).

Chemical analysis

In the present work, chromatographic column with C18 packing (250mm x 4.6 mm) was used in 35-55% AcN condition at 1 mL/min flow rate of mobile phase and a temperature of 30°C. The column elutes were monitored at 214, 220, 235 and 254 nm wavelengths, but the best identification was done at 254 nm wave length.

Table 4. Retention time and content of melittin from reverse-phase high performance liquid chromatography (three times) of melittin from Asian honey bee venom.

Run No.	Retention Time (min)	Peak Name	Height uAU	Area uAU * min	Relative Area
1.	2.44		1391.280	239.878	59.99%
2.	2.44	melittin	1341.760	229.895	58.19%
3.	2.44		1322.960	213.207	59.68%

A chromatogram of Asian honey bee venom is presented in Figure 5. The main peptides and proteins in bee venom is melittin and the second peak (RT = 2.44 min) is for melittin. These results were confirmed by comparing with melittin standards (Fig. 7), which showed almost similar retention times as the bee venom sac samples (Fig. 6). The average retention time of melittin is 2.44 minutes (Table 4).

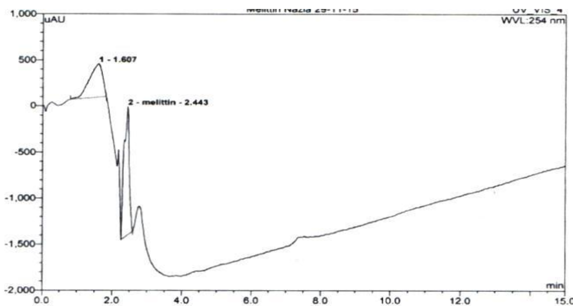

Fig. 6. *A. cerana* venom chromatogram: Reverse-phase high performance liquid chromatography was performed using C18 column ((Macherey-Nagel, EC 250/4.6 Nucleodur 100-5 C18ec).

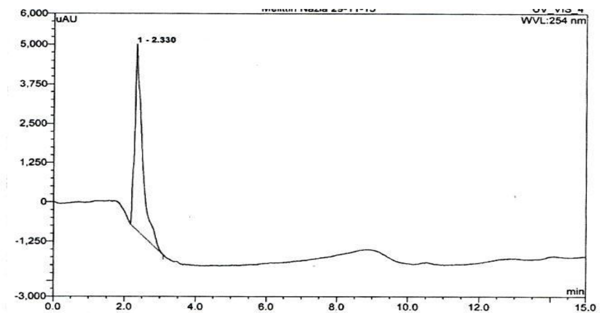

Fig. 7. Reverse-phase high performance liquid chromatography of melittin standard (Sigma-Aldrich).

The content of melittin in bee venom sample ranged from 58.2 to 60% with the mean value of 59.3% (Table 5). The accuracy and repeatability of the method as applied to the bee venom components, melittin was adequate (Fig. 8). The variation coefficients were 0 and 1.62% respectively for the retention time and quantity of melittin in the bee sample.

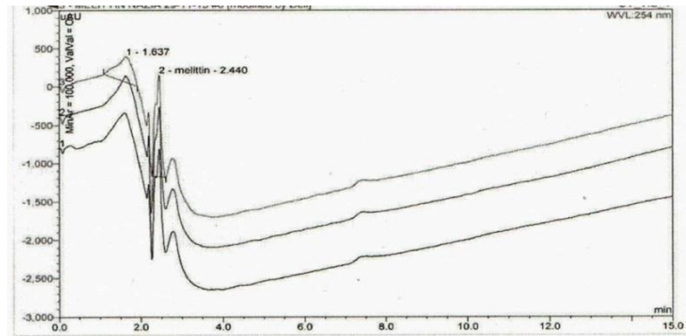

Fig. 8. Bee venom protein fraction chromatogram: for accuracy of the method.

The study was attempted to know the melittin content in various species of the honey bees. Finally the analysis was done only with *A. cerana.* According to the result the melittin content was found about 59.3% of the total venom content examined. Previous study showed about 64.40% melittin content in the venoms of *A. mellifera* honeybee species (Chmielewska and Szczena 2004). Projection can be made on the fact that, the large venom sac contains more melittin content in total. The present study finds that *A. mellifera* possesses larger sac than *A. cerana.* Dong *et al.* (2015) stated that melittin content is correlated with the age of the honey bees. The present investigation results that melittin content is correlated with the size of venom sac, in turn the body size of the bees.

Table 5. Detection time for melittin (in chromatographic condition) and Melittin protein fraction composition in honey bee sample.

	Average± SD	Variation Coefficient (%)
Retention Time for melittin separated by the chromatographic column (minute)	2.44± 0	0
Melittin protein fraction composition of honey bee venom	59.28± 0.96	1.62

From the different previous observations it can be said that *Apis dorsata,* the largest honey bee species, contains more quantity of melittin than the other species. Hence it would be more applicable to collect medicinal compound from a bigger species. Further work in the line is suggested to carry out on other species.

As bees are very powerful pollinators and known as dense pollinators (Bashar 2015) various works have been carried out on the question of 'honey bee-interactions' in the past (Begum and Hossain 2015). But so far, literature is concerned, the present work is the first attempt on melittin content analysis in the venoms of Asian honey bees (*A. cerana*) in Bangladesh. It could be said that this simple method followed in the present investigation may be used in the research purposes for the case of other bee species.

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