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Venom Glands Parameters, Venom Production and Composition of Honeybee *Apis mellifera* L. Affected by Substitute Feeding

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ABSTRACT

Two experiments were carried out during two successive years 2014 and 2015 at the period from July to October in the experimental apiary of Faculty of Agriculture, Benha University, Egypt to study the effect of honeybee (*Apis mellifera* L.) feeding with pollen substitute on bee venom production. As well as, the effect of venom gathering during various periods of the day on the number of dead bees and worker's brood rearing. After that, venom gland was microscopy examined and the collected venom was HPLC analyzed. Results indicated that the highest average of collected bee venom was 0.17 and 0.20 g/colony in July at 2014 and 2015, respectively. The highest venom amounts were collected at 7-9 pm. In addition, the higher mean numbers of dead bee's (18.7 and 24.0 dead bee/colony) were observed in October and August of 2014 and 2015 year, respectively. Venom gathering effect on worker's sealed brood area so it increased in control colonies by 4.0 and 13.0 % more than the colonies used for collection venom at different day times during 2014 and 2015, respectively. Results also showed that venom gland parameters were affected by feeding honeybee on pollen substitute. From HPLC analysis of the collected bee venom, results showed that the main components of venom were increased by feeding honey bee colonies on pollen substitute. The main components were melittin (48.7%), phospholipase A2 (16.4%) and apamin (2.0%).

Key words: Honeybee, Venom composition, Pollen substitutes, brood rearing, dead workers

Introduction

Honeybee venom (apitoxin), is a transparent liquid, ornamental pungent smell, a bitter taste, hydrolytic blend of proteins with basic pH between 4.5 to 5.5 (Ali, 2012). The active portion of apitoxin is a complex mixture of proteins, peptides and low molecular components (Bogdanov, 2016) which causes local inflammation and acts as an anticoagulant. The venom is produced in the abdomen of worker bees from a mixture of acidic and basic secretions. It is synthesized by the venom glands associated with the sting apparatus of worker and queens, stored in the venom reservoir and injected through the sting apparatus during the stinging process. Its production increases during the first two weeks of the adult worker's life and reaches a maximum when the worker bee becomes involved in hive defense and foraging (Roat *et al*., 2006). Many factors affecting honey bee venom production and its quality such as; honey bee race, age of bees, colony strength, season of collection, feeding supply, race, its defense behavior and method of collection (Haggag *et al.,* 2015). Besides the painful toxic effects, bee venom has many beneficial biological ones. The most important ones are: anti-inflammatory, anti-rheumatic, pain-soothing; anti-bacterial; immuno-suppressive; radiation-protective; improves hemoglobin synthesis, anti-coagulant; accelerates heartbeat, increase blood circulation, lowers blood pressure; lowers cholesterol levels; activates the central nervous system; stimulates building of endogenous cortisone (Ali, 2012).

Honeybee like any other organisms, have specific nutritional requirements. Necessary proteins, carbohydrates, fats, vitamins and minerals are available in natural sources which are pollen and nectar (Saffari *et al*., 2010). Protein plays a major role in honeybee longevity, brood rearing, production of honey and other bee products which will be reduced when protein availability is inadequate (Amdam and Omholt, 2002). Colonies that have no access to pollen, the bees' natural source of protein, have a reduced capacity to all life activities and may eventually die. Absence of pollen also affects the ability of honey bees to resist diseases (Matilla and Otis, 2006). As pollen is not always available, beekeepers feed honeybee colonies with either pollen substitutes (without pollen) or supplements (with pollen) as an adequate source for pollen which are necessary for maintaining healthy bee colonies during periods of pollen dearth (De Jong *et al*., 2009). To be of nutritional value for honeybee, the diet must comprise various feed ingredients as alternative sources

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of nutrition similar to their natural food sources (Wilson *et al*., 2005). Once ingested, the diet must have nutritional values, be free of any toxic compounds or anti-nutritional factors, have long shelf life in various conditions, be easily available and be economical (Saffari *et al*., 2010). The objectives of this research study the effect of pollen substitute on venom amounts collected at different day times during four months in the two studying years and its effect on some dead bee's number and sealed brood area. After that, microscopic examination of venom gland and HPLC analysis of the collected venom were studied.

Materials and Methods

Two experiments were carried out during two successive years 2014 and 2015 at the period from the beginning of July to the end of October in the experimental apiary of Faculty of Agriculture, Benha University, Qalubia, Egypt.

Honeybee colonies:

Fifteen Italian race honeybee colonies equal in strength were chosen for the experiments (10 honey bee combs housed in Langstroth hives), each treatment consists of 3 Colonies.

Pollen substitute:

The mixture of soybean flour, powdered yeast, and powdered sugar (0.5:0.5:2) was used as pollen substitute. All components were mixed with honey, citrus juice and volatile oils (Menthol; Camphor and Clove oil) for making pollen substitute cake. 300g/week from each pollen substitute cake were put on the top of the hives combs. Analysis of each component was presented in Table (1).

Table 1: Chemical analysis of each component of the used pollen substitute

Component	Analysis
Soybean flour (per 100g)	Humidity 6.58%, protein 50.88%, fats 5.41%, fibers 6.54%, 23.86% carbohydrates and ash 6.73% (Hammad, 2000)
Powdered yeast	Humidity 9.5%, crude protein 40.5%, 9% fats, 33.25% carbohydrates other extracts 1.5% and ash 6.25% in addition to vitamin B1, B2 and nicotinic acid (Atallah, 1975)
Fresh Citrus juice:	Humidity 88.3%, carbohydrates 10.4%, protein 0.7% and vitamins and minerals 0.6%. (Dagulo *et al*, 2010).

Bee venom

Time of gathering and its weight:

Venom was collected by electric current pulses device under honey bee combs as described by (Rybak et al. 1995) weakly of the four months under study (July, August, September and October), in different three times of the day (9-10 am, 3-5 pm and 7-9 pm) using collector frame. Collected bee venom was gathered, weighted using an electrical balance and store in the refrigerator (Benton et al., 1963).

Composition:

High performance liquid chromatography (HPLC) analysis was conducted in EuTech Scientific Services, Inc., Washington, USA, (www.eutechsci.com) to separate and identify the chemical composition of dry powdered bee venom as the method described by Rybak-chmielewska, and Szczêsna (2004).

Effect of venom gathering on brood area and dead workers:

The effect of bee venom collection process on worker's sealed brood area were studied by measuring it with a typical Langstroth wired frame divided into square inches at 13 day intervals during the study months (Haggag *et al*., 2015). As well as, dead workers of each colony were recorded by counting dead honey bee workers, every day, in each hive on the wire of collecting device, each three times a day (9-10 am, 3-5am and 7-9 pm) from the previously mentioned periods.

Effect of pollen substitute on venom gland structure:

New emerged workers (0:12 h. old) were colored marked on thorax in honey bee colonies which received pollen substitute and in control colonies (received only sugar syrup 1 sugar: 1 water). Five workers of each colony at the age of 18 and 21 days were collected. Then each worker was dissected, the acid gland was removed and transferred to a slide containing alcohol drop. The gland was stretched on the slide with stylets and removed the excess alcohol with filter paper, then dribbled a glycerin drop on the gland. The Length of acidic gland (L.A.G), Length of acidic sac (L.A.S.) and Width of acidic sac (W.A.S.) were determined and photographed.

Statistical analysis:

Data were analyzed using ANOVA test with LSD at 5% level (Little and Hills, 1978).

Results and Discussion

Effect of feeding honey bee colonies with pollen substitute on the amount of bee venom:

Data graphically illustrated by Fig (1) indicated that the feeding of honeybee colonies on pollen substitute and sugar syrup increase the amount of collected honey bee venom through the period of experiment study. This may be due to that pollen supplementary feeding plays an important role in honey bee colony life. Honey bees require many nutrients that found in the diet in a definite quantity for optimum nutrition. Honey bee colonies must be amended with these requirements by the beekeeper (Aly *et al*., 2014). Also, he proved that the supplementary feeding of honeybee improved the activity of their glands viz. venom gland. In addition, these results agree with Rashid *et al*. (2013) who reported that honey bee colonies treated with supplemental diet showed higher activities than colonies with ordinary feeding.

Additionally, it was clear that the amounts of bee venom were higher in July than August in both colonies fed on sugar syrup only (control) or colonies fed on pollen substitute and sugar syrup. This trend of results was the same in 2014 and 2015. Also, results showed that honeybee colonies fed on sugar syrup only gave the lowest amounts of bee venom collected in October of the two studying years.

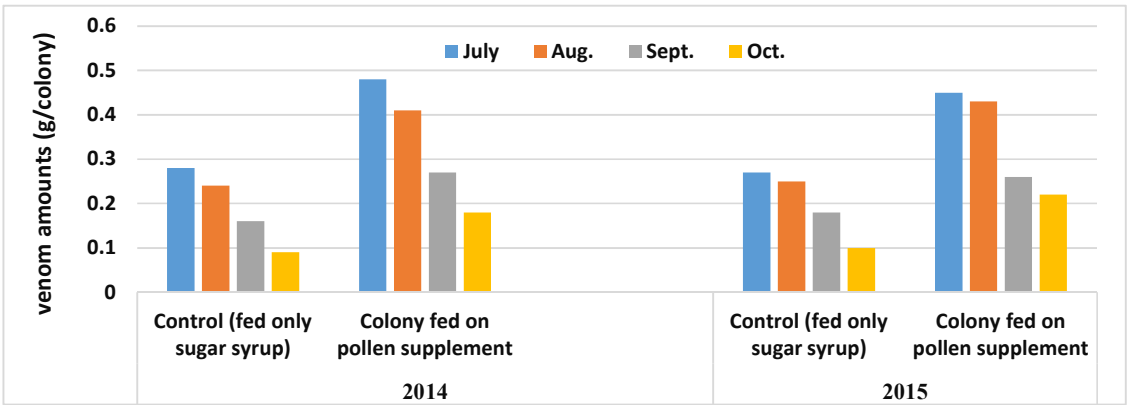

Fig. 1: Effect of feeding honey bee colonies with pollen substitute on the amount of bee venom during two studying years.

Effect of different day times on the amount of collected bee venom during 2014 and 2015:

Regarding the effect of month on the collected amounts of bee venom, data presented in Fig. 1 indicate that the highest amounts of bee venom were collected in July followed by August then September. On contrast, the lowest amounts were collected in October in the two years of study. The average of bee venom amounts collected in July was 0.17 and 0.20 g/colony during 2014 and 2015, respectively. In addition, there was no significant differences observed between bee venom amount collected in September in 2014 and 2015. Generally, the collected venom was higher during 2015 than 2014. Similar results were emphasized

by Sanad and Mohanny (2013) who stated that that the highest amounts of bee venom collected every 15 days during six months were recorded at August month (0.185 g/15day). Moreover, Khodairy and Omar (2003) stated that there was variability in the collected amounts of bee venom at different periods through the active season. Also, they reported that the amount of venom was high in July compared with that collected in May. Regarding the effect of the time of day, results in Table (2) showed that the amounts of honey bee venom were vary in the different collection times during the same day, where the highest amounts of bee venom were collected at 7-9 pm through the day followed by that collected at 10-12 Am and 3-5 pm, respectively during the two years of study. This could be due to that population density of bees was increase in the hive because the foragers bees return to the hive at that time of day. In this respect, Haggag *et al*., (2015) showed that honey bee venom production was affected by many factors one of these factors was season of collection.

Table 2: Effect of different day times on the amount of collected bee venom during 2014 and 2015.

Months	2014			Mean	2015			Mean
	10-12 Am	3-5 pm	7-9 pm		10-12 Am	3-5 pm	7-9 pm	
	Bee venom (g/colony)							
July	0.16	0.15	0.21	0.17	0.19	0.17	0.25	0.20
Aug.	0.14	0.12	0.19	0.15	0.18	0.15	0.20	0.18
Sept.	0.09	0.10	0.14	0.11	0.10	0.10	0.13	0.11
Oct.	0.07	0.07	0.09	0.08	0.08	0.07	0.09	0.08
Mean	0.12	0.11	0.16		0.14	0.12	0.17	
LSD 0.5	(Months): 0.014 (day times): 0.018				LSD 0.5	(Months): 0.007 (day times): 0.010		

Effect of different day times on the number of dead bees during 2014 and 2015:

Regarding to the effect of bee venom collecting month on the numbers of dead workers during the period of July to October of the two studying seasons, data in Table (3) indicate that the number of dead honey bee workers affected with bee venom collection and this affection was differing by months of collection. The highest average of dead bee's number was observed in October (18.7 dead bee/colony) and August (24.0 dead bee/colony) during 2014 and 2015, respectively. Generally, the number of dead workers were higher in 2015 than 2014. In similar results by Sanad and Mohanny (2013) who proved that there were significant differences among the studying months (March to November). Results revealed that the highest average numbers of dead bees were recorded at June (55.0 worker/day), and the lowest records were at November (14.1 worker/day).

Concerning the effect of different times of day, the highest dead bees number was observed at 7-9 pm (70 dead bee/colony) followed by 10-12 Am (65 dead bee/colony) and 3-5 pm (62 dead bee/colony), respectively during the 2014. Whereas, during 2015 the average of dead bee at different times of day were ranged from 88-69 dead bee/colony, the highest number was observed at 7-9 pm. These results agree with results by Sanad and Mohanny (2013) who revealed that the highest number of dead workers was recorded at 4-6 pm (49.32 worker/day).

Table 3: Effect of different day times on the number of dead bees during 2014 and 2015.

Bee venom collection time / Months	2014			Mean	2015			Mean
	10-12 Am	3-5 pm	7-9 pm		10-12 Am	3-5 pm	7-9 pm	
	No. of dead bees/colony							
July	15	13	17	15.0	22	18	22	20.7
Aug.	18	14	14	15.3	26	22	24	24.0
Sept.	14	18	18	16.7	17	14	20	17.0
Oct.	18	17	21	18.7	20	15	22	19.0
Mean	16.25	15.5	17.5		21.3	17.3	22	
LSD 0.5	(Months): 1.48 (day times): 0.81				LSD 0.5	(Months): 1.03 (day times): 0.8		

Effect of bee venom collection at different day times on the worker's sealed brood area during four months in 2014 and 2015:

Regarding the effect of bee venom collecting month, data graphically illustrated by Figs (2 a & b) indicated that bee venom collection has a negative effect on honey bee worker's brood rearing activity compared with control (untreated colonies). Also, Fig (2 a) showed that the highest average of worker's

sealed brood area in treated colonies was observed in August (2199 in^2) followed by July (2191 in^2) at different day times during 2014. Whereas, during 2015 the highest worker's sealed brood area was observed in July at different day times (2361 in^2 at 10-12 Am and 2350 in^2 at 3-5 Pm) except at 7-9 pm (2357 in^2) during 2015 (Fig. 2 b). From another view, it was clearly to indicate that untreated colonies (control) appear higher worker's brood rearing activity than colonies used for bee venom collection. Also, there was large distinction between the untreated colonies and all of bee venom collection treatment colonies in worker's brood rearing activity. Also, worker's sealed brood area in control colonies (2120 in^2) increased by 4.6% than the colonies used for collection venom at 10-12 Am and by 4.1% for the other two day times during 2014. On the other hand, during 2015 there was clearly that worker's sealed brood area in control colonies (2293 in^2) increased by 13.3, 13.2 and 13.5% than colonies used for collecting venom at 10-12 Am, 3-5 pm and 7-9 pm, respectively. Similar results were demonstrated by Sanad and Mohanny (2013) who reported that the highest and the lowest decrease in sealed brood area of the treated colonies compared to control ones, was recorded at July and November by 18.1 and11.3%, respectively.

Additionally, no significant difference recorded due to the effect of day times on worker's sealed brood area. Figs (2 a & b) showed that honeybee colonies used for venom collection gave worker's sealed brood area activity lower that control colonies. This trend of results was observed in the two studying years 2014 and 2015.

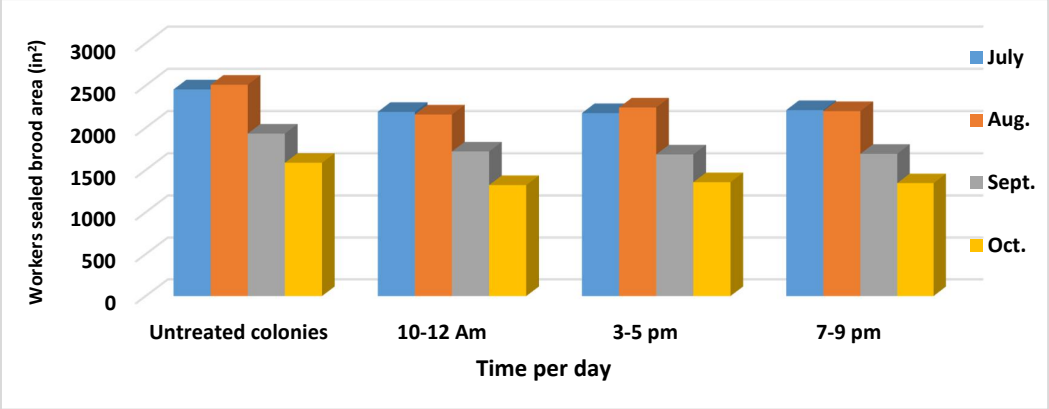

Fig 2 a: Effect of bee venom collection on worker's sealed brood area during four months in the year 2014.

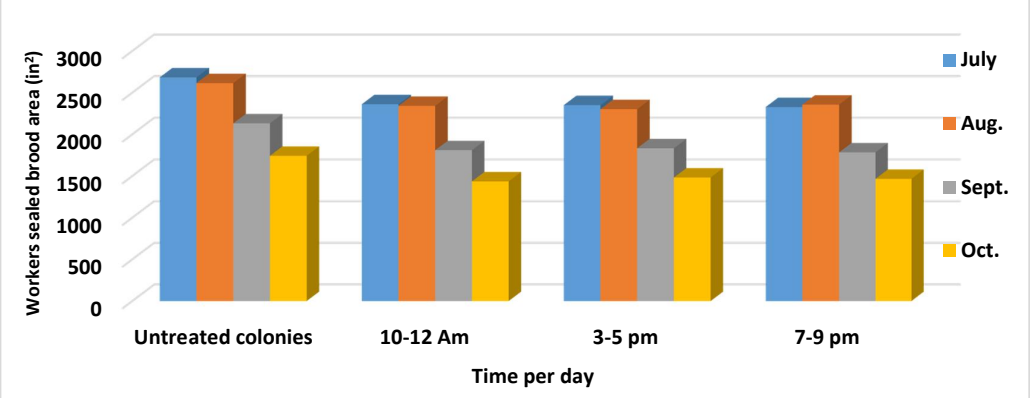

Fig. 2b: Effect of bee venom collection on worker's sealed brood area during four months in the year 2015.

Effect of supplementary feeding honey bee colonies on venom gland parameters

Data in Table (4) showed that feeding honey bee colonies on pollen substitute significantly increases the parameters of honey bee worker's venom gland and venom sac (length of acidic gland, length of acidic sac and width of acidic sac) compared with control honey bee colonies (only fed on sugar syrup). Regarding

the effect of honeybee age on venom gland parameters, data also indicated that length of acidic gland at 18-day old is bigger in that of 21-day old. While, the length of acidic sac and width of acidic sac was increased at 21-day old than at 18-day old. The width of acidic sac also increased at 21 worker's day old than at 18 worker's day old in control and feeding treatment. Alongside, Photos (1 & 2) showed the venom sac and venom gland diameters of honey bee worker's feeding on pollen substitute.

Table 4: Effect of supplementary feeding honey bee colonies on venom gland parameters

Parameters		Control		Pollen substitute Treat.	
		18-day old	21-day old	18-day old	21-day old
mm	L.A.G.	15.4	14.5	18.2	16.8
	L.A.S.	1.43	1.65	1.62	1.95
	W.A.S.	0.22	0.28	0.26	0.38
LSD 0.5 (L.A.G)	Age 0.16 Feeding 0.25	LSD 0.5 (L.A.S.)	Age 0.12 Feeding 0.2	LSD 0.5 (W.A.S.)	Age 0.16 Feeding 0.12

(L.A.G.) Length of acidic gland (L.A.S.) Length of acidic sac
(W.A.S.) Width of acidic sac

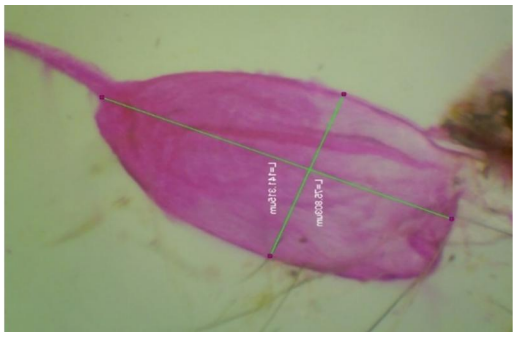

Photo 1: Venom sac

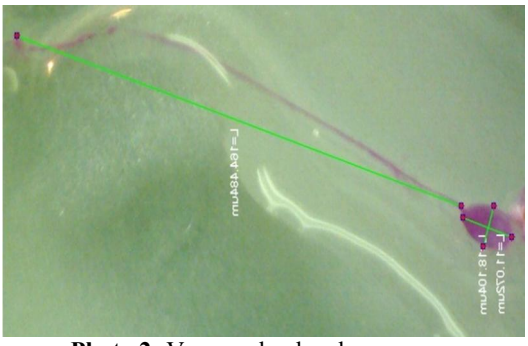

Photo 2: Venom gland and venom sac

Effect of supplementary feeding honey bee colonies on some powdered venom components:

In this part of study HPLC analysis for separation and identification of venom components of honeybee (*Apis mellifera* L.) was conducted to study the effect of feeding colonies with pollen substitute on powdered venom components. Data in Table (5) illustrated by Fig (3) showed that the main components of venom were apamin, phospholipase A2 and melittin. These results agree by Rybak-Chmielewska and Szczêsna (2004) who reported that the main components of venom fractions were identified as the enzymes phospholipase A2 and hyaluronidase, and the polipeptyde melittin.

Table 5: Effect of supplementary feeding honey bee colonies on some powdered venom components

Venom component	Concentration (%)	
	Control	Feeding treat.
Apamin	1.8	2.0
Phospholipase A2	11.3	16.4
Melittin	45.5	48.7

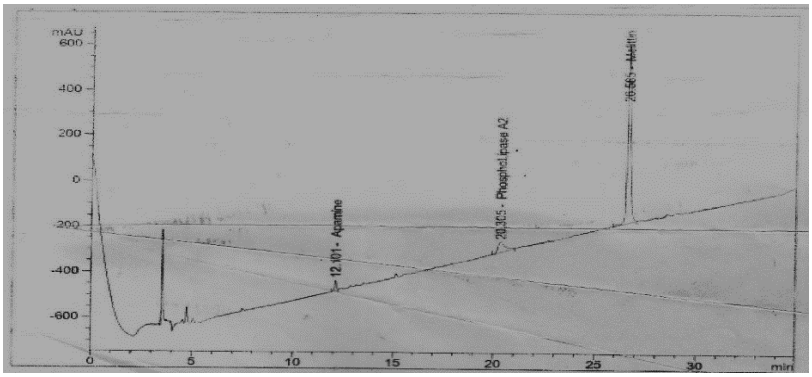

Fig. 3: HPLC analysis for honeybee venom collected from colonies feeding on pollen substitute

Generally, pollen substitutes gave higher concentrations of all separated components compared to control. In this respect, results indicated that pollen substitute gave the highest values of melittin being 48.7 % therefore it increased 3.2-fold comparing with control treatment. These results were confirmed by Zhou *et al*. (2010) who proved that the major protein fraction was melittin at range from 61.15 to 70.15%. Also, Ionete *et al*. (2013) reported that the average percent composition of major honeybee venom compounds is 65% mellitin. Moreover, honeybee venom has been demonstrated to contain some enzymes viz. phospholipase A2. In this trend, HPLC analysis showed that phospholipase A2 was the second major component at 16.4% followed by apamin at 2.0%. This trend of results agree with Ionete *et al*. (2013) and Matysiak *et al*. (2011) who proved that the average percent of phospholipase A2 and apamin were 13% and 3%, respectively. It is worthily to mention the apamin percentage in venom being 2.0% with increase of 0.2-fold than control and phospholipase A2 being 16.4% with increase of 5.1-fold than control. These results reflect the effect of honeybee feeding on pollen substitute.

Conclusion

It could be concluded that feeding honeybee with pollen substitute increase all activates of honeybee colonies such as worker's sealed brood area and the produced venom amounts. Also, bee venom collection was affected by month of collection and different day times, results concluded that July was the best month (due to the availability of corn pollen grains) and the day time at 7-9 pm were the best time for bee venom collection. Additionally, venom gland and venom sac were affected also by honeybee feeding and gave higher amounts of venom. The collected venom was HPLC analyzed, the major components were Apamin, Phospholipase A2 and Melittin.

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