

## Stimulation Effects of the Essential Oils on the Sensory and Defensive Behaviors of Egyptian Honey Bees towards Varroa Invasion

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### ABSTRACT

Stimulation effects on the sensory and defensive behaviors of Egyptian honey bees towards varroa invasion were studied through remedied honey bee colonies with the essential oils. Astonishing results to the grooming and hygienic behaviors consequence of the sensory responses enhanced the defense behavior of honey bees colonies against varroa mite.

**Key Words:** Honey bee, *Apis mellifera lamarckii*, Varroa mite, Essential oils, Sense organ.

### INTRODUCTION

The need to the genetic improvement and consolidation the immunity system of honey bees are important affair particularly at the last decade due to diseases and parasites surrounded to the bees environment and deterioration of the bee productivity. Using the acaricides within bee hives against the Varroa mites let to contaminate bees colony producer, further more developing resistance to used chemicals such as flumethrin, amitraz, cymiazole and bromopropylate which associated with toxic residues (Buchler, *et al.*, 1992, Wallner, 1995 and Kanga *et al.*, 2005). Recently reliance on natural extractions has played important role as remedy procedure particularly with the essential oils. *Nigella sativa*, *Thymus sp.*, *Eucalyptus sp.* and *Rosmarinus officinalis* L. (Allam *et al.*, 2004). The defensive behavior against Varroa mite involves two important mechanisms hygienic and grooming behaviors that minimized the parasitic threat. Antennae the main sites of the olfactory receptors in most insects have several kinds of small innervate setae and other minute sensory structures as chemo and mechanoreceptors (Abdel wahab *et al.*, 2006). The integration between the sensation centers and defense mechanism comfort worker bees to identify dead, injured or infested bees inside of capped brood cells. The breeding and selection from the local Egyptian bee race (*A.m. lamarckii*) showed very vocation for tolerant diseases particularly with its hybrid. Therefore, the aim of this study is to clarify the role of the essential oils as a factor to inspire the defensive behavior of the Egyptian honey bee race and to increase its tolerant to varroa mite.

### MATERIALS AND METHODS

#### 1- Selection of Egyptian Honey Bee Race

The selection from the Egyptian bee race for the higher bee colonies purity and productivity was carried out in Kerdasa region, Giza governorate in

two years. Naturally cross mating between selected Egyptian virgin queens and drons of the Carniolian race, *Apis mellifera lamarckii* X *A.m. carnica* were subjected for this study. Twelve honey bees colonies of the F1 Egyptian hybrid were subjected for this study. The efficiency of the essential oils on the hygienic behavior related to sensilla organs and haemolymph proteins were tested. The practical experiments of this investigation are finished at 2009 year.

#### 2- Tested Materials and Doses

Oil extraction of the black cumin seeds and mixture of some essential oils were used in this experiment (Three colonies/each treatment). Other three bee colonies were used as a check without any chemical treatment.

##### A- Oil Extraction of the Black Cumin Seeds (*Nigella sativa*, Fam: *Umbellifereae*)

The black cumin seed oil extraction was used as suspension and prepared by mixing 15 ml. of the crude oil with 1gm. of Triton-X (emulsifier) and sufficient water to obtain 100 ml. of the suspension.

##### B- Mixture of the Essential Oils

Mixture of the crude essential oils was prepared using cotton strips (20 x 2.5 x 0.5 cm) saturated with 5 ml of the black cumin oil added to 5 ml of Lemon oil and 2.5 ml of Jasmine oil (*Jasminwn grandiflorwn*) and placed in middle of the brood bee combs. The strips were replaced every week (one stripe/colony/each week) for 32 consecutive weeks.

##### C- Assessment of the Efficacy Levels

The infestation levels in all experimented colonies were determined before and after each application and dead Varroa mites fallen down on the bee hive bottom were recorded.

#### 3- Active Defense Behavior Measurements

##### A- Grooming Behavior Testing

Dead Varroa mites noted on the bottom of the bee colonies were recorded every 24 hours for 3 days/week/treatment. The biting characters

occurred on the body mites were detected and classified into 4 main symptoms representing biting or separating different parts of the Varroa body;

- 1- Gnathsoma and portions of the legs
  - a- Gnathsoma and distal segments of the legs.
  - b- Gnathsoma and portions of the legs from coxae.
  - c- Gnathsoma and portions from segments and from coxae differing in legs.
  - d- Gnathsoma , legs from coxae and parts of the abdomen .
- 2- Dorsal shield.
- 3- Biting Immature Varroa body.
- 4- Dropping immature Varroa mites.

Collected varroa mites were cleared by Nesbitts solution and mounted in Hoyer's medium (Krantz, 1978). The morphological specifications were clarified by a microscopic examination.

#### **B- Removal Dead Immature Brood Workers (Hygienic Behavior)**

The techniques used to determine the removal dead immature brood workers were carried out according to methods of Kefuss & Taber, (1996) and Harbo *et al.*, (1997).

#### **4- Assessment of Some Biological Activities**

Number of worker brood area (Inch<sup>2</sup>) and covered combs with bees were detected before and after treatment of bee colonies.

#### **5 - Scanning Electron Microscopic Studies (SEM)**

The Scanning Electron Microscopic technique (SEM) (Joel GM 4200) was used at the Applied Center for Entomonematodes (ACE), Faculty of Agriculture, Cairo University to test the antennal worker honey bees. This work was performed to correlate between the treatments and their effects on some sensory characteristic. Ten samples of each untreated and treated worker bees were used 32 weeks after treatments. The antennae were dissected out then dried to the critical point using Co<sub>2</sub> then mounted and coated with gold (Fashing *et al.*, 2000). The Sensilla organs were determined on the 10 flagellomeres and photographed by the (SEM). Trichodea types (A, B & C), Long Trichodea types (A&B), Basiconica, Placodea, Campaniformea and Coeloconica and counted/flagellomere then expressed as mean numbers/unit area (125x83.3 μm). The mean surface area (μm<sup>2</sup>) of the Placodea sensilla was estimated according to formula of Maurizio,(1954) =  $\Pi \times ab/2$  ( $\Pi=3.14$ , a= maximum length, b= maximum width). Length and width of the Trichodea type (B) and the length of the other sensilla organs were determined for all examined

antennae.

#### **6- Haemolymph Protein Analysis**

The haemolymph proteins of tested bee colonies were separated by Polyacrylamide Gel Electrophoresis (PAGE) according to the technique of Lammeli, (1970).

### **RESULTS AND DISCUSSION**

Data illustrated in Table 1 showed that the total fallen dead varroa mites through the experimental periods were 1209, 540 & 435 mites for black cumin oil, oils mixture, and control, respectively. They ranged between 20&130 mites/colony/month for oils mixture treatment, whereas treatments of black cumin oil and control ranged between 4 & 455and 3&174 mites/colony/month respectively.

As shown in Table 1, the biting behavior to Varroa body began to be noted after one month after treatments with oils mixture and after two months with black cumin oil and control, respectively. The percentage of cutting Varroa bodies with oils mixture treatment ranged between 15 & 81.42% of the dead mites, while with black cumin oil treatment it ranged between 11.11&- 92%. For control, it recorded the lowest value (8.33%) and did not increase than 35.07%. There were significant differences between attack behaviors (fallen dead Varroa mites and number of injured Varroa bodies) and treatment periods. The defensive mechanism behavior system began to be integrated with treatments progress corresponded with decreases in Varroa population reaching highest level after 8 weeks with oils mixture, and 16 weeks with black cumin oil & 20 weeks with control.

Different gnaw methods to Varroa mite bodies by adult worker honey bees through grooming behavior (Figs. 1-8) could be classified into 4 main categories as follows.

#### **1- Pull out Gnathsoma and Portions of the Legs**

Pulling out gnathsoma including chelicerae, palps, hypostome , epistome and portions of the legs;

- a- Removing out gnathsoma and terminal segments of the leg (pretarsus tarsus, tibia and genu) (Figs. 1, 2, 5and 6).
- b- Extracting out gnathsoma and portions of the legs from coxae (Fig. 3).
- c-Biting of gnathsoma and portions from segments and coxae (Figs. 1, 2 and 5).
- d- Plucking of gnathsoma and legs completely from coxae and parts of the ventral shield as sternal shield, endopodal shield and metapodal shield

(Figs. 6- 8). It could be concluded that:

- 1) Plucking of legs No. 1, 2 & 3 from trochanter considered the weakness point help bee workers to easy isolate rest legs (Figs. 1, 2, 5 and 6).
- 2) Discarding out the pretarsus empodium (capture organs) in legs saving the dorsal plate which protect the ventral apparatus that consider dangerous matter for mite alive and ovarian system kept (Figs. 6 and 8).
- 3) Biting of gnathsoma, legs and ventral shield except dorsal shield. This behavior may be attributed due to different genetic structures of the Egyptian hybrid.

## 2- Biting the Dorsal Shield

Separating the dorsal shield portions as shown in

(Fig. 7).

## 3-Biting Immature Varroa Mites

Removing out gnathsoma and portions of the leg segments and parts of the ventral shield as sternal shield, endopodal shield, genitoventral and metapodal shield (Table 1).

## 4- Dropping Immature Varroa Mites

Dropping immature varroa mites appeared in the first generation following genetic improvement applied on the Egyptian race. Allam *et al.*, (2004) reported that worker Egyptian hybrid which had shorter capping period of worker brood cells could negative effectly the biological varroa cycle while, Zakaria and Allam, (2007) did not record any fallen immature varroa mites during their experiments on

Table (1): Different gnaw methods of Varroa body followed essential oils treatment.

Treatment	Date/Month	DM	BT	Rate of gnaw Varroa body						
				1				2	3	4
				a	b	c	d			
Control	9/ 007	3	0(0 %)	0c	0c	0b	0d	0c	0b	0b
	10	3	0(0 %)	0c	0c	0b	0d	0c	0b	0b
	11	7	1(14.28 %)	1c	0c	0b	0d	0c	0b	0b
	12	11	2(18.18 %)	1c	1c	0b	0d	0c	0b	0b
	1/2008	60	3(8.33 %)	3c	0c	0b	0d	0c	0b	0b
	2	174	61(35.07 %)	20a	23a	1b	8a	2b	3a	4b
	3	122	33(27.04%)	7b	4b	2a	4b	4a	2ab	10a
	4	55	17( 30.9%)	9b	1c	0b	2c	0	3a	2b
	Total	435	117(26.89%)	41	29	3	14	6	8	16
	LSD <sub>0.05</sub>				2.66	2.11	0.86	1.05	0.86	1.49
Black cumin seed oil	9/2007	4	0	0f	0d	0d	0b	0c	0b	0b
	10	12	0	0f	0d	0d	0b	0c	0b	0b
	11	455	84(18.46%)	27bc	21a	3c	3a	15a	6a	9a
	12	170	60(35.94%)	26c	10b	4bc	4a	4b	8a	4b
	1/2008	75	69(92%)	35a	20a	10a	1b	1bc	1b	1b
	2	153	17(11.11%)	8d	4c	0d	0b	2bc	0b	3b
	3	290	46.5(16.03%)	28b	4c	4.5b	0b	4b	1b	5b
	4	50	14.33(28.66%)	2e	5c	0d	4a	1bc	1b	1.33b
	Total	1209	290.83 (24.055%)	126	64	21.5	12	27	17	23.3
	LSD <sub>0.05</sub>				1.498	1.49	1.22	1.22	2.52	2.73
Oils mixture	9/2007	22	0 (0 %)	0e	0e	0	0c	0	0c	0b
	10	20	3 (15 %)	2de	0e	0	0c	0	1bc	0b
	11	30	22 (73.3%)	3c	11d	1	2bc	2	3b	0b
	12	55	25(45.45%)	13b	1e	0	5ab	1	3b	2a
	1/2008	98	28(28.57%)	9c	4e	0	7a	2	5a	1ab
	2	130	37(28.46%)	11bc	15c	0	5ab	2	3b	1ab
	3	70	57(81.42%)	18a	33b	0	3bc	0	3b	0b
	4	115	65(56.52%)	17a	41a	0	2bc	2	1bc	2a
	Total	540	237(43.9%)	73	105	1	24	9	19	6
	LSD <sub>0.05</sub>				2.447	2.52	F=1.0	2.28	F=1.89	1.36

1- Biting Gnathsoma and portions of the legs

a- Gnaw of gnathsoma and distal segments of the legs.

b- Separation of gnathsoma and portions of the legs from coxae.

c- Gnaw of gnathsoma and portion of legs from segments and from coxae.

d- Cutting of gnathsoma , legs from coxae and parts of the abdomen .

2- Biting the dorsal shield.

3- Biting immature Varroa mites.

4- Cutting out immature Varroa mites.

DM: Mean numbers of the fallen dead mites.

BT : Mean numbers of bitten Varroa bodies.



Fig. (1): Plucked off gnathsoma, portions of the legs and portion of the dorsal shield (The right and left legs) and lateral setae.



Fig. (2): Gnawed gnathsoma and portions of the legs and portion of the dorsal shield.

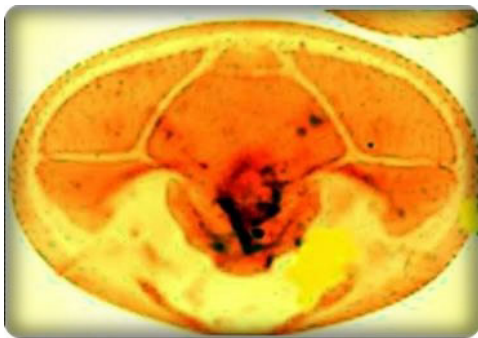


Fig. (3): Bitten gnathsoma and legs from coxae and separated some lateral setae.



Fig. (4): Separated legs from coxae.



Fig. (5): Bitten of gnathsoma and portions of the legs, causing pit ventral, endopodal and genital shields (anal, exopodal, metapodal parts).

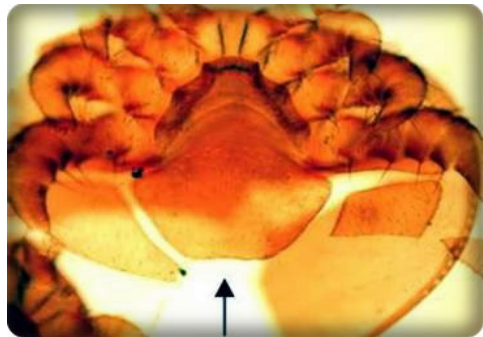


Fig. (6): Plucked off gnathsoma and parts of the ventral shield (anal & metapodal) and cut the pretarsal-empodium in the legs.



Fig. (7): Gnawed body, dorsal shields portion of sternal and exopodal plate.

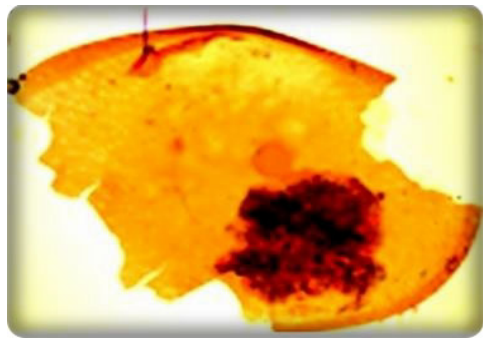


Fig. (8): Plucked off gnathsoma, anal plate and legs pretarsal-empodiae of pre-sexually developed female.

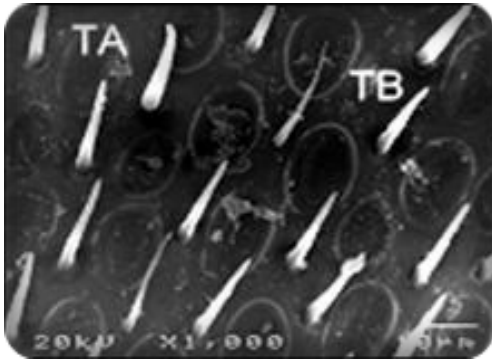


Fig. (9): TA: Trichodea type (A).  
TB: Trichodea type (B).

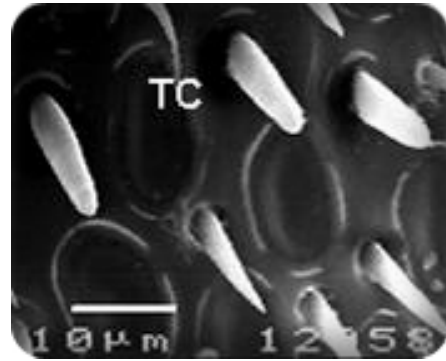


Fig. (10): TC: Trichodea type (C).

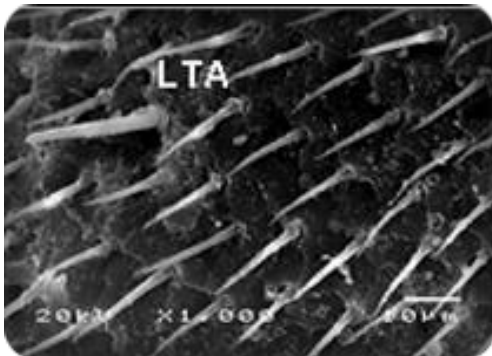


Fig. (11): LTA: Long Trichodea type (A).



Fig. (12): LTB: Long Trichodea type (B).

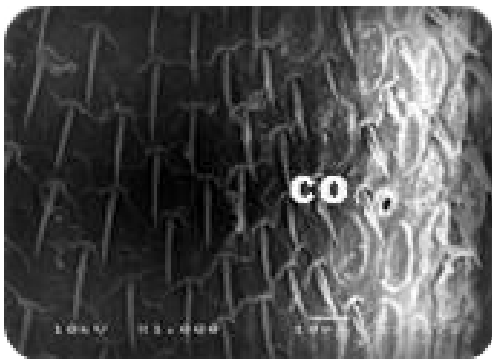


Fig. (13): CO: Coeloconica sensilla.

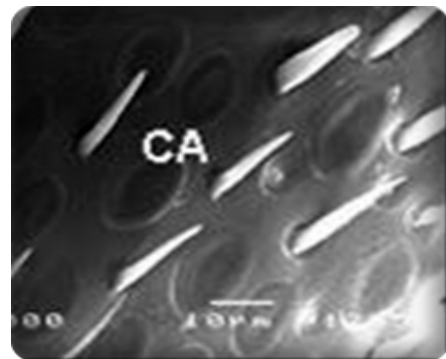


Fig. (14): CA: Campaniforme sensilla.

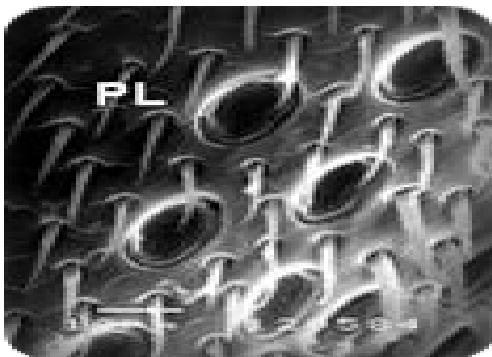


Fig. (15): PL : Placodea sensilla.

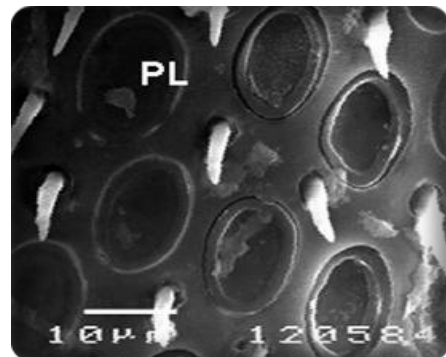


Fig. (16): Sensilla Placodea during treatment with the mixture of the essential oils.

Some of them were produced up than recorded with the control one as shown in Fig.(15).

the Carniolian hybrid which had not been subjected to any procedure of the genetic improvement.

## 2- Removal Dead Immature Brood Workers and Biological Activities

Removal dead immature worker brood bees did not show any significant differences between oils mixture treatment and control, while treatment with black cumin oil recorded the lower values (Table 2). Number of covered combs with bees was higher only with the mixture of the essential oils.

## 3-Scanning Electron Microscopic Studies (SEM)

The Scanning Electron Microscopic studies revealed presence of eight types of the sensilla organs in *Apis mellifera* worker antennae. They were; Trichodea types (A, B & C), Long Trichodea types (A & B), Basiconica, Placodea, Campaniformea, and Coeloconica (Figs. 9-14). The mean number and type of the sensilla organs were determined in all flagellomeres (10 segments) of bee workers untreated and treated with black cumin oil and oils mixture. Clearly significant differences in the mean numbers of all tested sensilla organs were recorded untreated and treated worker bees by the essential oils as shown in tables 3&4 as well as with measurement of the Trichodea type (B) and

Placodea sensilla organs. It could be observed that the dimensions of these organs were smaller size than those recorded with the control. This may be attributed to the changes occurred on these sensilla organs due to irritation state effect on the sensory centers. Stort and Rebutini, (1998) found correlation between numbers of the Campaniformea and Coeloconica sensillae organs and the defense behavior of the Africanized honey bees. Rath and Drescher, (1990) and Rosenkranz *et al.*, (1993) found relationship between the hygienic behavior and the infestation with varroa mite as well as within sealed worker brood cells. Salem *et al.*, (2001) found higher numbers of some sensilla organs in antennal tolerant worker bees to Varroa disease. Flottum, (1997) reported that the allogrooming behavior (between bee on bee) the nestmates inspect the hole body of the bee infested with mites, by their antennae, paying greater attention to the petioles region and the wing bases. Foreign particles are picked with the mandibles and eventually chewed.

## 4 -The Haemolymph Protein Analysis

Haemolymph of worker bees treated with oils mixture had more proteins with higher volume than those treated with the black cumin oil and control. Further more new proteins were recorded (bands No.

Table (2): Number of removed dead worker brood and some biological activities with different essential oils treatments.

Treatments	Closed worker brood area (inch <sup>2</sup> )			No. of covered combs with bees		RMV
	BT	AT	increasing level %	BT	AT	
Control	190	255	25	5	9	19.04
Black cumin seeds	240	300	20	6	7	9.75
Oils mixture	200	400	50	6	7	20

BT: Before treatments. AT: After last treatment.  
RMV: Number of removed dead brood bees after 48hr. of the 1<sup>st</sup> treatment

Table (4): Mean measurements of Trichodea type (B) and Placodea sensilla organs of antennal worker honey bees treated with black cumin seed oil and essential oils mixture ( $\pm$ SD).

Treatment	Trichodea type (B)	Placodea sensilla organs		
		Length $\mu$ m	Width $\mu$ m	Area ( $\mu$ m <sup>2</sup> )
Control	17.947a $\pm$ 0.1909	16.095b $\pm$ 0.1283	11.091a $\pm$ 0.04317	280.26a $\pm$ 2.6484
Black Cumin seed oil	17.329b $\pm$ 0.1909	17.222a $\pm$ 0.1283	10.11c $\pm$ 0.04317	273.35b $\pm$ 2.6484
Oils mixture	13.856c $\pm$ 0.1909	14.285c $\pm$ 0.1283	10.742b $\pm$ 0.04317	240.91c $\pm$ 2.6484
LSD <sub>0.05</sub>	0.00125	6.352	0.0070	2.6484

Table (3): Mean numbers of the sensilla organs of worker honey bees antennae treated with some essential oils mixture ( $\pm$  SD).

Item	Antennae sensilla organs								
	Trichodea					Basiconica	Placodea-	Campani- formea	Coeloco- nica
	Type A	Type B	Type C	Long Trichodea Type A	Type B				
Control	11.11 $\pm$ 0.11 <sup>a</sup>	20.20 $\pm$ 0.20	0.6 $\pm$ 0.1	0.0 $\pm$ 0.0	0.20 $\pm$ 0.01	0.0 $\pm$ 0	19.5 $\pm$ 0.1	0.2 $\pm$ 0.01	0.4 $\pm$ 0.01
Black Cumin seed oil	4.7 $\pm$ 0.2	55.9 $\pm$ 0.9	0.6 $\pm$ 0.2	0.6 $\pm$ 0.2	0.0 <sup>c</sup>	0.0 $\pm$ 0	3.5 <sup>c</sup> $\pm$ 0.1	0.0 <sup>c</sup>	0.0 <sup>c</sup>
Essential oils mixture	4.2 $\pm$ 0.2	35.7 $\pm$ 0.7	1.0 $\pm$ 0.1	0.0 $\pm$ 0.0	0.10 $\pm$ 0.01	0.1 $\pm$ 0.01	20.7 $\pm$ 0.7	0.1 $\pm$ 0.086	0.70 $\pm$ 0.01
LSD <sub>0.05</sub>	0.3461	1.3352	0.2825	0.230	0.0163	0.0115	0.8237	0.0163	0.016



Table (5): The haemolymph proteins analysis of worker honey bees treated with essential oils.

Band	Control		Oils mixture		Black cumin seed oil	
	Volume	MW	Volume	MW	Volume	MW
1	95.397.00	155817	199.831.00	154974	325.876.00	56308
2	185.271.00	138404	50.288.00	141669	474.677.00	44018
3	261.166.00	86331	45.348.00	137961	541.342.00	38252
4	256.869.00	79069	247.161.00	59164	1.346.612.00	35622
5	226.298.00	75285	322.818.00	55425	436.788.00	24697
6	860.109.00	59156	898.770.00	45104	585.638.00	21331
7	337.085.00	55639	1.173.702.00	29749	788.319.00	19865
8	691.521.00	44078	350.150.00	25269		
9	202.602.00	28817	408.116.00	21817		
10	836.891.00	20444	1.144.317.00	20189		

MW: Molecular weight.

7, 8 & 9) as shown in Table (5). These proteins may be responsible for the hygienic behavior recorded with the essential oils especially with its mixture. Gatchev and Kantchev, (1998) found changes in the haemolymph protein concentration, Isozyme amount and the pH of bee intestine depends on different active substances of the drug (Amitraz, Cumaphos and Fluvalinate).

It could be established that treated honey bee colonies with essential oils used in controlling Varroa mites can be affected on the hygienic behavior. Essential oils treatment caused dropping higher numbers of dead Varroa mites particularly with black cumin oil treatment in comparison with the other treatments. Force relationship between treatments with black cumin oil, oils mixture and the hygienic behavior especially cutting up varroa mite bodies. The biting behavior started to occur, with oils mixture, faster than black cumin oil and control by one and two months, respectively. In spite of crossing between *Apis mellifera lamarckii* x *A.m. carnica* did not receive any essential oils treatments it recorded higher percentage of the mechanical defense behavior towards Varroa mite infestation almost of effect of the black cumin oil treatment. The removal behavior of dead worker brood cells was not significant in the different treatments. The oils mixture and black cumin oil treatments increased the biological activities of treated bee colonies (number of covered combs with bees & sealed worker brood area). Cutting Varroa mites from legs and coxae may be attributed to selected gene. Separating ventral shield of the Varroa mite prevented the Varroa eggs from development and exercise to deprivation chance of the fertilization. It could be summarized that Separating gnathosoma with portions of the legs from coxae followed with

biting up gnathosoma with segments of legs are considered important grooming behavior recorded with essential oils treatments (Figs 1-8). Also damage to setae responsible for transport sensation to Varroa mite happened (Figs. 1-8). Bogansky, (2007) reported that some bees appearance hygienic behavior properties towards Varroa invasion, thus ensuring another passing bees. Salem *et al.*, (2001) found significant differences in the damage percentage of Varroa bodies among tolerant bee colonies.

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