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EVALUATION OF THE LARVICIDAL ACTIVITY AND EFFECT ON PROTEIN CONFIGURATION OF TWO *BACILLUS* SPECIES AND SIX PLANT EXTRACTS TESTED AGAINST *CULEX PIPIENS* AND *AEDES CASPIUS* LARVAE

*AISHA MOHAMMED ALQAHTANI; * ZAMZAM MOHAMMED ALDHAFAR
**NAEMA B.EL KASSAS AND ** MAGDA HASSAN ABDUL AZIZ

** Faculty of Science –Dammam University - Saudi Arabia*
*** Faculty of Science – Ain-Shams University- Egypt*.
E.mail: drmagdaradi@yahoo.com

Abstract

All tested bacteria and plant extracts proved larvicidal activity against third instar larvae of both mosquitoes *Culex pipiens* and *Aedes caspius* .*Bacillus sphaericus*, indigenous strain (dammam) showed higher activity against *Cx. pipiens* larvae, with LC50 = 0.35x10-7 ppm. Than to *Ae. caspius* (LC50= 4.5x10-7 ppm) .*Bacillus thuringiensis* H-14 (Bactimos) has higher toxicity against *Ae. caspius*, followed by *Cx. pipiens* (LC50=8.0x10-7 and 1.4x10-6 ppm respectively. Both mosquito larvae were susceptible to all tested native plant extracts, *Cleome arabica*, *Fagonia mollis*, *Gomphocarpus sincaicus*, *Origanum syriacum*, *Trichodesma africanum* and *Artemisia judaica* with median lethal doses equal to 125.09, 135.1, 203.03 , 289.5, 310.8 and 450.08 ppm when tested against *Ae. caspius* larvae and 225.07, 188.2, 305.5, 390.4, 420.2 and 650.2 ppm *when tested against C. pipiens* larvae. All treatments led to protein disconfiguration of treated larvae. Fractionation of native proteins, disappearance of some peptides and appearance of new bands are signs obviously recorded after larval treatment with the tested microbial agents or plant extracts.

Key Words: Toxicity of *B.t.i* , *B. Sphaericus* plant extracts. mosquito control. protein profile.

Introduction

Entomopathogenic bacteria and plant extracts are now widely used to control insect pests. During the past five decades many industrial formulations of the famous bacteria *Bacillus thuringiensis* H-14 and *Bacillus sphaericus*, beside some plant extracts proved to have mosquitocidal activity. New strains of the environmentally safe bacteria; *B.t.i* and *B. sphaericus* are daily added to the known strains (Sun, *et al.,* 1996; Vilarinhos, *et al.,* 1996; Thiery & Hamon, 1998; Lecadet, *et al.,* 1999 ; and Park, *et al.,* 2007). The toxic effect varied according to mosquito species and bacterial crystal structure (Gupta, *et al.,* 1991; Theiry, *et al.,* 1992; Nicolas, *et al* , 1992; Krieger, *et al.,*1999; Lecadet, *et al.,* 1999 and Otieno-Ayayo, *et al.,*2008).

However these microbial agents faced some constrains during field application, especially the effect of sun light and U.V. (Saleh, 1989; Walton & Mulla, 1991; Theiry, *et al.*, 1999; Khalaf, 1999b; Dominic Amalaraj, *et al*., 2000; Tawfik, *et al.*, 2000 and Setha, *et al.*, 2007).

Extracted parts from plants appeared to be good candidate in controlling insect pests which may be involved in pest control programs. Different plant extracts were tested against mosquito species (Shalaby, *et al.*, 1998; Khalaf, 1999a; Choochote, *et al.*, 1999; Ansari, *et al.*, 2000; Mansour, *et al.*, 2000; Amer & Mehlhorn, 2006 and Pushpalatha & Muthukrishnan, 2008). *B.t.i*. and *B. sphaericus* are known to destruct epithelial cells of mosquito gut (Nelson-Leroux & Charles, 1992; Ravoahagimalala & Charles, 1995; Charles, *et al.*, 1997; Silva-Filha, *et al.*, 1997& 1999; Krieger, *et al.*, 1999 and Hafez, 2000) While the pathological effect of plant extracts varies, including destruction of gut cells (Koua, *et al.*, 1998) cuticular melanization and lesions (Zebitz,1984). Protein disconfiguration was detected after treatment of mosquitoes with *Bacillus* and some plant extracts (El-Bokl & Moawad, 1996 and Aisha, 2005). Our study compared the toxic effect of *B.t.i, B. sphaericu* and six native plant extracts on larvae of *Culex pipiens* and *Aedes caspius* as vectors of filaria and dengue fever.

Materials and methods:

Tested mosquitoes:

Field larval samples were collected from El-Dammam region –Eastern Zone of Saudi Arabia (KSA) and used to raise laboratory colonies of both *Culex pipiens* and *Ae. caspius* mosquitoes,following the method of Christophers (1960).

1-Bacterial strains :

i- *Bacillus thuringiensis israelensis* Bactimos flowable powder produced by Biochem, Belgium, (1000 IU / mg).

ii- *Bacillus sphaericus* (Local strain- dammam) isolated and identified by Aisha (2005) from KSA. habitat.

Aqueous suspension from both bacteria was used in all bioassays .

Tested plants :

Six native plants collected from the saudian habitats were used, Boyceran (*Artemisia judaica* L.– Compositae), Shaka'ah (*Fagonia mollis* Dell. –

Zygophyllaceae), Hargal (*Gomphocarpus sinaicus* Boiss – Asclepiadaceae), Za'ater (*Origanum syriacum* L.–Labiatae), Himhim (*Trichodesma africanum* L.– Boraginaceae) and Ziyeta (*Cleome arabica* L.– Cleomaceae). To prepare extracts , each tested plant (whole plant)was washed, dried and grinding to prepare aqueous extract .

Bioassays :

Bacillus spp. and plants extracts were tested using the standard method of bioassays recommended by deBarjac & Large,(1979), in which five concentrations of each tested material were prepared and applied to cups containing 20 ml. dist water and twenty 3rd instar mosquito larvae / cup. all experiments were incubated at 27 ± 2 C° for 24-48 hr. Mortality readings were recorded to draw the regression line. Values of LC_{50} & LC_{90} were calculated in ppm through calculating the slope function of the regression line according to Finney(1971).

Protein analysis :

Bio-Rad, protein assay kit was used to estimate the total protein content of both bacterial and plant extract treated larvae as well as healthy ones, as a control.

SDS polyacrylamide gel electrophoretic technique was used to study the protein configuration of treated and untreated 3rd instar mosquito larvae according the method described by Ibarra and Federici (1996).

Results

Effect of *B.t. i.* and *B. sphaericus* on mosquito larvae :

B. sphaericus (dammam isolate) showed high toxicity against both *C. pipiens* and *Ae. caspius* larvae with LC_{50} values ($0.35x10^{-7}$ and $4.5x10^{-7}$ ppm) respectively . Measuring *B.t.i.* toxicity, *Ae. caspius* larvae were more susceptible than *C. pipiens* larvae (LC_{50} = $8.0x10^{-7}$ and $1.4x10^{-6}$ ppm.) as shown in Table (1) .

Susceptibility of mosquito larvae to plant extracts :

Table (2) proved the toxic effect of the used plant extracts against the two tested mosquito larvae. *Fagonia mollis* recorded the highest larvicidal activity against *Cx. pipiens* larvae (LC_{50}: 188.2 ppm) followed by *Cleome arabica*, *Comphocarpus sinaicus*, *Trichodesma africanum*, *Origanum syriacum* and *Artemisia judaica* (LC_{50} values, 225.07, 305.5, 350.3, 390.4 and 650.2 ppm) respectively. Comparing LC_{50}

values after testing toxicity of plant extracts against *Ae. caspius* larvae (Table 2), It seems that *Cleome arabica* induced the highest larvicidal properties (LC_{50}: 125.09 ppm) followed by *Fagonia mollis* (135.1 ppm), *Comphcarpus sinaicus* (203.03 ppm), *Origanum syriacum* (289.5 ppm), *Trichodesma africanum* (310.8 ppm) finally *Artemisia judaica* recorded the lowest activity toward *Aedes* larvae (LC_{50}: 450.08 ppm). LC_{90} values assured the degree of toxicity of used plant extracts toward mosquito larvae.

Effect of tested bacteria and plant extracts on mosquito protein profile :

SDS - PAGE and their analysis, as shown in tables (3&4) proved protein disconfiguration after larval treatment with pathogenic bacteria or the plant extracts.

The total protein analysis of untreated *Cx. pipiens* larvae (Table 3, Lane 9) revealed eight bands of calculated molecular weights 212, 146, 95, 76, 18, 15, 12 and 2.0 KD). Protein fractions, after larval treatment with *B. sphaericus* had altered molecular weights to (76, 44.5, 19, 12, 9.6 KD) as shown in (Table 3, Lane 1). Larval treatment with *B.t.i* fractionate *Culex*, proteins to fourteen bands of (115, 95, 86, 59, 31, 20, 17, 15, 13, 9.4, 8.4, 6.3 and 2.3 KD) as shown in (Lane 7).

Treatment with *Artemisia judaica* (Lane 2) reduced larval proteins to five bands (114, 89, 14, 12, 9.9 KD). Treated larvae with *Cleome arabica* extract has seven protein bands ranged from 160 to 13 KD (Table 3-lane 3). Treatment with *Origanum* reduced protein fractions to six bands with molecular weight ranged from 131 to 3.1 KD (Lane 4). In case of testing *Fagonia* extract (Lane 5) thirteen protein bands appeared, their molecular weights ranged from (205–2.6 KD). Lane 6 and 8, showed protein configuration of *Culex* larvae after treatment with *Gromphocarpus* and *Trichodesma*, the first treatment increased protein fractions to be thirteen subfractions ranged from 197 to 2.3 KD), while second treatment altered protein to nine bands of molecular weight ranged from 131 to 3.5 KD.

Both tested bacteria and plant extracts reduced the number of protein fractions of treated *Aedes caspius* larvae than normal except the treatment with *Artemisia judaica* extract. Table (4-lane2) showed body protein configuration of untreated *Aedes* larvae, consisted of twelve bands of molecular weights 175.14, 43.88, 32.95, 30.41, 20.4, 18.01, 15.15, 11.9, 6.15, 2.65, 0.4 and 0.3 KD. Bacterial treatment reduced larval proteins to four bands, using *B. t. i.* (25, 17.4, 10.9, 0.4 KD) and four bands, using *B. sphaericus* (31.78, 17.42, 15.2 and 2.4 KD) as shown in table (4–

Lanes 3 &4). Treatment with *Fagonia* extract reduced larval body proteins to seven fractions of molecular weights ranging between 280.57 to 0.4 KD (Lane 5) while treatment with *Trichodesma* extract (Lane 6) revealed eight fractions of larval proteins ranged from 212 to 0.6 KD. *Gomphocarpus* extract highly affects larval proteins (Lane 7) it reduced protein fraction to four bands of low molecular weights 47.77, 35.3, 18.35 and 16.9 KD. While treatment with *Artemisia* (Lane 8) increased larval protein fractions to thirteen bands, with molecular weights ranged from 321.7 to 0.31 KD. Lane (9 & 10) in table (4) revealed protein fractions after larval treatment with *Cleome arabica* and *Origanum syriacum* extracts, the first altered larval proteins to nine bands of molecular weights ranged from 253.14 to 0.4 KD, the second reduced proteins to eight fractions of molecular weights ranging between 273.71 to 0.4 KD.

Discussion

Bacillus thuringiensis proved its higher toxicity against *Ae. caspius* larvae than *Cx.pipiens*, this observation was previously confirmed by (Thiery & Hamon, 1998; Silva-Filha, *et al*., 1999 ; Sharma, *et al*., 2008 and Giraldo *et al.*, 2008).

These results are explained by comparing the effect of *B. t. i.* on protein profile of both mosquitoes. Comparing protein profile of *B. t. i*. treated and untreated *Culex* larvae (table 3 Lanes 7 ، 9) we realized splitting of high molecular weight proteins (212, 146 KD) to smaller proteins. New proteins of relatively low molecular weights appeared in *B. t. i*. treated larvae (59, 31, 9.4, 8.4, 6.3 KD). But after treatment of *Aedes* larvae with *B. t. i.* , the number of protein sub fractions reduced to four subfractions only comparing with twelve bands for untreated larvae (Table 4 Lanes 2 ، 3), all larval proteins of high and moderate molecular weights disappeared completely (157.14, 43.88, 32.95, 30.41 and 20.4 KD). No common proteins could be detected between treated and non treated larvae. (Porter, *et al*., 1993; Sun *et al*., 1996; Charles, *et al.*, 1997 and Krieger *et al.*, 1999).

The appearance of protein band of 27 KD may be related to the protein profile of *B.t.i*. not to larval proteins (Lane 8). It characterized and proved the splitting of *Bti* protoxin to the toxic fraction within the larvae of *Aedes caspius* (Sriram and Jayaraman, 1986).

Comparing LC_{50} values, we found that *Culex pipiens* larvae were more susceptible to *B. sphaericus* than *Ae*. larvae (Table 1) as previously mentioned by

Berry, *et al.* (1987) de Barjac, *et al.* (1988), Gupta, *et al.* (1991), Nelson-Leroux & Charles, (1992), Thiery & de Barjac, (1989), Thiery, *et al*. (1992) and Thiery & Hamon, (1998). Protein profile of *B. sphaericus* treated larvae (Table 3 Lane 1) cleared the disappearance of protein bands of high molecular weights that detected in untreated *Cx*. larvae (212, 146 & 95 KD). Two bands were found common between treated and non treated larvae of mol.wt (76 & 12 KD), three protein bands of low molecular weights could not be detected in treated larvae when compared with untreated (18, 15, & 0.2 KD) ones.

Treated *Culex* larvae with *B. sphaericus* reduced larval proteins to four bands. Three new bands appeared during treatment (44.5, 19, 9.6 KD). High denatured protein profile could be detected after larval treatment with *B. sphaericus.* The protein of 44.5 KD which appeared in treated *Culex* larvae may be the characterized crystal protein of *Bacillus sphaericus* (Table 3 - Lane 1). As this protein is known to be a part of the toxic binary proteins of *B. sphaericus* (Nelson – Lerous & Charles 1992).

Extract of *Fagonia mollis* induced high toxicity to both mosquitoes (LC_{50} = 188.2 ppm for *Culex* and 135.6 ppm for *Aedes* larvae). *Fagonia* fractioned *Culex* larval proteins to thirteen bands instead of eight fractions for untreated larvae (Table 3, Lane 5 and 9). Fractions of molecular weights (108, 111, 5.3, 2.6 KD) appeared only after *Culex* treatment with *Fagonia*, while treatment of *Aedes* larvae reduced larval body proteins to seven bands . Protein bands of MWt (43.8, 30.4, 20.4, 18.01, 10.15 KD) disappeared (Fig. 4 Lanes 5, 9). New high mol. weight protein bands appeared after larval treatment (280.57, 218.86 KD). These new proteins may be belonging to the plant proteins.

Cloeme arabica extracts could be considered a promising candidate in mosquito control LC_{50} are 225.07 and 125.09 ppm after treatment of *Culex* and *Aedes* respectively. Protein analysis explains the higher potency against *Aedes* larvae. Protein bands are reduced to four bands (Table 4 – Lane 3), All protein bands of high molecular weights (253.14, 61.98, 35.5 KD), in control larval proteins (Table 4 – Lane 9) were disappeared completely. One low molecular weight protein band of 0.4 KD could be detected between *Cleome* treated and control *Aedes* proteins. Treatment of *Culex* with *Cleome* (Table 3 Lane 2) affect normal proteins of low molecular weights only (18, 2.0 KD – Lane 9).

Artemisia sp. reduced *Culex* proteins to five bands, no identical bands with control samples could be detected. While treatment of *Aedes* increased the number of protein subfractions to twelve with one common band with normal larvae (0.4 KD). Although *Artimisia* sp. recorded the least toxicity against both mosquito larvae, (table 2) .

Origanum sp. extract does not induce great changes within *Culex* or *Aedes* protein configuration. In case of *Culex* proteins, many bands appeared common between treated and untreated larvae (Table 3, Lane 4 ، 9). In case of *Aedse* larvae two proteins are common (Table 4 Lane 4 ، 9). This plant extract is more toxic to *Aedes* than *Culex* (LC_{50} 289.5 ppm for *Aedes* and 390.4 ppm for *Culex* larvae).

Aedes larvae were more susceptible to *Comphocarpus* extract than *Culex* larvae (LC_{50} 203.03, 305.5 ppm) respectively. The plant extract reduced body proteins of *Aedes* to four fractions instead of 12 bands in normal larvae (Table 4, Lanes 7 ، 9). All proteins of low molecular weights disappeared completely, while after treatment of *Culex* larvae, thirteen protein fractions appeared (Table 3 Lanes 6 ، 9). Splitting of some proteins could be detected.

Trichodesma sp. extract has moderate lethal effect on both mosquito larvae. *Culex* larvae were less susceptible (LC_{50}420.2 ppm) with slight changes of body proteins but *Aedes* larvae showed great reduction of body proteins after treatment (Table 4, Lanes 8 ، 9). All high and moderat molecular weight proteins disappeared. This may explain the relatively low LC_{50} (310.8 ppm – Table 2) for *Aedes* larvae.

Adverse effect of microbial agents and plant extracts on insect proteins was proved previously by (Singh & Kumari, 2003).

Conclusion

Our results proved the toxicity of tested plant extracts against larvae of *Culex pipiens* and *Aedes caspius*. In addition to the well known *Bacilli* species (*Bti & B.sphaericus)*. *Cleome arabica*, *Fagonia mollis* and *Comphocarpus sinaicus* extract, proved its toxicological and biochemical effect against mosquito larvae., so we recommend these plants as potential larvicidal agents, beside, these plants are considered ideal eco-friendly approach in biological control. Plant extracts as safe tools for mosquito control agents were recommended previously by Abdul Rahuman, *et al.*, 2007; Pandey, *et al.*, 2007; Abdel Rahman & Venkatesan , 2008.

Table 1: Susceptibility of 3rd instar larvae of *Cx. pipiens* and *Aedes Caspius to Bacillus thuringiensis* and *B. sphaericus.*

bacterial species Con. ppm	*.B.t.i*			*B. sphaericus* (local strain)			
	C. pipiens	*Ae. Caspius*		*Ae .caspius*		*C. pipiens*	
	.M %	.Conc	.M %	.Conc	% .M	.Conc	.M %
$10^{-6} \times 2.0$	8o.3	$\times 2.0\ 10^{-6}$	92.3	$\times 8.0\ 10^{-6}$	90	$\times 1.5\ 10^{-7}$	92.3
$10^{-6} \times 1.5$	55.8	$10^{-} \times 1\ 6$	72.5	$\times 3.0\ 10^{-6}$	80	$\times 0.7\ 10^{-7}$	86.7
$10^{-6} \times 1$	31	$\times 5.0\ 10^{-7}$	40	$\times 7.0\ 10^{-7}$	70	$\times 0.3\ 10^{-7}$	50
$10^{-6} \times 0.7$	11	$\times 2.5\ 10^{-7}$	14.1	$\times 5.0\ 10^{-7}$	56.7	$\times 0.7\ 10^{-8}$	35.3
$10^{-6} \times 0.4$	5.3	$\times 0.8\ 10^{-7}$	6.6	$\times 3.0\ 10^{-7}$	26.7	$\times 0.3\ 10^{-8}$	16.5

Table 2: Susceptibility of 3rd instars larvae of *Cx. pipiens* and *Ae. caspius* to plant extracts

Plant exstract	Conc. Ppm	% M.		LC_{50}		LC_{90}		X^2		Significance P value	
		C. pipiens	*Ae. caspius*	*C. pipiens*	*Ae. caspius*	*C. pipiens*	*Ae. caspius*	*C. pipiens*	*Ae. caspius*	*C. pipiens*	*Ae. caspius*
Cleome arabica	600	96.7	95.7	225.07	125.09	420.02	320.9	6.3	5.8	< 0.0001 sig.	< 0.0001 sig.
	400	93.2	92								
	300	60	80								
	200	45	61								
	100	19	42.5								
Artemisia judaica	800	63	97	650.2	450.08	1052	715.5	1.19	1.67	< 0.0001 sig.	< 0.0115 sig.
	600	42.8	85.1								
	500	36.5	63.2								
	400	25.5	43.4								
	250	16.5	20.5								
Fagonia mollis	350	84	89	188.2	135.1	390.2	340.1	2.4	1.9	< 0.0006 sig.	< 0.0003 sig.
	250	71	85.2								
	150	43.5	72								
	120	40	55.9								
	100	29	49								
Comphocarpus sinaicus	600	92	95	305.5	203.03	550.0	550.6	7.4	5.2	< 0.0003 sig.	< 0.0001 sig.
	500	89	88								
	400	65.6	71								
	250	40.9	55.8								
	100	25	30								
Origanum syriacum	600	92.5	79.9	390.4	289.5	590.2	620.5	2.99	4.52	< 0.0001 sig.	< 0.0002 sig.
	500	57.2	71								
	300	48.8	55.5								
	200	42.1	43.9								
	100	20	15.2								
Trichodesma africanum	500	72.4	75	420.2	310.8	640.1	625	2.6	0.7	< 0.0001 sig.	< 0.0002 sig.
	400	52	60.02								
	300	38.2	45.5								
	200	25.5	41.8								
	100	15	25								

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Table (3) : Protein configuration of *Culex pipiens* larvae treated with bacterial spp. and plant extracts.

Lanes / **Rows**	**Molecular weight (KD)**									
	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7	Lane 8	Lane 9	Lane 10
1									212	212
2					205					
3						197				
4			160							
5									146	
6						136				
7				131				131		
8										116
9							115			
10		114								
11					111					
12			110							
13								108		
14					106					
15				98						
16					95	95	95		95	95
17			92							
18		89								
19							86			
20					85	85				
21					79					
22	76			76					76	
23							68	68		
24										66
25						64				
26					63					
27			61							
28							59			
29										58
30					49					
31	44.5									
32										40
33							31			31
34								29		
35								27		
36			23							
37						20	20			20
38	19									
39									18	
40				17		17	17	17		17
41			16		16					
42						15	15	15	15	
43		14		14						14
44			13		13	13	13	13		
45	12	12			12				12	
46		9.9								
47	9.6					9.6				
48							9.4			
49							8.4			
50							6.3			
51						5.8				
52					5.3					
53						4.1				
54				3.8						
55								3.5		
56					2.6					
57						2.3	2.3			
58									2.0	

Lane (1) : Treatment with *B. sphaericus* , Lane (2) : Treatment with *Artemisia judaica* extract , Lane (3) : Treatment with *Cleome arabica* extract , Lane (4) : Treatment with *Origanum syriacum* extract , Lane (5) : Treatment with *Fagonia mollis* extract , Lane (6) : Treatment with *Gomphocarpus sinaicus* extract , Lane (7) : Treatment with *B. t. i.* , Lane (8) : Treatment with *Tricodesma africanum* extract , Lane (9) : Proteins of untreated *Culex* larvae , Lane (10) : Standard molecular weight proteins .

Table (4) : Protein configuration of *Aedes caspius* larvae treated with bacterial spp. and plant extracts.

Lanes Rows	Lane 1 (molw)	Lane 2 (molw)	Lane 3 (molw)	Lane 4 (molw)	Lane 5 (molw)	Lane 6 (molw)	Lane 7 (molw)	Lane 8 (molw)	Lane 9 (molw)	Lane 10 (molw)
								321.71		
					280.57					
										273.71
									253.14	
					218.86					
	212					212				
		175.14						198.29		
	116									
								101.6		
	97.4									
						75.56				
	66.2									
								61.45	61.98	
										52.9
						51.01				
							47.77	47.13		
		43.88								
							35.3	35.30	35.5	35.1
					33.93	33.73				
		32.957								
	31			31.78						
		30.411								
								26.289		
			25							
									23.344	
	20.4	20.4								
								19.461		19.376
		18.01				18.607	18.351		18.095	
			17.4	17.42				17.327		
	16.9					16.15	16.9			
		15.15		15.2						
	14.4								14.9	14.15
								12.9		
		11.9								
			10.9							10.9
					9.4			9.4		
						8.4			8.4	
		6.15			6.65					
	4.15									
								3.9	3.9	
	2.65	2.65		2.4	2.65					
										1.15
						0.6				
		0.4	0.4		0.4				0.4	0.4
		0.3						0.31		

Lane (1) : Molecular weight standard proteins , Lane (2) : Treatment with *Artemisia judaica* extract , Lane (3) : Treatment with *Cleome arabica* extract , Lane (4) : Treatment with *Origanum syriacum* extract , Lane (5) : Treatment with *Fagonia mollis* extract , Lane (6) : Treatment with *Tricodesma africanum* extract , Lane (7) : Treatment with *Gomphocarpus sinaicus* extract , Lane (8) : Treatment with *B. t. i.*, Lane (9) : Proteins of normal *Aedes* larvae , Lane (10) : Treatment with *B. sphaericus* .

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