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Sublethal concentrations of *Beauveria bassiana* affect biochemical aspects of *Culex pipiens* larvae

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Abstract
The biochemical effects of entomopathogenic fungi, *Beauveria bassiana* were studied in larvae of *Culex pipiens* in laboratory. Results revealed that the mean total glucose, protein and lipid contents decreased after treatment of second instar larvae with LC_{25}, LC_{50} and LC_{90} compared with control larvae 96 h post treatment. The decreasing effect was dose dependent. A noticeable decrease in the activity of acetylcholine esterase, alpha esterase and beta esterase compared with control was recorded. Additionally, the activity of Glutathione- S-transferase was reduced after treatment with LC_{25}, LC_{50} and LC_{90} of *B. bassiana*. Similarly, data showed that *B. bassiana* treated larvae induced a significant decrease of peroxidase activity and ascorbic acid compared to that of control larvae.
Changes in electrophoretic protein pattern of untreated and treated second instar larvae of *C. pipiens* were analyzed after 96 hrs of treatment using (SDS-PAGE) and significant changes in the total protein profile of *C. pipiens* larvae were observed.

Keywords: *Beauveria bassiana*, *Culex pipiens*, acetylcholine esterase, alpha esterase, beta esterase, glutathione- S-transferase, peroxidase and SDS-PAGE

Introduction
Mosquitos are medically important insects closely related to the life of human beings. The harm of mosquitoes to human beings is not only because of the harassment and blood feeding habits, but also due to their transmission of various diseases, such as malaria (Wood *et al* 2010) [51], filariasis (Pedersen *et al* 2009) [37], yellow fever (Salvemini *et al* 2011) [39], dengue (Wu *et al* 2010) [52], and Japanese encephalitis (Dutta *et al* 2011) [16]. Enormous efforts have been required to control these diseases including environmental management, the use of insecticides and repellents, vaccine research and biological mosquito control (Itokawa *et al* 2011) [26]. Insecticides play a central role in controlling mosquitoes, but recently, more serious insecticide resistance has appeared in mosquitoes against every chemical class of insecticides, such as organochlorine, carbamate, organophosphate, pyrethroid and insect growth regulators (Wang and Pantopoulos 2011) [50]. Biological control is confident in pest management using different predators, parasites and pathogens. Among different micropathogens, entomopathogenic fungi are considered unique due to their wide host range, way of their pathogenicity and specificity to juice sucking pests such as mosquitoes, aphids and chewing pests (DeFaria and Wraight, 2007) [12]. Entomopathogenic fungi can be used for pest control without affecting other non-target organisms (Khetan, 2001) [29]. They are currently being used for the control of several insect pests as alternatives or supplements to chemical insecticides (Fan *et al*., 2007) [19, 53]. The genus *Beauveria* contains at least 49 species of which approximately 22 are considered pathogenic (Kirk, 2003) [31]. *Beauveria bassiana*, a white muscardine fungus, is the most commonly used fungi in this genus known as the causative agent of a white (later yellowish or occasionally reddish) muscardine disease in domestic silkworms (Furlong & Pell, 2005; Zimmermann, 2007) [21, 54].
B. bassiana fungus grows naturally in soils throughout the world and acts as a pathogen on various insect species (Sandhu and Vikrant 2004; Jain *et al*., 2008) [40, 27]. An interesting feature of *Beauveria* sp. is the high host specificity of many isolates. Hosts of agricultural and forest significance include the Colorado potato beetle, the codling moth, and several genera of termites, American bollworm, *Helicoverpa armigera* (Thakur and Sandhu 2010) [47]. *B. bassiana* can easily be isolated from insect cadavers or soil in forested areas (Beilharz *et al*., 1982) [6], as well as by baiting soil with insects. In laboratory it can be cultured on simple media (Roberts & Hajek, 1992; Goettel & Inglis, 1997) [38, 24].

B. bassiana is considered one of the most effective entomopathogenic fungi due to their cosmopolitan distribution (Bidochka *et al.,* 1998) [7], ability to infect any life stage of its host, wider host range than the other Deuteromycetes (Roberts and Hajek, 1992) [38] and can infect certain plant tissues (Bing and Lewis, 1992) [8].

There are few studies examined the biochemical effects of *B. bassiana* against mosquitoes. Therefore, the objective of this study was to investigate the biochemical effects of *B. bassiana* after treatment of second instar larvae of *C. pipiens* with sub lethal concentrations under laboratory conditions. The present investigation is an effort to elucidate mosquito physiological reactions following *B. bassiana* treatment.

Materials and methods

Insect culture and bioassays

C. pipiens used in the present study was obtained from susceptible reared strain of Research Institute of Madical Entomology, Dokki, Egypt. The colony was maintained under laboratory conditions of 27±2 ^{0}C and 75± 5% R.H. according to El-bokl and Moawad (1996) [17]. Second instar larvae were collected for bioassay tests. Different concentrations of *B. bassiana* were prepared by dissolving the powder in water. In each test, larvae were put in a plastic cup with100 ml tap water and then treated with *B. bassiana*. Each test was replicated three times. Control experiments were performed using water only. The percentage of mortality (Data not published here) and resultant LC_{25}, LC_{50} and LC_{90} were calculated according to Finney (1971) [20], using "Ldp line" software by (Bakr, 2000) [5]. After 96 hours of treatment, larvae were separated and used to subsequent analysis.

Biochemical assays

Preparation of insects for biochemical assays

Insects were homogenized in distilled water (50 mg /1 ml) in a chilled glass Teflon tissue homogenizer (ST – 2 Mechanic-Preczyina, Poland) as described by Amin (1998) [3]. Homogenates were centrifuged at 8000 r.p.m. for 15 min at 2 ºC in a refrigerated centrifuge. The deposits were discarded and supernatants were stored at -20 ºC till use for biochemical assays. Double beam ultraviolet/visible spectrophotometer (spectronic 1201, Milton Roy Co., USA) was used to measure absorbance of colored substances or metabolic compounds.

Quantitative determination of total glucose, proteins and lipids

Total proteins were determined according to Bradford (I976) using Coomassie Brilliant blue G-250(sigma chemical co.) as protein reagent. Total glucose was extracted and prepared for assay according to Crompton and Birt (1967) [11]. Total lipids were estimated by the method of Knight *et al*. (1972) [30] using phosphovanillin reagent.

Quantitative determination of acetylcholinesterase and non-specific esterases

Acetylcholinesterase (AchE) activity was measured according to Simpson *et al*. (1964) using acetylcholine bromide (AchBr) as substrate. Alpha (α) esterases and beta (β) esterases were determined according to Van Asperen (1962) using α-naphthyl acetate or β-naphthyl acetate as substrates (respectively,).

Quantitative determination of Glutathione S-Transferase (GST), peroxidase and ascorbic acid

Glutathione S-transferase (GST) was detected as described by the method of Habig *et al*. (I974) [25]. Peroxidase activity was determined according to Vetter *et al*. (I958) [49]. Ascorbic acid method (A.O.A.C., 1975) [4] is based on measurement of the extent to which a 2, 6-dichlorophenol-indophenol dye solution is decolorized by the presence of ascorbic acid.

Protein electrophoresis by SDS-PAGE

Preparation of the gels followed the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970) [33]. The gel was prepared from monomer solution of 30% acrylamide and 0.8% N-N-bis-methylene-acrylamide. The denatured gels prepared as 12 % of separating gel in 1.5 M Tris-HCl buffers (pH 8.8) and 3 % of stacking gel in 0.5 M Tris-HCl buffer (pH 6.8).

Statistical analysis

All experiments contained 3 replicates and the results were analyzed by one way analysis of variance (ANOVA) using SPSS statistical software, Version 21.

Results

The effect of sub-lethal concentrations of *B. bassiana* on treated second instar larvae of *C. pipiens* on total glucose, protein and lipid content were recorded in Table (1). Total glucose contents were 11.17, 11.57 and 11.77 mg/gm in larvae of *C. pipiens* treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively as compared with 12.27 mg/gm in control larvae revealing a non-significant decrease. The total protein contents were 34.4, 25.77 25.1 mg/gm in larvae of *C. pipiens* treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively as compared with 36.4 mg/gm in control larvae. Similarly, total lipid contents were 9.34, 8.38 and 6.1 mg/gm in larvae of *C. pipiens* treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively as compared with 14.34 mg/gm in control larvae showing significant decrease after treatment with sub-lethal concentrations of *B. bassiana*. The decreasing effect in the three main body metabolites was dose-dependent.

The impacts of sub-lethal concentrations of *B. bassiana* on the activity of acetylcholine esterase, alpha esterase and beta esterase were demonstrated in table (2). The activities of acetylcholine were 4.87, 4.83 and 2.98 (ug AchBr/min/mg protein) in larvae of *C. pipiens* treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively, as compared with 5.28 (ug AchBr/min/mg protein) in control larvae. Acetylcholine esterase activity significantly decreased after treatment with sub-lethal concentrations of *B. bassiana*. Alpha esterase activity insignificantly decreased after treatment with sub-lethal concentrations of *B. bassiana* and the activity was 10.60, 10.94 and 10.98 ((*ug α- naphthol/min/mg protein*)) in larvae of *C. pipiens* treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively as compared with 16.33 ((*ug α-naphthol/min/mg protein*)) in control larvae. The activities of beta esterase were 20.50, 15.79 and 14.51 ((*ug β - naphthol/min/mg protein*)) in larvae of *C. pipiens* treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively as compared with 27.23 (*ug β - naphthol/min/mg protein*)) in control larvae showing significant decrease after treatment with sub-lethal concentrations of *B. bassiana*.

The activity of glutathione- S-transferase, peroxidase and ascorbic acid was recorded in table (3). The activities of Glutathione- S-transferase were 6265, 5717 and 3521 (*n-mol-sub. conjugated/min/mg protein*) in larvae of *C. pipiens*

treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively as compared with 6745 (*n-mol-sub. conjugated/min/mg protein*) in control larvae. Glutathione- S-transferase activity significantly decreased after treatment with sub-lethal concentrations of *B. bassiana*. The activity of peroxidase was 264.67, 252 and 245.67(Δ*O.D. x103/min/mg protein*) in larvae of *C. pipiens* treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively as compared with 276.67 (Δ*O.D. x103/min/mg protein*) in control larvae (Table 3). Peroxidase activity significantly decreased after treatment with sub-lethal concentrations of *B. bassiana*. The activity of ascorbic acid was 9.65, 9.47 and 8.67(*ug Ascorbic acid/g.b.wt.*) in larvae of *C. pipiens* treated with LC_{25}, LC_{50} and LC_{90} *B. bassiana*, respectively as compared with 10.07 (ug *Ascorbic acid/g.b.wt.*) in control larvae (Table 3). Ascorbic activity significantly decreased after treatment with sub-lethal concentrations of *B. bassiana*.

SDS- PAGE results

Changes in electrophoretic protein pattern of untreated and treated second instar larvae of *C. pipiens* were analyzed after 96 hrs of treatment using (SDS-PAGE). Electrophoretic protein patterns are shown in Fig. (1). The SDS protein pattern of larval proteins showed different numbers of protein bands according to their molecular weights and revealed differences between untreated and treated larvae (Table 4). The control larvae were separated into 20 protein bands with molecular weight ranged from 275.50 to 14.18 kDa. The number of bands of larvae treated with LC_{25}, LC_{50} and LC_{90} of *B. bassiana* were 14, 11 and 10 bands, respectively compared with 20 bands in control larvae. The molecular weight of bands in larvae treated with LC_{25} of *B. bassiana* was 211.05, 188.37, 136.61, 111.61, 97.94, 88.92, 76.80, 69.40, 55.60, 43.64, 39.73, 33.89, 28.81 and 25.40 kDa**.** The molecular weight of bands in larvae treated with LC_{50} of *B. bassiana* was 211.05, 136.61, 111.61, 97.94, 76.80, 55.60, 43.64, 39.73, 33.89, 28.81 and 25.40 kDa. The molecular weight of bands in larvae treated with LC_{90} of *B. bassiana* was 211.05, 136.61, 111.61, 97.94, 76.80, 55.60, 43.64, 33.89, 28.81 and 25.40 kDa. There were 9 common bands between control and treated larvae with molecular weight approximately 211.05, 136.61, 111.61, 97.94, 76.80, 33.89, 43.64, 28.81 and 25.40 kDa. There was 1 common band between control and larvae treated with LC_{25} of *B. bassiana* with molecular weight 88.92. There were 8 characteristic bands for the control larvae with molecular weight 275.50, 254, 226.19, 61.21, 51.72, 46.90, 22.35 and 14.18 kDa. There was one characteristic band for larvae treated with LC_{25} and LC_{50} of *B. bassiana* with molecular weight 69.40 and 39.73 kDa, respectively. Treatment of larvae with LC_{25} of *B. bassiana* caused the disappearance of 9 bands and appearance of three unique bands while treatment LC_{50} of *B. bassiana* caused the disappearance of 10 bands and appearance of one unique band. Additionally, treatment of larvae with LC_{90} of *B. bassiana* caused the disappearance of 10 bands with no appearance of any new bands indicating a disturbance in the immune system and proteins. The obtained results indicated that the application of entomopathogenic fungi *B. bassiana* as larvicidal agents against mosquito larvae caused significant changes in the total protein profile of *C. pipiens* larvae.

Discussion

As indicated from the obtained results, the protein contents of larvae treated with LC_{25}, LC_{50} and LC_{90} of *B. bassiana* were significantly reduced compared to control larvae. The obtained result is in agreement with Abdou *et al* (2017) [1] who studied the biochemical effects of two entomopathogenic fungi, *B. bassiana* and *Metarhizium anisopliae*, in the 3rd instar larvae of *C. pipiens* in laboratory and observed a significant reduction in the total proteins of the treated larvae compared to control. In accordance with this study, Sree and Joshi (2015) [45] who indicated that, inoculation of fungal pathogen *B. bassiana* in silkworm larvae resulted in a significant reduction in protein content of haemolymph. Sahayaraj and Borgio (2010) [41] found that *B. bassiana* and *M. anisopliae* reduced total body protein content in *Dysdercus cingulatus*. The reduction in protein level resulted from damage of protein molecules and alteration of certain amino acid side chains, which leads to alteration in its properties to the point where it can no longer serve its usual purpose (Spikes and Macknight, 1970) [44]. Callaham *et al*., (1977) [10] suggested that this decrease might be due to accumulative energy stress on the organism.

Detoxification enzymes in insects such as acetylcholine esterase and glutathione- S-transferase can effectively metabolize the exogenous toxic compounds (Zhang *et al*. 2001) [26]. GST conjugates endogenous glutathione with toxic pro-electron material to form a nucleophilic center that protects substances such as proteins and nucleic acids and acts as a means of excreting toxic substances. Also, they play important roles in maintaining the normal physiological activities in the body (Kontogiannatos *et al*. 2011) [32]. GST serves a variety of physiological and metabolic functions (Su *et al*. 2007) [46]. GST is able to catalyze harmful or polar compounds with glutathione and can discharge various potential toxic compounds and some carcinogens from the body in a non-enzymatic pattern (Ding 2007) [13]. In this study, the activity of GSTwas determined in *C. pipiens* larvae 96 h following treatment with *B. bassiana.* The decreased GST activity could be due to the damage caused by the fungal toxins. In accordance with these results, Ding *et al*. (2005) [14] studied the effects of 4 strains of 2 species of *Beauveria* against larvae of *Xylotrechus rusticus* and reported that the activity of GST (first increased after treatment and then slowly decreased. The enzyme activity peaked at 72 to 96 h post-infection.

Acetylcholine esterase is an important insect hydrolase for maintaining normal functions of the nervous system through rapid hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid to stop nerve impulse transmission. Organophosphate and carbamate pesticides function primarily by binding to AchE and inhibiting its catalytic activity (Niu *et al*. 2005; Machado *et al*. 2012) [36, 35]. When AchE is suppressed, acetylcholine cannot be decomposed in a timely manner and is accumulated in the synaptic cleft, resulting in neural hyperactivity, convulsions, poisoning, and death. In the present results, AchE activity in treated larvae was suppressed. This may be due to release of fungal toxin in the body gradually reducing the efficacy of larvae defense system directly impacting the larvae nervous system.

The present findings are in agreement with Ali, *et al*. (2017) [2] who showed that AChE activity decreased in *Bemisia tabaci* when treated with the fungus *Lecanicillium muscarium*. Also, the activity of AChE in *L. migratoria* under different treatment conditions with the fungus *M. anisopliae* increased during the early period but decreased during the later period (Jia, *et al.,* 2016) [28]. Additionally, Ding *et al*. (2005) [14] studied the effects of 4 strains of 2 species of

Beauveria against larvae of *X. rusticus* and reported that the activity of AChE first increased after treatment and then slowly decreased. The enzyme activity peaked at 72 to 96 h post-infection.

Under stress conditions, the insect produces large quantities of reactive oxygen species such as superoxide anion hydroxyl radical. These compounds are cytotoxic and help the insect to kill pathogens and parasites. However, excessive reactive free oxygen radicals can damage the organism itself (Li *et al*. 2006) [34]. The protective enzyme system in the insect body includes superoxide dismutase, catalase, and peroxidase. All these enzymes work coordinately to maintain the organism in a state of dynamic equilibrium by keeping free radicals in the cell at low levels to prevent the cells from damage (Gao *et al*. 1995) [23]. Studies have shown that, after infection by fungi, the insect's protection systems are activated to ward off infection and to maintain the normal physiological activities (Song *et al*. 2002; Zhang *et al*. 2003) [43, 55]. In the same context in accordance with the present results, Ding *et al*. (2005) [14] reported that the enzyme activities were peaked at 72 to 96 h post-infection where larvae produced toxic oxidation substances that induced the synthesis of peroxidase by the immune system to maintain normal physiological functions. However, at 96 h post-infection, possibly due to the toxins, peroxidase enzyme synthesis was affected, and its activity decreased (Ding *et al* 2007) [13]. Furthermore, due to the proliferation of *B. bassiana* toxins in the larval body, the organ functions in the larvae were blocked and their normal physiological activities were affected resulting in the inhibition of protein synthesis and protein transportation.

The electrophoretic analysis of SDS-PAGE protein of control and treated larvae of *C. pipiens*, with sub-lethal dose of *B. bassiana* showed changes in protein pattern. Similar results were observed by Abdou *et al* (2017) [1]. They indicated that the total numbers of bands of control larvae were 21 with molecular weights ranged from 176.79 to 4.88 kDa, while 14 bands in treated samples with *B. bassiana*. The present results are in agreement with El-Sonbaty *et al*., (2016) [18] who conveyed similar analysis on haemolymph protein profile of *Spodoptera littoralis* larvae and showed 14differentially expressed protein bands ranging from 9.6 - 116.2 KDa post treatment with entomopathogenic fungi. The infection distinctly affected protein profiles and can be manipulated in the proteins of molecular weight in the range of 56.9-82.6 KDs of prophenoloxidases and phenoloxidases. They concluded that entomopathogenic fungi treatment greatly affected cellular immune system and protein expression consequently, result into death of insect due to disturbance in the immune system and proteins. Gabarty *et al*., (2013) [22] reported that SDS protein analysis of the *S. littoralis* larvae revealed that the immune enzymes activity and protein concentration were significantly decreased at second, third, and fourth day of treatment with *B. bassiana* and *M. anisopliae*. Abdou *et al*. (2017) [1] concluded that the application of entomofungi as larvicidal agents against mosquito larvae caused significant changes in the total protein profile of *C. pipiens* larvae suggesting that toxins secreted by these pathogens caused damage to the larval proteins which finally leads to larval death.

The current study concluded that the examined *B. bassiana* sublethal concentrations had a potent inhibitory response against main body metabolites, detoxification and antioxidant enzymes of *C. pipiens* larvae proceeded until the fourth day after treatment. Hence, it could be successfully used in biological control of *C. pipiens* larvae.

Table 1: Effect of *B.bassiana* on total glucose, proteins and total lipids of the 2nd instar larvae of *C. pipiens*

Conc. (ppm)	Total glucose (mg/g.b.wt.)	Total proteins (mg/g.b.wt.)	Total lipids (mg/g.b.wt.)
0.0	12.27 ±0.2 [a]	36.4 ±0.1 [a]	14.43 ±0.5 [a]
LC_{25}	11.17 ±0.5 [b]	34.07 ±0.4 [b]	9.43 ±0.3 [b]
LC_{50}	11.57 ±0.3 [b]	25.77 ±0.7 [c]	8.83 ±0.6 [c]
LC_{90}	11.77 ±0.9 [b]	25.1 ±0.2 [d]	6.1 ±0.8 [d]
F	133.4	5.7	71.3

Data was expressed as mean ± standard error (SE). Means with different letters within column are significantly different. $P<0.05$ considered significant.

Table 2: Effect of *B. bassiana* on acetylcholine esterase, Alpha esterase and Beta esterase of the 2nd instar larvae of *C.pipiens*.

Conc. (ppm)	Acetylcholine esterase (ug AchBr/min/mg protein)	Alpha esterase (ug α- naphthol/min/mg protein)	Beta esterase (ug β- naphthol/min/mg protein)
0.0	5.28 ±0.2 [a]	16.33 ±0.5 [a]	27.23 ±0.9 [a]
LC_{25}	4.87 ±0.7 [b]	10.60 ±0.8 [b]	20.5 ±0.3 [b]
LC_{50}	4.83 ±0.3 [c]	10.94 ±0.6 [b]	15.79 ±0.7 [c]
LC_{90}	2.98 ±0.6 [d]	10.98 ±0.4 [b]	14.51 ±0.5 [d]
F	59.21	70.43	60.00

Data was expressed as mean ± standard error (SE). Means with different letters within column are significantly different. $P<0.05$ considered significant.

Table 3: Effect of *B. bassiana* on glutathione- S-transferase, peroxidase and Ascorbic acid of the 2nd instar larvae of *C. pipiens*.

Conc. (PPm)	Glutathione- S-transferase (*n-mol-sub. conjugated/min/mg protein*	Peroxidase (Δ*O.D. x103/min/mg protein*	Ascorbic acid (ug *Ascorbic acid/g.b.wt.*)
0.0	6745 ±2.1[a]	276.67 ± 1.1 [a]	10.07 ± 0.18 [a]
LC_{25}	6265 ±2.6[b]	264.67 ±1.3 [b]	9.65 ± 0.08 [b]
LC_{50}	5717 ±2.9[c]	252 ±0.8 [c]	9.47 ±0.33 [c]
LC_{90}	3521 ±2.7[d]	245.67 ±0.4 [d]	8.67 ±0.19 [d]
F	244.10	5.40	7.67

Data was expressed as mean ± standard error (SE). Means with different letters within column are significantly different. $P<0.05$ considered significant.

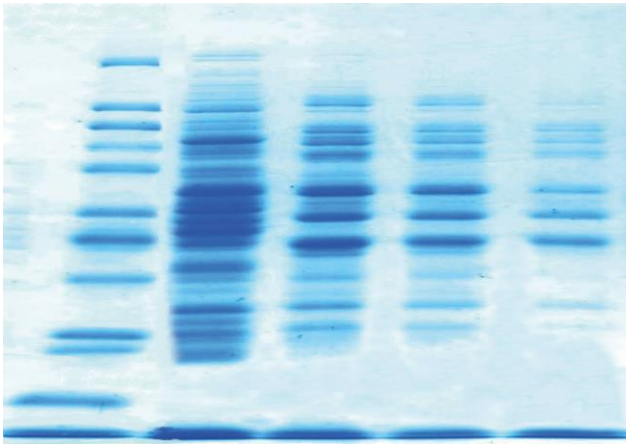

Fig 1: Electrophoretic protein pattern of control and treated 2nd instar larvae of *C. pipiens* after 96 hrs. M: Marker, Lane 1: Control samples, Lane 2: larvae treated with LC_{25} *B. bassiana*, Lane 3: larvae treated LC_{50} *B. bassiana* and Lane 4:l arvae treated with LC_{90} of *B. bassiana*.

Table 4: Molecular weight analysis of Electrophoretic protein pattern of control and treated larvae of *C. pipiens*. Lane M: protein marker. 1: Control larvae, 2: larvae treated with LC_{25} *B. bassiana*, 3: larvae treated LC_{50} *B. bassiana* and Lane 4: larvae treated with LC_{90} of *B. bassiana*.

M. Wt. of bands	RF	1	2	3	4
		Band %	Band %	Band %	Band %
275.50	0.1168	2.0	--	--	--
254.00	0.1629	0.9	--	--	--
226.19	0.2144	2.7	--	--	--
211.05	0.2251	--	11.3	8.0	3.2
188.37	0.2390	6.1	2.8	--	--
136.61	0.2787	2.5	4.9	7.2	7.8
111.61	0.3076	12.3	9.6	8.8	5.7
97.94	0.3344	0.7	10.2	5.7	5.4
88.92	0.3687	2.0	2.5	--	--
76.80	0.4180	6.9	16.1	16.3	21.3
69.40	0.4405		2.9	--	--
61.21	0.4609	1.8	--	--	--
55.60	0.4770		6.5	16.6	21.5
51.72	0.4845	1.0	--	--	--
46.90	0.5091	1.2	--	--	--
43.64	0.5338	0.6	18.1	21.8	26.3
39.73	0.5563		2.0	0.6	--
34.84	0.5895	2.9	--	--	--
33.89	0.6152	0.1	1.5	1.5	0.5
30.87	0.6431	0.1	--	--	--
28.81	0.6795	9.7	6.8	9.3	6.3
25.40	0.7063	1.5	4.8	4.2	2.1
22.35	0.7320	0.9	--	--	--
14.18	0.7792	11.6	--	--	--

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