

***In Vitro* and *In Vivo* Induction, and Characterization of Beauvericin Isolated from *Beauveria bassiana* and Its Bioassay on *Galleria mellonella* Larvae**

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ABSTRACT

Entomopathogenic fungi produce secondary metabolites which may bioactively help fungus in its virulence toward insect hosts. *Beauveria bassiana* produces several toxic low molecular compounds *in vitro* as well as *in vivo*, the most important of them is Beauvericin. The BEH isolate of the fungus was selected for Beauvericin assay. Beauvericin was obtained from surface and submerged cultures of the fungus in PDB and PDA, culture filtrates, and *in vivo* conidia harvested from insect cadavers. Results indicated that *in vivo* fungal conidia contained the most Beauvericin, causing a higher mortality to *Galleria mellonella* larvae as compared with *in vitro* fungal products in their different concentrations. Beauvericin chromatogram revealed that Beauvericin was in its greatest quantity in comparison with the other secondary metabolites of BEH isolate. The impact of Beauvericin on mean larval survival and on paralysis time was in agreement with bioassay data showing lower ST₅₀ vs. higher PT₅₀ in larvae treated with metabolites of the insect-derived conidia.

Keywords: *Beauveria bassiana*, Bioassay, *Galleria mellonella*, Mass spectrometry.

INTRODUCTION

In general, entomopathogenic fungi involve an infective spore stage, in which it germinates on the host cuticle, forming a germ tube that penetrates the cuticle and invades the haemocoel of the insect host (Hajek and St. Leger, 1994). The fungus then multiplies within the insect body and kills it. Death occurs due to toxin production by the fungus and/or multiplication to inhabit the entire insect body (Goettel *et al.*, 2010). Such circumstantial evidences as reduced activity, reduced microbiota on the host cadaver, paralysis and some other phenomena are consistent with the involvement of fungus-derived biocides in pathogenesis (Charnley, 2003). Entomopathogenic fungi are prolific producers of bioactive secondary

metabolites (Isaka *et al.*, 2003, 2005; Molnar *et al.*, 2010), which are predicted to play key roles as virulence factors for fungi, infecting arthropods (Rohlf and Churchill, 2011). Metabolites produced by entomopathogenic fungi would serve one or more of the following functions: (1) toxic to the host and help to cause death; (2) to aid the fungus overcome host defence; (3) to suppress competition from other pathogens and saprophytes on the insect cadaver; (4) to provide a defence outside the host against mycophagous organisms (Charnley, 2003). Nonribosomal peptides, alkaloids, terpenes, and polyketides are the main classes of fungal secondary metabolites the expression and secretion of which appear to be controlled by various genetic and cellular regulatory mechanisms (Chanda *et al.*, 2009; Hoffmeister and Keller, 2007). These are not

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needed for growth or development of the producing organism under laboratory conditions, but are thought to aid the fungus in successfully competing with other organisms in its natural habitat. Accordingly, many secondary metabolites tend to be compounds that bear toxic or inhibitory effects on other organisms (Shwab and Keller, 2008). Only a few studies to date have unequivocally demonstrated the significance of entomopathogen secondary metabolites, including toxins, in the disease process (Rohlf and Churchill, 2011).

The entomopathogenic fungus, *Beauveria bassiana* (Balsamo-Crivelli) Vuillemin (1912) (Ascomycota: Cordycipitaceae) is a ubiquitous fungus which has been found and isolated from a wide variety of insects of different orders (MacLeod, 1954; Leatherdale, 1970; Li, 1988; Goettel *et al.*, 1990) and is the most widely used fungal species available commercially (Goettel *et al.*, 2010). It is generally found on infected insects both in temperate and tropical areas throughout the world (Zimmermann, 2007). Some molecular studies have recently confirmed a teleomorph, *Cordyceps bassiana* for *B. bassiana* (Huang *et al.*, 2002). The biosynthesis of secondary metabolites is an outstanding hallmark of the fungal organisms. They may operate as either defensive or offensive chemical weapon against animals (Rohlf and Churchill, 2011).

Beauveria bassiana produces several toxic compounds *in vitro* and *in vivo* (Strasser *et al.*, 2000; Vey *et al.*, 2001). A majority of these insecticidal molecules are low molecular weight secondary metabolites (Zimmermann, 2007). Beauvericin, bassianin, bassianolide, beauverolides, beauveriolides, tenellin, oosporein (Strasser *et al.*, 2000; Vey *et al.*, 2001), oxalic acid (Roberts, 1981) bassiacridin (Quesada-Moraga and Vey, 2004) are some of the important metabolites of *B. bassiana*. Among them, Beauvericin is the most important compound which was reported first from *B. bassiana*. Beauvericin is a toxic

cyclic hexadepsipeptide and comprising a cyclic repeating sequence of three molecules of N-methyl phenylalanine that alternate with three molecules of 2-hydroxyisovaleric acid. Not all isolates of *B. bassiana* produce beauvericin *in vitro* (Frappier *et al.*, 1975; Peczynska-Czoch *et al.*, 1991; Zimmermann, 2007). Beauvericin carries moderate insecticidal properties (Suzuki *et al.*, 1977; Kanaoka *et al.*, 1978; Champlin and Grula, 1979; Qadri *et al.*, 1989; Zizika and Weiser, 1993; Gupta *et al.*, 1995). Nevertheless, there are some reports of no toxicity against certain insects (Champlin and Grula, 1979).

Many works have been directed towards fungal penetration into host cuticle, but there is little knowledge as regards the role of toxins in fungal pathogenesis. In this study, *in vitro* and *in vivo* production of Beauvericin, as an important fungal toxin of *B. bassiana*, was investigated and its virulence assayed against the greater wax moth larvae.

MATERIALS AND METHODS

Fungal Isolate

In this study, an isolate of *B. bassiana* (BEH) was utilized. BEH had been isolated by the author from the soil in a corn field in Behshahr, Iran in 2005 (Safavi, 2010). It was initially passed through larvae of the greater wax moth to maintain the original potential of virulence. Re-isolate of the fungus was cultured on PDA (Potato Dextrose Agar) medium (Merck, Germany). Cultures were kept at 27°C in darkness for 15 days and then transferred to fridge (4°C) until further use.

Insect Rearing

Greater wax moth, *Galleria mellonella* L. (Lepidoptera: Pyralidae), was selected for bioassays regarding fungal toxins. The insects were reared in glass jars (15 cm

diameter vs. 25 cm height), at 28°C and 40% relative humidity (RH) in darkness. They were fed with their natural diets of honeybee nest debris. Bioassays were performed with last instar larvae. Selection of *G. mellonella* as experimental host was due to its active cellular and humoral reactions against fungi (Vey and Vago, 1971; Vey *et al.*, 2002; Seitz *et al.*, 2002; Schuhmann *et al.*, 2003). Moreover, this moth has been previously used to study the effects of fungal toxins, and is highly resistant to trauma occurring through injection process (Fuguet and Vey, 2004).

***In vitro* Beauvericin Induction**

Conidia harvested from cultures were suspended in sterile distilled water containing 0.03% Aq Tween-80® and 2 ml of the suspension (1×10^8 conidia ml⁻¹) used to inoculate 330 ml Potato Dextrose Broth medium (PDB, Sigma) in a 500 ml conical flask. The inductive medium autoclaved at 121°C for 20 minutes before inoculation. Cultures were incubated at 27°C, in a rotary shaker at 120 rpm for 8 days. There were two replicates per treatment while the whole experiment being repeated three times.

Toxin Extraction from Mycelia and Filtrates

Fungal mycelia were separated through filtering over a vacuum in Buchner funnel through a No. 1 Whatman® filter paper. Air-dried mycelia were incubated with 100 ml of methanol (Fisher Scientific) for one hour. The solvent was evaporated in distillation column at 55°C. Residuals were passed through a 0.2 µm sterile filter (Sartorius, Germany) equipped with a sterile syringe and re-dissolved in 5 ml of methanol for collection and dried again in cupboard into powder in a 30 ml glass container on a hot plate (45°C) and with nitrogen current involvement.

Crude filtrates (separated from mycelia) were filtered through a 0.2 µm sterile filter (Sartorius, Germany) equipped with a sterile syringe being freeze dried to dryness and used in HPLC-ESI/MS analysis and in bioassays.

Toxin Production within Insect Bodies

Toxin induction within insect bodies was carried out using method described by Fuguet and Vey (2004) along with some modifications. To achieve the necessary inoculum for bioassays, conidial suspension was prepared by scraping conidia from well sporulated fungal medium, and dispersal of spores in distilled sterile water. Conidial concentration was finally adjusted to 1×10^3 conidia ml⁻¹. Batches of 25 last instar larvae of *G. mellonella* were injected into the haemocoel with 8 µl of suspension using a Desaga micro-injector. The treated insects were maintained at 25°C. Haemolymph from 300 larvae, was sampled after 96 hours. Haemolymph was obtained through puncture of proleg of larvae. To prevent melanization, collected haemolymph was transferred to chilled (0°C) Eppendorf tube containing 25 µl of a solution of cysteine hydrochloride (Sigma-Aldrich). A centrifugation at 12,000g was then immediately performed for a duration of 10 minutes. The supernatant was taken with a syringe and filtrated on 0.2 µm sterile filter (Sartorius, Germany) to obtain a sterile serum. The prepared serum was immediately injected to *G. mellonella* larvae. Haemocoel of control larvae were injected with distilled sterile water instead of with fungal suspension, while sampled and treated in the same way. Cysteine hydrochloride was also injected as control.

***In vivo* Bioassays with Toxins**

Each powdered toxin of mycelia and dried crude filtrates was re-dissolved in 1 ml double distilled sterile water with injection of



solutions of fungal toxins performed through a Desaga micro-injector to greater wax moth. The fresh *in vivo* prepared serum, containing toxins was injected to *G. mellonella* larvae in the same way. The toxicity of all mentioned fungal materials was tested by injection of 5, 8, and 11 μl into the haemocoel of each larvae of *G. mellonella*. Technical Beauvericin (Sigma) was diluted to 1 mM with double-distilled water and directly injected to larval haemocoel. Three replicates of twenty larvae were treated with each fungal preparation. Control insects were injected with sterile broth, except mentioned otherwise. Larvae were placed in sterilized 500 ml glass containers and held at 25°C. Signs of toxic effects on insect host were observed and the mortality recorded for 12 days.

Toxin Production by Conidia

Pure conidia from artificial medium (*In vitro* conidia) and conidia from sporulated cadavers (*In vivo* conidia) of *G. mellonella* were extracted with 1:1 acetonitrile/water at 0.2 ml of solvent per mg of conidia (Leland *et al.*, 2005). Extracts were then passed through 0.2 μm sterile filter (Sartorius, Germany) and diluted 10,000-fold with double distilled water before 10 ml of the extract being directly injected into the HPLC-ESI/MS and analyzed as described below. These conidial extracts were also used for bioassays.

HPLC-ESI/MS Analysis

Freeze dried toxin was reconstituted in 1 ml of double distilled water. A 1 ml 100 mg C_{18} (endcapped) SPE cartridge (Isolute) was conditioned with 1 ml of methanol followed by 1 ml of water. The sample was then applied to the cartridge and allowed to pass through. Following 2 washes with 1 ml of 50/50 methanol/water with 0.1% formic acid, the cartridge was eluted with 1 ml of acidified methanol (0.1% formic acid). The eluent was concentrated to dryness using an

Eppendorf speed vac before being reconstituted in 20 μl of 50/50 methanol/water with 0.1% formic acid ready for LC/MS analysis. Each sample was freeze dried and purified in triplicate, with each aliquot of culture media analysed separately in order to obtain an average level. Five microliters of the sample was then injected using a FAMOS autosampler (Dionex, UK) onto a self packed C_{18} column containing C_{18} pepmap stationary phase (Dionex, UK) with dimensions of 15 cm \times 320 μm I.D. This column was run at a flow rate of 4 $\mu\text{l min}^{-1}$ using an Ultimate HPLC gradient pump (Dionex, UK) and a gradient that held the mobile phase composition at 50/50 methanol/water (0.1% formic acid) for 5 minutes before being altered to 95% methanol/5% water (0.1% formic acid) over 20 minutes, holding this mobile phase composition for 45 minutes and then having it returned to the starting conditions withing 5 minutes. An equilibration time of 20 minutes was allowed between each LC/MS experiment. The eluent was passed into an LCQ-DECA XP ion trap mass spectrometer via a low flow adapted needle (preventing excessive dead volume in the electrospray source). The ion trap was operated in positive ion mode with a spray voltage of 3.5 kV, a sheath gas supply of 30 (arbitrary units), a capillary voltage of 23 V and a heated capillary temperature of 200°C. The mass spectrometer was scanned between 600 and 1,200 Da throughout the HPLC run with data dependent MS/MS spectra collected from each full mass spectrometric scan. The levels of Beauvericin were determined depending upon the area of the peak produced by the protonation of the cyclic peptide at m/z 784 and the identity confirmed as that of Beauvericin by a comparison of the ions retention time through a commercial standard.

Statistical Analysis

Bioassay data were subjected to ANOVA and means compared using Tuckey's_(0.05)

Honestly Significant Difference test. All analyses were carried out through SAS (6.12) software.

RESULTS

HPLC-ESI/MS Analysis

Before the samples being extracted, recovery experiments were performed using culture media spiked with Beauvericin to test the use of the extraction procedure. Beauvericin was added in order to give a final concentration of 8 pmoles μl^{-1} of peptide while the extractions being performed in triplicate. The comparative area of the protonated peptide peak (analyzed in single ion monitoring mode) was then utilized to determine the recovery of the peptide through the extraction procedure. The recovery achieved was 55.19% of the expected level of Beauvericin

with a CV of 5.87%. Although approximately half the peptide is lost during purification, the consistency of the media implies that the purification is essential prior to LC/MS analysis and as the recovery is shown to be reproducible, the levels of Beauvericin detected were still comparable among samples. The identity of the toxin was based upon its retention time as compared to the Beauvericin standard (Figure 1). The MS/MS data showed common losses of amino acids following ring opening as expected.

Beauvericin Bioassays

Corrected mortality data indicated that there were significant differences among various sources of Beauvericin production in 5 μl ($F_{3,16} = 95.09$; $P < 0.0001$), 8 μl ($F_{3,16} = 126.45$; $P < 0.0001$) and 11 μl ($F_{3,16} = 201.91$; $P < 0.0001$) concentrations (Table 1). In 5 μl concentration, there was no

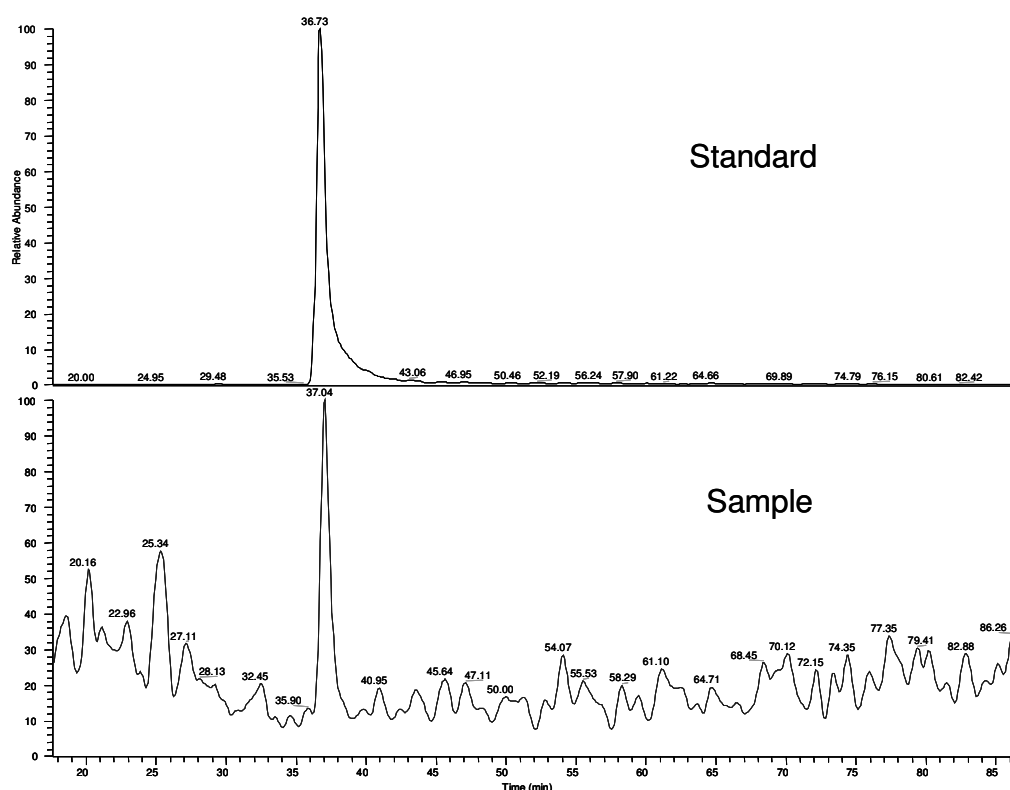


Figure 1. Extracted ion chromatograms of Beauvericin standard vs. Beauvericin extracted through SPE from culture filtrates of *B. bassiana* (BEH isolate).



significant difference observed between mortality level of greater wax moth larvae treated with culture filtrate toxin and *in vitro* conidial toxins. But toxins from *in vitro* mycelia and *in vivo* conidia, sporulated on cadavers, resulted in significantly higher mortality rate (percent) (10.2 and 22.7, respectively). In 8 μ l concentration, the lowest (0.15) and the highest (36.2) mortality rates were obtained for *in vitro* conidia produced on PDA and *in vivo* conidia on dead insects, respectively (Table 1). There was a significant difference observed among all treatments in this concentration, as also observed in 11 μ l treatments. In recent concentration, metabolites from cadaver derived conidia caused the highest mortality rate (percent) (48.8) to greater wax moth larvae as compared with the other three treatments by *in vitro* produced toxins. Bioassays with technical Beauvericin showed 14.52, 24.26, and 37.35 percent mortalities to greater wax moth larvae in 5, 8, and 11 μ l concentrations, respectively.

Mean Survival Time (ST₅₀) and Mean Paralysis Time (PT₅₀)

Survival of greater wax moth larvae was monitored for 12 days in 11 μ l concentration of each Beauvericin source. Control insects were approximately survived for all this time. Insect larvae treated with toxin from *in vitro* conidia showed the highest survival time among all treatments. They survived about 10.4 days. However, toxin from insect

derived conidia lowered mean survival time to 3.2 days for 50 percent of the treated population (Table 2). ST₅₀ for hosts treated with culture filtrate and mycelia derived toxin (cultured *in vitro* on PDB) were 8.7 and 6.2 days, respectively. Mean survival time for technical Beauvericin was approximately equal to PDB-produced mycelia (6.1 days).

Mean paralysis time (PT₅₀) was zero for control and as well for insects treated with metabolites of pure conidia. Culture filtrates from PDB medium showed 52.5 minutes of paralysis in greater wax moth larvae, but mycelia-derived and cadaver-produced conidial Beauvericin imposed 116.4 and 131.2 minutes of paralysis upon 50% of host insects, respectively. Technical Beauvericin revealed 123.5 min paralysis in half of the exposed larval population (Table 2).

Beauvericin Production

Beauvericin level was estimated in different sources of toxin production using HPLC/MS as shown in Table 2. These values varied between 0.01 and 136 ng ml⁻¹. Control exhibited no toxin production. The lowest Beauvericin production (0.01) was recorded in conidia harvested from PDA medium. Culture filtrate from PDB produced 0.57 ng ml⁻¹ of Beauvericin, but mycelial bodies within PDB contained much more of it (6.18 ng ml⁻¹). Conidia from sporulated cadavers of insect host exhibited the highest (136 ng ml⁻¹) Beauvericin production within tested materials (Table 2).

Table 1. Mortality (%)±SD of *G. mellonella* larvae in different sources and concentrations of secondary metabolites of *B. bassiana*.

Toxin Source	Mortality (%)±SD in concentration		
	5 μ l	8 μ l	11 μ l
<i>In vitro</i> conidial extracts	0.038±0.03 ^a	0.15±0.06 ^a	1.014±0.22 ^a
Culture filtrate of PDB	3.02±1.91 ^a	10.58±2.31 ^b	21.38±3.35 ^b
Mycelia from PDB	10.2±2.32 ^b	19.14±3.52 ^c	39.78±3.4 ^c
Technical Beauvericin	14.52±2.74 ^{bc}	24.26±3.81 ^c	37.35±3.69 ^c
<i>In vivo</i> conidial extracts	22.7±3.52 ^c	36.2±4.36 ^d	48.8±4.64 ^d

Means within a column labeled with the same letter are not significantly different (Tukey HSD, P≥ 0.01).

Table 2. Beauvericin production from different sources of *B. bassiana* cultures, along with mean survival and paralysis times of *the exposed G. mellonella*.

Toxin Source	Beauvericin (ng ml ⁻¹)	Mean Survival Time (ST ₅₀) days	Mean Paralysis Time (PT ₅₀) minutes
<i>In vitro</i> conidial extracts	0.01	10.4±1.6	0±0
Culture filtrate of PDB	0.57	8.7±1.8	52.5±14
Mycelia from PDB	6.18	6.2±1.5	116.4±25
Technical Beauvericin	-	6.2±1.4	123.5±18
<i>In vivo</i> conidial extracts	136	3.2±0.7	131.2±38

However, control exhibited no toxin production.

DISCUSSION

The present experiments revealed that BEH isolate is a potential Beauvericin-producing isolate (Figure 1). It had previously been revealed that not all isolates of *B. bassiana* secrete Beauvericin (Frappier *et al.*, 1975; Peczynska-Czoch *et al.*, 1991; Zimmermann, 2007). Figure 1 shows that BEH produces higher rates of Beauvericin as compared with technical Beauvericin (standard). Standard Beauvericin peak occurred at 37.04 minutes. The peak for isolated Beauvericin was observed to occur approximately within an equal time (36.73 minutes). Moreover, a comparison between standard Beauvericin and BEH metabolites' chromatogram (Figure 1) revealed that BEH produced some metabolites other than Beauvericin with different retention times. These metabolites may be other products released by *B. bassiana*, some of which may help fungus to overcome insect defenses in the disease process on host insect. Although secondary metabolites other than Beauvericin were not identified during this research, there are such likely compounds as bassianin, bassianolide, beauverolides, beauveriolides, etc., previously reported by researchers (Strasser *et al.*, 2000; Vey *et al.*, 2001).

The level of Beauvericin produced by the fungal isolate on surface and in submerged cultures on various media was different (Table 2). It has been previously emphasized that the quantities and types of exocellular metabolites

of *B. bassiana* vary according to the media and growth stage (Boucias *et al.*, 1995). *In vivo* and *in vitro* produced conidia were at the extremes of Beauvericin production. While conidia harvested from insect cadavers contained the highest level of Beauvericin (136 ng ml⁻¹), conidia harvested from artificial (PDA) medium contained nearly no Beauvericin (0.01 ng ml⁻¹). This reveals that Beauvericin is produced as response to a liquid medium. No or very low levels of Beauvericin production by *in vitro* conidial suspensions have been reported for nine isolates of *B. bassiana* (Leland *et al.*, 2005). When fungal conidia cultured in artificial liquid medium (PDB), they produced Beauvericin as well as some other secondary metabolites (Figure 1). The secreted Beauvericin into the culture medium (0.57 ng ml⁻¹) was lower than that within the fungal mycelia (6.18 ng ml⁻¹). The results showed that most of the Beauvericin produced by fungus was within the fungal hyphae and the toxin gradually secreted into the medium. It may be the case in insect body, too. Mean Survival Time (ST₅₀) as well as mean Paralysis Time (PT₅₀) were proportional to Beauvericin production. Mean survival time decreased with increase in Beauvericin. Mean paralysis time increased with increase in Beauvericin level. For example, *in vitro* conidial extracts that approximately contained no (0.01 ng ml⁻¹) Beauvericin, caused no paralysis in the tested larvae allowing them to live longer than (10.4 days) in other treatments. Conversely, *in vivo* conidial extracts containing the highest Beauvericin content (136 ng ml⁻¹) caused longer paralysis (131.2 minutes) and lower (3.2 days) longevity.



Mortality data showed the role of secondary metabolites in fungal pathogenesis. Mortality rate (percent) obtained for larvae treated with extracts of *in vitro* conidia was markedly low, nearly equal to zero. This reveals that fresh conidia harvested from solid artificial medium contained considerably very low metabolites (Figure 1) and virulence (Table 1) in its different concentrations. Conversely, extracts of *in vivo* produced conidia carried a high content of Beauvericin and other secondary metabolites (Figure 1) that caused the highest mortality, and mean paralysis time to greater wax moth larvae. Besides, larvae underwent statistically lower mortality in technical Beauvericin in 8 and 11 µl of concentrations. This emphasizes that: (1) the produced Beauvericin type is most likely different as regards technical compound and BEH isolate; (2) there are other metabolites besides Beauvericin in natural conditions that help intensify virulence. Percent mortality observed for toxins from culture filtrate and mycelia grown in PDB were considerably lower when compared with larval mortalities caused by technical Beauvericin and *in vivo* conidial extracts. This may be explained by lower concentration of Beauvericin in culture filtrate and mycelia grown in PDB. This research was directed to identify and assess the Beauvericin role in fungal virulence, but the role of other secondary metabolites of this isolate of *B. bassiana* remains to be clarified in the next and future investigations.

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القا و شناسایی بووریسین قارچ *Beauveria bassiana* در بدن میزبان و محیط غذایی مصنوعی، و زیست سنجی آن روی شب پرهی موم خوار بزرگ

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چکیده

قارچ های بیمارگر حشرات متابولیت های ثانویه فعالی را تولید میکنند که نقش عمده ای به عنوان عوامل بیماریزا در قارچ های آلوده کننده ی حشرات ایفا می کنند. قارچ *B. bassiana* چند ترکیب سمی با وزن ملکولی پایین را در بدن میزبان تولید می کند که مهم ترین آنها بووریسین می باشد. جدایه ای از قارچ مذکور (BEH) برای بررسی بووریسین انتخاب گردید. بووریسین با چند روش مختلف کشت قارچ شامل محیط غذایی مصنوعی جامد و مایع، عصاره ی خام و کنیدی های برداشت شده از لاشه ی حشرات تهیه شد. نتایج نشان داد که بیشترین مقدار بووریسین از کنیدی های جمع آوری شده از حشرات مرده بدست آمده و این سم مرگ و میر بیشتری را در مقایسه با متابولیت های جدا شده از کشت های مصنوعی به لاروهای پروانه ی موم خوار *G. mellonella* تحمیل کرد. کروماتوگرام بووریسین نشان داد که بووریسین بیشترین متابولیت ثانویه ی جدایه ی BEH بود. تاثیر بووریسین روی میانگین بقای لاروها و میانگین فلج شدن آنها نتایج زیست سنجی را تایید کرد که نشان دهنده ی مقادیر پایین ST_{50} و مقادیر بالای PT_{50} در لاروهای تیمار شده با کنیدی های جدا شده از حشرات بود.