BIOACTIVE METABOLITES OF *Beauveria bassiana* (BALSAMO) VUILLEMIN AND ITS EFFICACY ON TOBACCO CATERPILLAR *Spodoptera litura* (FAB.)

By

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(2020-11-120)

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THESIS

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DEPARTMENT OF AGRICULTURAL ENTOMOLOGY
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2023

DECLARATION

I, Kavya K. S. (2020-11-120) hereby declare that the thesis entitled **"Bioactive metabolites of *Beauveria bassiana* (Balsamo) Vuillemin and its efficacy on tobacco caterpillar, *Spodoptera litura* (Fab.)"** is a bona fide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

Place: Vellanikkara
Date: 20/09/2023

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CERTIFICATE

Certified that this thesis entitled **"Bioactive metabolites of *Beauveria bassiana* (Balsamo) Vuillemin and its efficacy on tobacco caterpillar, *Spodoptera litura* (Fab.)"** is a bona fide record of research work done independently by **Ms. Kavya K. S. (2020-11-120)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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INTRODUCTION

1. INTRODUCTION

Global climate change poses new challenges to agricultural production by increasing crops' vulnerability to pests and diseases. Additionally, environmental and human hazards associated with the preponderant use of chemical pesticides for plant protection lead to strategies based on safer pest management. Biocontrol has been receiving increased attention during the last few decades in the quest to regulate pest populations through the use of bioagents, especially entomopathogenic organisms. Among the microbial pathogens, entomopathogenic fungi are a major component of integrated pest management techniques as biological control agents (Kidanu and Hagos, 2020). Unlike bacteria and viruses, fungi have a unique mode of entry through the host cuticle, making them a potent candidate for microbial control of insects of different feeding habits. More than 750 species of fungi belonging to 85 genera are reported to be entomopathogenic to more than 1000 species of insect pests (Paschapur *et al.,* 2021). The most popular and widely used entomopathogenic fungi are *Beauveria bassiana* (Balsamo) Vullemin, *Metarhizium anisopliae* (Metschn) Sorokin, *Lecanicillium lecanii* (Zimmermann) Zare and Gams, *Isaria fumosorosea* (Wize) Brown and Smith, *Hirsutella thompsonii* (Fisher) and *B. brongniartii* (Sacc).

Beauveria bassiana is a ubiquitous insect pathogen with broad spectrum bioactivity and endophytic ability, which offers great potential for its utilization in microbial pest management. *Beauveria bassiana* NBAIR-Bb-5a is an indigenous strain isolated from coffee berry borer in coffee plantations in Kodagu district, Karnataka (Haraprasad *et al.,* 2001). It is pathogenic to a wide range of pests, including sucking pests, lepidopteran pests, and malaria vectors, *Anopheles stephensi* (Nirmala *et al.,* 2006; Poornesha and Ramanujam, 2020; Renuka *et al*., 2023). It is being currently mass produced and used against a broad range of insect pests in the diverse agroecosystems of Kerala.

Entomopathogenic fungi are a treasure trove of secondary metabolites with diverse chemistry and distinct mode of action. Metabolomics brings attention to the host-pathogen relationship and plays a role in the infection process by impeding enzymes crucial for the insects' physiological activities. A wide array of metabolites were reported from *Beauveria* spp., such as beauverolide (Elsworth and Grove, 1980),

oosporein (Strasser *et al.,* 2000), bassiacridin (Moraga and Vey, 2004), bassianolide (Xu *et al.*, 2009), beauvericin (Safavi, 2013), *etc.*

In the present agrochemical scenario, structure-based virtual screening plays a crucial role in pinpointing potent molecules with a specific mode of action (Loza-Mejia *et al.,* 2018). The molecular docking of secondary metabolites of entomopathogenic fungus is a relatively unexplored field of study that has garnered limited attention so far. Moreover, metabolite profiling of *B. bassiana* (NBAIR-Bb-5a) might ease the way to produce safer insecticidal molecules that could be commercialised directly for pest management or serve as templates for the synthesis of insecticidal compounds.

The present investigation on "Bioactive metabolites of *Beauveria bassiana* (Balsamo) Vuillemin and its efficacy on tobacco caterpillar, *Spodoptera litura* (Fab.)" was therefore focused on gathering information on the following aspects:

- Extraction and identification of secondary metabolites of *B. bassiana* grown in potato dextrose broth and Czapek-Dox medium
- Extraction and identification of secondary metabolites of *B. bassiana* from host insect, *S. litura*
- Bioefficacy of crude toxin of *B. bassiana* on larvae of *S. litura*
- *In silico* molecular docking studies with secondary metabolites of *B. bassiana*
- Acetylcholinesterase assay in *S. litura* treated with crude toxin of *B. bassiana*

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Agrochemicals are being used in pest management around the world. Excessive use of harmful chemicals results in environmental pollution, insecticide resistance, pest resurgence and negative impacts on the natural enemy population in the agroecosystem. Additionally, these hazardous chemicals pose a threat to the health of birds, animals, and humans through biomagnification. As a result, innovative eco-friendly pest control approaches came to light, which helped reduce the usage of synthetic insecticides. Among these approaches, microbial control measures are the best alternative to conventional insecticides and an important tool in biointensive pest management (BIPM). Their unique mode of infection through integument of the insect and the subsequent release of mycotoxins into the haemocoel, render them as a potential bioagent. Entomopathogenic fungi (EPF) are rich source of bio-insecticide molecules that can revolutionize the pesticide industry either directly as insecticide molecules or as templates for synthesis of compounds with novel mode of action.

2.1 Taxonomy of *Beauveria bassiana*

Beauveria bassiana (Balsamo) Vuillemin is an entomopathogenic fungus belonging to Division Ascomycota, Order Hypocreales and family Cordycipitaceae.

Petch was the first among scientists to attempt classification of the genus *Beauveria* at the species level and classified it into eight different species, *i.e., B. effuse, B. densa, B. brongniartii, B. bassiana, B. globulifera, B. delacroixii, B. vexans* and *B. stephanodesis,* based on the shape of conidia (Rehner, 2005). Sevim *et al.* (2010) recognised the six species of the genus, *viz., B. bassiana, B. brongniartii* (Sacc.), *B. caledonica* (Bissett and Widden), *B. bassiana* cf. Clade C, *B. vermiconia* (de Hoong and Rao) and *B. amorpha*. In addition, six new species *viz., B. varroae, B. kipukae, B. sungii, B. asiatica, B. australis* and *B. pseudobassiana* Rehner and Humber were described (Rehner *et al.*, 2011; Wang *et al.*, 2020).

Systematic position of *B. bassiana* according to Sung *et al.* (2006) and Halouane (2008) is as follows:

- Kingdom: Fungi
- Phylum: Ascomycota
- Class: Sordariomycetes

- Order: Hypocreales
- Family: Clavicipitaceae
- Genus: *Beauveria*
- Species: *B. bassiana* (Balsamo) Vuillemin

2.2 Host range of *Beauveria bassiana*

Beauveria bassiana is one of the most extensively studied entomopathogenic fungus and has a broad host range of 700 insect species (Meyling *et al.,* 2009). It is known as the white muscadine fungus and is pathogenic to a wide range of insects belonging to Lepidoptera, Hemiptera, Coleoptera, Isoptera and Thysanoptera (Goettel and Glare, 2010).

Beauveria bassiana infecting auger beetle, *Sinoxylon anale* Lesne (Coleoptera: Bostrichidae) was reported for the first time from Kerala (Kumar *et al.,* 2016). Two indigenous isolates of *B. bassiana* (Bb-m2 and Bb-m5) from rice ecosystem of Kerala, found pathogenic to larvae of rice leaf roller, *Cnaphalocrocis. medinalis* Guen. and nymphs and adults of rice bug *Leptocorisa acuta* Thunb (Malini and Sudharma, 2017).

The insecticidal potential of *B. bassiana* as mycopesticides has been reported by many authors (Jaber and Ownley, 2018). According to Dannon *et al.* (2020), the insecticidal activity of *B. bassiana* is faster than other entomopathogenic agents and the conidia can persist in the environment through the spread of enzootic or epizootic diseases. Moreover, its effect on beneficial insects and other non-target organisms is limited.

2.3 *Beauveria bassiana* NBAIR-Bb-5a

Beauveria bassiana (NBAII-Bb-5a) is an indigenous strain of the entomopathogenic fungus isolated from coffee berry borer (*Hypothenemus hampei*) in Kodagu district, Karnataka (Haraprasad *et al.,* 2001). Nirmala *et al.* (2006) investigated the pathogenicity of Bb-5a in comparison with *Metarhizium anisopliae* (Metschinikoff) Sorokin and *Lecanicillium lecanii* (Zimmerman) against *Aphis craccivora* Koch, *A. gossypii* Glover and *Rhopalosiphum mallis* Fitch. Bb-5a, caused the highest per cent mortality in *A. gossypii* (80.8%) followed by *R. mallis* (50%).

Ramanujam *et al.* (2017) assessed the field efficacy of three isolates of EPF, *B. bassiana* (NBAIR-Bb-5a), *M. anisopliae* (NBAIR-Ma-4) and *L. lecanii* (NBAIR-Vl-8), for the management of the cabbage aphid, *Brevicoryne brassicae*. Findings revealed a lower incidence of 13.9–17.1 aphids per plant in *B. bassiana* (NBAIR-Bb-5a) treated plots compared to a higher incidence of 107.5 in the untreated control, which proved the superiority of the strain in the suppression of the cabbage aphid under field conditions.

Borkakati *et al.* (2019) studied the efficacy of six different strains of entomopathogenic fungi against sucking pests of chilli. Among all, *B. bassiana* (NBAIR-Bb-5a) efficiently managed *A. gossypii*, *Scirtothrips dorsalis* Hood and *Bemisia tabaci* (Genn.) at the treated concentration of 5 g L^{-1}.

Poornesha and Ramanujam (2020) evaluated *B. bassiana* (NBAIR-Bb-5a) against *Plutella xylostella* (L.) under laboratory conditions. Findings revealed a significantly higher mortality of 77.36 per cent at a concentration of 10^8 conidia mL^{-1}.

In addition to crop pests, Bb-5a was effective in vector management also. Renuka *et al*. (2023) studied the bioefficacy of *B. bassiana* (Bb5a and Bb-NBAIR) and *M. anisopliae*. Bb5a showed a prodigious effect in controlling adult mosquitoes, *Anopheles stephensi* (Diptera: Culicidae).

2.4 Importance of secondary metabolites of EPF

The metabolites secreted by entomopathogenic fungi are a rich source of bioactive compounds. Based on their chemical structure, the metabolites obtained from EPF can be broadly grouped into three classes: polyketides, terpenes and peptides. The synthesis of bioactive metabolites is the result of the polymerization of primary metabolites by core enzyme groups such as polyketides, which are produced by polyketide synthases and non-ribosomal peptides by non-ribosomal peptide synthetases (NRPSs) (Keller *et al.,* 2005).

EPF have evolved highly specialized mechanisms to produce secondary metabolites and enzymes with immunosuppressive or otherwise toxic functions, that help them in the invasion of their insect hosts by overcoming cellular and humoral defense systems (Rohlfs and Churchill, 2011). There are thousands of secondary metabolites reported from hundreds of EPFs, but their exact role in the host infection

process is still unknown. Genomic studies on *Metarrhizium* (Gao *et al.,* 2011) and *Beauveria* (Xiao *et al.,* 2012) suggested the existence of unique and vast arrays of gene pools associated with metabolite production. These metabolites will have multiple roles to play in establishing a successful infection in an insect host.

2.5 Role of secondary metabolites in fungal pathogenesis

The main route of infection for *B. bassiana* is through the insect cuticle, which represents the first encounter and barrier between the fungus and host (Agarwal *et al.,* 2012). Upon adhesion to and recognition of the insect surface, *B. bassiana* deploys a combination of biochemical and mechanical tools to make its way through the insect integument and into the hemocoel (Mascarin and Jaronski, 2016).

Once the fungus reaches the nutrient-rich environment, the mycelium switches to a specialised hyphal body, or blastospores. At this stage, the insect host has very little chance of surviving the fungal infection despite the activation of the immune response (humoral and cellular) as a last-ditch attempt to overcome the fungal infection (Butt *et al.,* 2016)

Successful fungal pathogenesis will then depend on the concerted combination of several events, one being the production of a plethora of toxic secondary metabolites that can either facilitate the fungal invasion or act as immunosuppressive compounds, fighting against host defences (Lu and Leger, 2016).

2.6 Production of secondary metabolites of *Beauveria bassiana*

Beauveria bassiana is well known for its ability to produce a wide array of biologically active metabolites (Kucera and Samsinakova, 1968; Kishore *et al*., 2007) that are pivotal in pathogenesis and virulence. These secondary metabolites have potential industrial, pharmaceutical and agricultural uses (Xu *et al.,* 2009). These secondary metabolites have different chemical natures and include cyclooligomeric nonribosomal peptides, cyclic peptides, diketomorpholine, polyketides and many more (Patocka, 2016).

The important secondary metabolites and their role in entomopathogenicity are briefly reviewed below.

Beauvericin

Beauvericin is a cyclic hexadepsipeptide that belongs to the enniatin antibiotic family and is produced by fungi, such as *B. bassiana* and *Fusarium* spp. (Wang and Xu, 2012). Beauvericin has moderate antibacterial, antifungal, and insecticidal activities (Hamil *et al.,* 1969; Gupta *et al.,* 1991; Gupta *et al.,* 1995), as well as potent cytotoxic activity against human cell lines (Ivanova *et al.,* 2006).

Through diffusion, it transports divalent cations across biological membranes and acts as an ionophore; beauvericin increases cytoplasmic Ca^{2+} concentration, causes ATP depletion, and activates calcium-sensitive cell apoptotic pathways (Jow *et al.,* 2004; Chen *et al.,* 2006).

Bassianolide

The octodepsipeptide bassianolide is a cyclic tetrameric ester of the dipeptidol monomer D-hydroxyisovaleric acid-N-methylleucine. Kanoka *et al*. (1978) isolated the toxin bassianolide from the mycelia of *L. lecanii* and *B. bassiana.* Bassianolide is insecticidal and inhibits acetylcholine induced smooth muscle contractions independent of ionophoric interactions (Nakajyo *et al.,* 1983). Xu *et al.* (2009) reported bassianolide as a highly significant virulence factor in *B. bassiana* and *L. lecanii*. The entomotoxicity of bassianolide was attributed to its ionophoric nature, which was internally hydrophilic and externally hydrophobic.

Oosporein

Oosporein is a red-coloured pigment of dihydroxybenzoquinone, which belongs to the group of non-reduced polyketides. Oosporein was first reported as a dye from the endophytic fungus *Oospora colorans* (Beyma) by Kogl and Wessem in 1944. It was then described as a mycotoxin from different entomopathogenic fungi, *viz., B. bassiana* (Vining *et al.,* 1962), *Chaetomium trilaterale* (Ames) (Cole *et al*., 1974), *L. psalliotae* (Nagaoka *et al*., 2004) and *L. saksenae* (Kushwaha) Kurihara and Sukarno (Sreeja *et al*., 2023).

Oosporein was reported to be an inhibitor of the Ca-dependent ATPase of the erythrocyte membrane at relatively high concentrations of 200 µg mL^{-1}. Immune interference studies by Feng *et al*. (2015) revealed that injection of oosporein inhibited prophenoloxidase (PPO) activity and down-regulated the antifungal peptide

gallerimycin gene in insects following activation by fungal spores. Fan *et al.* (2017) reported that oosporein protected the host cadaver from bacterial infection and helped the fungus to utilise the host nutrients and complete its life cycle.

Beauverolide

The beauverolides, which belong to a family of cyclic tetradepsipeptides featuring 3-hydroxy-4-methyl alkanoic acid units, were isolated from *B. bassiana, B. tenella* and *Isaria fumosorosea*. Beauverolide H is a cyclotetradepsipeptide isolated from the UICP32 strain of the entomopathogenic fungus *B. bassiana*. Beauverolide I is isolated from the same and identified as a homologue derived from P-hydroxyundecanoic acid (Elsworth and Lamchen, 1966). It exhibited moderate insecticidal properties against the leaf caterpillar *Spodoptera litura* and the pulse beetle *Callosobruchus chinensis* (Linn.), (Mochizuki *et al.*, 1993).

Tenellin and bassianin

Tenellin and bassianin are yellow 1,4-dihydroxy-2-pyridone pigments isolated from several species of *Beauveria*. They are formed from a chain of polyketides reduced (pentaketide in the case of tenellin and hexaketide for bassianin), with the amide portion of the tyrosine (Molnár *et al.,* 2010). These pigments were produced by *B. bassiana* and *B. tenella* (Patocka, 2016).

Bassianin differs from tenellin by one chain extension in the ketide moiety. Eley *et al.* (2007) described the production of the acyltetramic acid tenellin by a hybrid PKS-NRPS gene cluster that shows much similarity to the gene cluster encoding NG39x in *Metarhizium* and fusarins in *Fusarium* spp. The tenellin knockout mutants showed no loss of virulence in assays against *Galleria mellonella,* suggesting that tenellin is not involved in pathogenesis.

Bassiacridin

Bassiacridin is a toxic protein purified from a strain of the entomopathogenic fungus *B. bassiana,* which was isolated from a locust, using chromatographic methods (Moraga and Vey, 2004).

Bassiatin

Bassiatin is a cyclized monomeric unit of the trimer involved in beauvericin biosynthesis and thus it might be a shunt product of that pathway (Süssmuth *et al.*, 2011).

2.6.1 Factors affecting metabolite production

In vitro metabolite production by *B. bassiana* is affected by many factors such as culture medium, temperature, pH and incubation time. The precise interaction among those factors will provide an optimum condition for fungal growth and the production of secondary metabolites.

Media conditions *viz.*, pH, media composition, nutrient supplements, *etc*. have varying effects on the production of fungal secondary metabolites (Miao *et al.*, 2006). Often, the variation of culture conditions is used to optimise the yields of a specific compound, such as the active metabolite (Xu *et al.*, 2008).

2.6.1.1 Culture conditions

Submerged cultures of entomopathogenic fungi were reported to produce higher mycelial biomass and secondary metabolites in a more compact space, within a shorter period when compared with those cultivated on solid media (Kim *et al.* 2003). Molen *et al.* (2013) evaluated the usefulness and reliability of different media types, including six liquid culture media and five solid media. The findings revealed that the potato dextrose broth supported the highest production of secondary metabolites.

Roswanjaya *et al.* (2021), investigated the suitable culture media (potato dextrose broth, yeast and malt extract broth, malt extract broth and *Fusarium* defined medium) and incubation time (6 and 12 days) for growth and production of beauvericin by *B. bassiana*. The highest production of biomass and beauvericin was observed in Malt Extract Broth (MB) medium at 12 days.

2.6.1.2 Composition of culture media

The production of *B. bassiana* pigments has been reported by Amin *et al.* (2010), who obtained a red extract from a culture medium consisting of 40 g L^{-1} of glucose and 5.0 g L^{-1} of yeast extract and enriched with several salts ($NaNO_3$ 1 g, KH_2PO_4 2 g, KCl 0.5 g, $MgSO_4.7H_2O$ 0.5 g and $FeSO_4.7H_2O$ 0.02 g).

The synthesis of red pigment by the filamentous fungi *Isaria farinosa* under submerged culture conditions has been reported by Velmurugan *et al.* (2010). The pigment production was found to be optimal under the following conditions *viz.* sucrose and glucose as carbon source, yeast extract, meat peptone and monosodium glutamate at a fixed concentration of 3% as nitrogen source. The culture medium supplemented with 10 mM $CaCl_2$ was reported to enhance the biomass and red pigment production of *I. farinosa*.

Most fungal strains are capable of using various carbon and nitrogen sources, as these nutrients influence their growth and the types and yields of pigments produced (Celestino *et al.,* 2014). To produce pigments, glucose is the ideal carbon source, and nitrogen sources such as ammonium and peptone confer good growth and increase the concentration of the pigments (Kumar, 2015). In addition, there are reports on the production and characterization of the pigment oosporein obtained from *L. aphanocladii* where potato dextrose broth was used as culture media (Costa Souza *et al.,* 2016).

Sanghi *et al*. (2019) studied the effect of media components for improved linoleic acid production by *B. bassiana* MTCC 5184. Findings revealed the glucose yeast extract (GYE) medium supplemented with 1.5 per cent (w/v) peptone yielded maximum linoleic acid (0.32±0.01 g L^{-1}). Further the addition of 1 per cent (w/v) oleic acid, the precursor of linoleic acid enhanced the production by 12 folds (1.24±0.03 g L^{-1}), in comparison to those in the basal (GYE) medium.

2.6.1.3 Temperature

Temperature is capable of altering the physiology of entomopathogenic fungi and biosynthesis of important secondary metabolites in an unpredictable way (Borisade and Magan, 2014). Borisade *et al.* (2016) reported the production of destruxin A was maximum at 35°C and lowest at 25°C. The findings revealed the increase in temperature from 30-35°C caused approximately 13 per cent increase in the production of destruxin A.

2.6.1.4 pH

Margaritis and Chahal (1989) studied the different pH levels for the production of cyclosporin A (CyA) by *B. nivea* ATCC 34921 in a fructose based

medium. A maximum of 170 mg of CyA per litre of broth was obtained at pH 5.5 in 8 days of fermentation whereas 109 mg of CyA was concentrated at pH 7.5. Dhar *et al.* (2016) reported that the maximal growth of *B. bassiana* strains was optimum at the pH of 6-7.

Chen *et al.* (2022) revealed the pH signaling transcription factor BbPacC is involved in the regulation of oosporein production. Overexpression of *BbPacC* promotes oosporein production in *B. bassiana* at pH 6.0 or under alkaline conditions (pH 8.0), but deletion of this gene abolished oosporein production. Under acidic conditions (pH 4.0), no oosporein production was observed in the wild-type and *BbPacC* overexpression strains.

2.6.2 Isolation of secondary metabolites of *Beauveria bassiana*

During the infection process, entomopathogenic fungi produce toxins in host insects that hamper their immune systems and act as insecticidal molecules. They also produce secondary metabolites when cultured in a specific nutrient-rich medium, which can be retrieved from the culture filtrate.

Recent advances in analytical techniques facilitated easier separation, identification and structural determination of biomolecules. GC-MS is a powerful tool for the identification and quantification of volatile molecules based on their retention indices and mass spectral fragmentation patterns.

Insecticidal cyclodepsipeptide, bassianolide, was extracted from the mycelia of two entomopathogenic fungi, *B. bassiana* and *L. lecanii*, cultured in Czapek-Dox medium containing two per cent yeast extract. The toxin was extracted using methanol and purified through different chromatographic techniques (Kanaoka *et al.*, 1978).

Gupta *et al.* (1995) isolated two novel beauvericin analogues, beauvericin A and beauvericin B, containing 2-hydroxy-3-methylpentanoic acid as one of the constituent residues and as insect toxic principles from a mycelial extract of *B. bassiana*.

Takahashi *et al.* (1998) isolated two novel metabolites, pyridovericin and pyridomacrolidin, from *B. bassiana* EPF-5, a strain isolated from an adult Mulberry small weevil (*Baris deplanata* Roelofs). The active toxins were extracted from the

mycelia through a series of purification steps, including silica-gel column chromatography and preparative HPLC, which yielded a pale yellowish powder.

Moraga and Vey (2004) purified the toxic protein bassiacridin from a strain of the entomopathogenic fungus *B. bassiana* that was isolated from a locust, *Locusta migratoria*. The crude filtrate was precipitated by ammonium sulphate and submitted to gel filtration through G25 sephadex exclusion chromatographic methods for purification of toxin bassiacridin.

Two new destruxins, [ß-Me-Pro] destruxin E chlorohydrin and pseudodestruxin C, as well as five known cyclic depsipeptides were isolated from the growth media of the marine-derived fungus *B. felina* (Lira *et al.,* 2006).

Xu *et al.* (2007) extracted and purified the beauvericin from *B. bassiana* ATCC 7159 cultured on potato dextrose broth *via* Sepharose LH-20 column (850 mm × 20 mm) chromatography. Stock solutions (1.0 M) of amino acid and hydroxycarboxylic acid analogues of D-Hiv and L-Phe, 2-oxovaleric acid, β-chlorolactic acid, and (S)- and (R)-(+)- hexahydromandelate were supplemented to the main cultures one day after inoculation as nutrient supplements for the metabolite production. Xu *et al.* (2009), extracted bassianolide from the ATCC 7159 strain for studying the insecticidal virulence factor of *B. bassiana*.

GC-MS analysis of the ethyl acetate fraction of mycelia from *B. bassiana* identified n-hexadecanoic acid, 9, 12, octadecadienoic acid, squalene, and octadecanoic acid (Ragavendran *et al*., 2017). Vivekanandhan *et al*. (2018) reported hexadecanoic acid from mycelia as the major compound of pathogenicity in *B. bassiana* 28. Ragavendran *et al*. (2019) identified mosquitocidal compounds such as 1-octadecene, 1-nonadecene, 9-octadecenoic acid and cyclobutane through GC-MS analysis of *Penicillium* sp. GC-MS analysis of the methanolic extract from *Cladosporium cladosporioides* (Fresen) yielded 26 compounds, while that from *P. lilacinum* yielded 19 compounds. The most bioactive compounds were linoleic acid and palmitic acid (Elbanhawy *et al*., 2019).

An *et al.* (2021), purified and identified the compounds from *Beauveria* sp. LY2 cultures in Czapek-Dox medium *via* extensive chromatographic techniques, NMR and MS. One new cerebroside, cerebroside F and nine known compounds, *viz.*, cerebroside B, bassiatin, methyl 1,4-dihydro-4-oxo-2-quinolinecarboxylate,

cerevisterol, 9-hydroxycerevisterol, 6-dehydrocerevisterol, (22E,24R)-ergosta-8(14), 22-diene-3β,5α,6β,7α-tetrol, melithasterol B and ergosterol peroxide were isolated.

2.6.3 Isolation of fungal metabolites from host insects

Entomopathogenic fungi have a fascinating set of mechanisms that allow them to degrade and assimilate host components while tackling host-resistance mechanisms. Secondary metabolites released by entomopathogenic fungi can impair the host's cellular recognition and defense reactions.

Strasser *et al.* (2000) monitored the distribution of secondary metabolites produced by three commercial isolates of the entomogenous fungus *B. brongniartii* with reference to oosporein in cockchafer larvae, *Melolontha melolontha* (Coleoptera: Scarabaeidae).

Skrobek *et al.* (2008) investigated the destruxin production by the entomogenous fungus *M. anisopliae* in mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae) and greater wax moth, *Galleria mellonella* (Lepidoptera: Pyralidae) and discovered that the amount and type of destruxin produced are highly dependent on the fungal strain and insect host. Destruxin A was produced relatively high in *G. mellonella* whereas, destruxin E was predominant in *T. molitor*.

Nithya *et al.* (2021) investigated the ditrophic interaction between *B. bassiana* and *Plutella xylostella*. The metabolome profile of *P. xylostella* infected by *B. bassiana* revealed the presence of non-volatile organic compounds (NVOCs) like phthalate esters, hydroxyquebrachamine and lactones. However, the metabolite profile was different in healthy larvae and consisted of docosene, nonadecene, palmitic acid and heneicosane.

2.6.4 Bioactivity of metabolites of *Beauveria bassiana*

The insecticidal activity of beauvericin from *B. bassiana* was first discovered by Hamil *et al.* (1969), confirming that the active compound in *B. bassiana* possess insecticidal activity.

Grove and Pople (1980) evaluated the insecticidal activity of beauvericin and the enniatin complex produced by *F. lateritium* against *Calliphora erythrocephala* and *A. aegypti*. Enniatin A and the enniatin complex were less active than beauvericin

against mosquito larvae but more active than beauvericin in the assay against adult blowflies.

Oller-López *et al.* (2005) investigated the antimicrobial activity of bassianolone cephalosporolides E and F extracted from *B. bassiana.* Results revealed that bassianolone at the concentration of 100 ug mL^{-1} completely inhibited the growth of gram-positive bacteria, *Staphylococcus aureus* and fungus, *Candida albicans*.

Gurulingappa *et al.* (2011) demonstrated the insecticidal activity of metabolites in mycelial and ethyl acetate extracts of *L. lecanii* and *B. bassiana* against *A. gossypii.* At 0.25 to 2 per cent concentrations, the methanolic fractions of *L. lecanii* and *B. bassiana* mycelia caused mortality ranging from 24 to 82 per cent and 45 to 97.5 per cent, respectively.

Sahab (2012) studied the antibacterial and antifungal activity of a crude ethyl acetate extract of *B. bassiana* and found that the crude toxin exhibited antibacterial activity at 100 ug mL^{-1} and moderate antifungal activity at a concentration of 1200–1600 ug mL^{-1}.

The secondary metabolites produced within the insect as part of the infection process exhibit different functions, *viz.,* beauverolides are involved in killing the host and destruxins mainly function as antimicrobials (Bekker *et al*., 2013).

The insect-toxic protein (Bb70p) purified from *B. bassiana* 70, caused high mortality in *G. mellonella* by intra-haemocelic injection, with an LD_{50} of 334.4 g g^{-1} body weight (Khan *et al.* 2016). N-hexadecanoic acid from *B. bassiana* was found to be insecticidal against first, second, third and fourth instar larvae of *Anopheles stephensi* (Liston), *Culex quinquefasciatus* and *A. aegypti* (Ragavendran *et al.,* 2017).

Keppanan *et al.* (2018) evaluated the toxicity of bassianolide on third-instar larvae of *P. xylostella* by the leaf assay, where a leaf was injected with 5 µL solution at concentrations of 0.5, 0.1 and 0.01 mg mL^{-1} using a microsyringe. The results revealed that a greater dose of 0.5 mg mL^{-1} had a substantial maximal mortality (88.8 ± 9.6 %) at 120 h after treatment.

Namara *et al.* (2019) investigated the insecticidal, anti-feedant and immunomodulation effects of oosporein produced by *B. caledonica* on the forestry pest *Hylobius abietis* and the model insect *G. mellonella*. Findings revealed that

oosporein extracted from *B. caledonica* cultures exhibits immunosuppressive properties that have the potential in pest control by promoting faster and greater mortality.

Nithya *et al.* (2019) studied the metabolome heterogeneity in the isolates of the entomopathogenic fungus, *B. bassiana* (Balsamo) Vuillemin. Metabolite profiles of the isolates with several biological functions were observed, including insecticidal and antimicrobial activity, lipid and fatty acid metabolisms.

Al Khoury *et al.* (2019) investigated the lethal activity of beauvericin against the two-spotted spider mite, *Tetranychus urticae* Koch. This study revealed the acaricidal activity of beauvericin at 100 µg g^{-1} concentration and the inhibition of egg hatching up to 83.3 per cent at 10 µg g^{-1} concentration.

2.7 Bioefficacy of fungal secondary metabolites

Beauveria bassiana produces secondary metabolites that disable several immune mechanisms, allowing the fungus to overcome and then kill its host. This characteristic makes *B. bassiana* a promising model for biological control of insect pests.

Wang *et al.* (2007) investigated the contact toxicity of crude *L. lecanii* toxins (toxinV_{3450} and toxin Vp_{28}) against the sweet potato whitefly, *B. tabaci*. The findings demonstrated ovicidal action, with LC_{50} values of 447 and 629 mg L^{-1}, respectively. Toxin V_{3450} had an LC_{50} of 111 mg L^{-1} in nymphs and 178 mg L^{-1} in adults. On the other hand, toxin Vp_{28} had an LC_{50} of 216 mg L^{-1} in nymphs and 438 mg L^{-1} in adults.

Zibaee *et al.* (2009) investigated the effects of *B. bassiana* spores and its secondary metabolite on *Eurygaster integriceps via* topical bioassay. LD_{50} values were 9.4 µL mg^{-1} adult and 22.5 µL mg^{-1} adult for spore and metabolite-treated adults, respectively, in comparison with 5.9 µL mg^{-1} adult for control individuals, which are significantly different from each other.

Zibaee *et al.* (2011) investigated the effect of the secondary metabolites of the entomopathogenic fungus *B. bassiana* on the cellular immune defences of *Eurygaster integriceps* by injecting toxins (Burkard syringe). The results showed that the fungal secondary metabolites inhibited the phagocytic activity of *E. integriceps*

hemocytes, hampered nodule formation and caused a reduction in phenoloxidase activity.

Fan *et al.* (2013) studied the effect of *B. brongniartii* and its secondary metabolites on the detoxification enzymes of the pine caterpillar, *Dendrolimus tabulaeformis*, *via* topical bioassay at three concentrations *viz.*, 5.5, 55 and 550 µg mL^{-1}. The LC_{50} was 2.80×10^2 µg mL^{-1} and the high dose (550 µg mL^{-1}) showed the highest GST activities from 24-48 h. This result indicated that the immune reaction of the *D. tabulaeformis* larvae treated with the fungal metabolite was faster than that of those infected with the fungal spores.

Vivekanandhan *et al.* (2018) evaluated the toxicity of *B. bassiana*-28 ethyl acetate extracts on different larval stages and pupae of *Culex quinquefasciatus* mosquitoes. The LC_{50} values for first to forth-instar larvae and pupae were 11.538, 6.953, 5.841, 3.581 and 9.041 mg L^{-1}, respectively. This study showed that *B. bassiana*-28 ethyl acetate extracts have strong insecticidal activity against the larval and pupal stages of *C. quinquefasciatus*.

An *et al.* (2021) studied the insecticidal effectiveness of *Beauveria* crude toxin, *i.e.,* bassiatin, methyl 1,4-dihydro-4-oxo-2-quinolinecarboxylate and 6-dehydrocerevistero, against *B. tabaci* via contact and feeding assays. The LC_{50} values were 10.59, 19.05 and 26.59 µg mL^{-1} respectively, in contact as well as 11.42, 5.66 and 5.65 µg mL^{-1} respectively, in the feeding bioassay experiment.

2.8 Molecular docking of fungal metabolites with insect protein

Entomopathogenic fungi are potential resource for biologically active metabolites. *In vitro* screening of these metabolites to identify a novel insecticidal molecule is a cumbersome process. Hence, structure-based virtual screening is the most widely used strategy to identify the most promising compounds for a biological assay.

In silico molecular docking analysis is a powerful tool for the study of the structure-activity relationship between two molecules and the prediction of their interaction. The binding affinity and the 3D structure of the complex obtained designate the degree of interaction between them. It is an essential component of the

modern agrochemical industry to identify a potent molecule with a specific mode of action.

Harel *et al.* (2000) investigated the three-dimensional structures of *Drosophila melanogaster* acetylcholinesterase and its complexes with two potent inhibitors, 1,2,3,4-tetrahydro-N-(phenylmethyl)-9-acridinamine and 1,2,3,4- tetrahydro-N-(3-iodophenyl-methyl)-9-acridinamine.

Keppanan *et al.* (2017) conducted docking studies with the protease of Tk6 isolate and the moricin-like peptide C4 and purified the extracellular protease enzyme from three isolates of *M. anisopliae*. The energy and thermal stability of the cuticle-degrading protease enzyme were enhanced by the interaction between the template proteins (moricin-like peptide C4). Based on the results, the selected protease enzyme from the Tk6 isolate has the maximum ability to bind and degrade the host protein naturally.

Keppanan *et al.* (2018) examined the ligand-target interaction of secondary metabolites of *L. lecanii*, binding with the immune receptor proteins of the target insect, *P. xylostella* and anticipated the function of toxicity against the insect. Findings revealed that the ligand-receptor interaction improved the binding energy and thermal stability of chemical activity against the pest, *P. xylostella*.

Yoneyama *et al.* (2022) studied the antimicrobial metabolites of *Cordyceps tenuipes*, targeting MurE ligase and histidine kinase via an *in silico* docking. Docking studies revealed that the binding affinities of beauvericin against MurE and HK proteins ($\Delta G = -8.021$ and -8.585 kcal mol^{-1}, respectively) of the best conformers were relative to the co-crystallised ligand ($\Delta G = -11.808$ and -6.364 kcal mol^{-1}, respectively) and ampicillin as a reference drug. Sreeja (2020) studied the insecticidal metabolites of *L. saksenae* targeting AChE through *in silico* molecular docking studies. The findings revealed the six metabolites showed highest interaction with acetyl cholinesterase with the binding energy varying from -111.21 to -133.24 kcal mol^{-1}.

2.9 Acetylcholiesterase enzyme assay

Acetylcholinesterase (AChE) is a key enzyme in the insect nervous system, which terminates nerve impulses by catalysing the hydrolysis of the neurotransmitter

acetylcholine (Wang *et al.,* 2004). Inhibition of AChE resulted in an increase in the level of ACh at the synapses, causing the postsynaptic membrane to enter a state of long-lasting stimulation, ultimately leading to ataxia and insect death (Singh and Singh, 2004).

2.9.1 Role of secondary metabolites as enzyme inhibitors

Fungi have been at the forefront of producing enzyme inhibiting secondary metabolites. This includes acetylcholinesterase, nuclear factor-kappa B, protein kinase, tyrosine kinase, aromatase and sulphatase, matrix metalloproteinases, cyclooxygenase, DNA polymerase/topoisomerases, glycosidases, *etc.* Pseurotin A, metabolite of *M. anisopliae*, exhibits inhibitory activity against monoamine oxidase (Maebayashi *et al.,* 1985). Pseurotin A and 8-0-demethylpseurotin A displayed chitin synthase inhibitory activity (Wenke *et al.,* 1993).

Paecilaminol, an antitumor metabolite of the strain *Paecilomyces* sp. FKI-0550, exhibited an IC_{50} value of 5.1 μM against *Ascaris suum* NADH-fumarate reductase (Ui *et al*. 2006). Song *et al.* (2019) studied the biological activity of a novel konjac glucomannan oligosaccharide (KGO) derivative, konjac glucomannan oligosaccharide kojic acid (KGOK). Findings revealed that the metabolite, Paecilaminol exhibited antibacterial and tyrosinase inhibitory activities.

Wei *et al.* (2022) examined the glycosidase inhibitory activity of two compounds, metarrhiziumoids A (R-enantiomer) and B (S-enantiomer), isolated from *M. flavoviride*. The results of α-glycosidase inhibitory tests showed that both enantiomers were competitive inhibitors of α-glycosidase.

2.9.2 AChE activity by fungal spore

Bilal *et al.* (2017) studied the enhanced AChE activity in the hemolymph, intestine and fat bodies of *H. armigera* exposed to higher concentrations of *M. anisopliae* Ma-4.1 and *B. bassiana* Bb-08 @ 7×10^8 spores mL^{-1}. The AChE activity in samples of fat body of *H. armigera* treated with *B. bassiana* Bb-08 was 1.5 μM min^{-1} mg protein. Whereas, AChE activity was high in haemolymph (1.7 μM

min^{-1} mg protein) and intestine (0.9 μM min^{-1} mg protein) of *H. armigera* treated with *M. anisopliae* Ma-4.1.

Wu *et al.* (2020) explored the biological impact and enzyme activities of *S. litura* (Lepidoptera: Noctuidae) in response to *B. brongniartii*. The results indicated an inhibitory effect, as the detoxification activity of enzymes in the fat body and hemolymph of *S. litura* were significantly lower than those of the control group.

Wu *et al.* (2021) evaluated the effect of the entomopathogenic fungus *Akanthomyces attenuatus* (Zare & Gams) against *Megalurothrips usitatus*. The results indicated that AChE activity in treatment (1.78 U mg^{-1} protein) was significantly lower than control (3.5 U mg^{-1} protein).

2.9.3 AChE activity of fungal toxins

Zibaee *et al*. (2009) studied the effect of secondary metabolites of *B. bassiana* on acetylcholine esterase inhibition in the sunn pest *Eurygaster integriceps* (Heteroptera: Scutellaridae). Findings revealed that the *B. bassiana* secondary metabolite had an adverse effect on the AChE activity of adults that decreased its activity level.

Ali *et al.* (2017) evaluated entomopathogenic fungus *L. muscarium* against *B. tabaci* (Gennadius). This study revealed that the production of bassianolide affected the AchE activities of the whitefly.

Zhang *et al.* (2022) conducted the toxicological and biochemical studies of *B. bassiana* against *Megalurothrips usitatus* (Bagrall). Findings revealed the activity of AChE was significantly reduced compared to the control, which indicates decreased AChE activity is linked with the production of the secondary metabolite bassianolide.

Sbaraini *et al*. (2022) studied the interactions between *G. mellonella* AChE and pseurotin A, isolated from *M. anisopliae* strain QS155. The inhibition exerted by the compound was related to interactions with hydrophobic residues, *viz.,* tyrosine, phenylalanine and tryptophan, which were present in the active site pocket. Findings

showed that the AChE-pseurotin A complex was stable and the effects was attributed to AChE activity modulation.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study on "Bioactive metabolites of *Beauveria bassiana* (Balsamo) Vuillemin and its efficacy on tobacco caterpillar, *Spodoptera litura* (Fab.)" was carried out at the Department of Agricultural Entomology, College of Agriculture, Vellanikkara, during 2020-2022. The facilities at the Pesticide Residue Testing Laboratory and the Centre for Plant Biotechnology and Molecular Biology were utilised for the research programme.

This chapter describes the materials, procedures and techniques used for the conduct of different experiments.

3.1 Extraction and identification of secondary metabolites

3.1.1 Maintenance of fungal isolate - *Beauveria bassiana* (NBAII-Bb-5a)

The culture of *B. bassiana* maintained in the fungal repository of the Department of Agricultural Entomology, College of Agriculture, Vellanikkara was used for the study. It was subcultured and maintained in Potato Dextrose Agar (PDA) medium for further studies.

PDA media was prepared by dissolving 39 g of commercial Potato Dextrose Broth (PDB) and 20 g of agar in 1000 mL of distilled water. The antibiotic, chloramphenicol (25 mg) was added to the medium to prevent bacterial contamination, and the final pH was adjusted to 5.6±0.2 at 25°C. The media was sterilised by autoclaving at 15 pounds of pressure (121°C) for 15 min and then cooled to 45-50°C.

3.1.2 Preparation of fungal cultures

Beauveria bassiana was cultured in two different growth media, PDB and Czapek Dox Broth (CDB). The PDB was prepared as in 3.1.1 without adding agar and the final pH was adjusted to 5.6±0.2 at 25°C. Czapek Dox Broth was prepared by suspending 30 g of sucrose, 2 g of sodium nitrate, 1 g of dipotassium phosphate, 0.5 g of magnesium sulphate, 0.5 g of potassium chloride and 0.01 g of ferrous sulphate in 1000 mL of distilled water. The antibiotic chloramphenicol (25 mg) was added to the

medium to prevent bacterial contamination, and the final pH was adjusted to 7.3±0.2 at 25°C. The media was transferred to 250 mL Erlenmeyer flasks @ 100 mL/flask and was sterilised as specified in 3.1.1.

A mycelial disc of 8 mm was cut using a sterile cork borer from seven-day-old *B. bassiana* grown on PDA plates and inoculated into 250 mL Erlenmeyer flasks containing 100 mL of PDB and Czapek-Dox broth, respectively, under aseptic conditions and incubated at room temperature (28±2°C) for 14 days (Plate 1A and B).

3.1.3 Extraction of secondary metabolites from fungal culture

3.1.3.1 From PDB

After 14 days of incubation, the culture filtrate of *B. bassiana* from PDB (1000 mL) was harvested by filtering the culture through four layers of sterilised cheese cloth under aseptic conditions. Then, the collected culture filtrate was transferred to 250 mL centrifuge bottles and centrifuged at 1500 g for 20 min at 10°C using a cooling centrifuge (Thermo Fisher Scientific^{TM}). The supernatant was filtered through a glass funnel lined with Whatman filter paper No. 1 under aseptic conditions to completely remove conidia and hyphal debris (Bandani *et al.*, 2000).

The culture filtrate was divided into different aliquots of 100 mL each and added with an equal quantity of ethyl acetate (100 mL) in the ratio of 1:1 (v/v) and kept overnight on a rotary shaker at 200 rpm at room temperature (28±2°C) (Plate 2A). The mixture was transferred to a separatory funnel, shaken vigorously for 10 min, and kept undisturbed for 10 min to separate the layers based on their relative solubilities (Plate 3). Once the layers were separated, the ethyl acetate and culture filtrate fractions were collected separately in conical flasks. The above extraction process was repeated thrice for accuracy and dried over Na_2SO_4 to remove water content from the ethyl acetate fraction before feeding to a rotary vacuum evaporator.

The ethyl acetate fraction was evaporated under reduced pressure at a vacuum of 178^{mbar} and 45°C (Heidolph®, Hei-VAP Precision) (Plate 4). After complete evaporation, the concentrated crude toxin in the round-bottom flask was collected and

recovered using 4 mL of HPLC-grade methanol. The crude toxins were stored in the refrigerator at 4°C for further analysis.

3.1.3.2 From CDB

The culture filtrate (Plate 2B) of *B. bassiana* from CDB (1000 mL) and crude toxin were obtained as per the procedure in 3.1.3.1.

3.1.4 Extraction of secondary metabolites from host insects

3.1.4.1 Rearing of *Spodoptera litura*

The host insect *S. litura* was reared on a semi-synthetic diet and castor leaves to ensure adequate availability of test insects for different laboratory studies.

3.1.4.1.1 Preparation of semi-synthetic diet for *Spodoptera litura*

The semi-synthetic diet for *S. litura* was prepared as per the procedure of Mani and Rao (1998). Kidney beans (65 g) soaked in water for 12 h. were ground in a mixer to get a fine paste. Wheat bran (65 g) and yeast extract powder (25 g) were added to the paste and ground for 5 min. The ingredients *viz.*, casein (3 g), ascorbic acid (4 g), sorbic acid (0.9 g), methyl para hydroxy benzoate (0.4 g), cholesterol (0.3 g), streptomycin sulphate (0.1 g), multivitamin (1 tablet) and formaldehyde (2 mL) were added. Agar powder (12 g) was boiled in distilled water up to the required consistency and was mixed with all the ingredients. The mixture was ground for 30 sec, and the molten medium was poured into the Petri plates to solidify (Plate 5A). Once solidified, the media-containing Petri plates were stored inside the refrigerator for future use.

3.1.4.1.2 Mass rearing of *Spodoptera litura* in semi-synthetic diet

Egg masses of *S. litura* were collected from banana fields of Banana Research Station, Kannara, Thrissur. Egg masses along with banana leaves were placed in plastic boxes (9 cm in diameter and 5 cm in height) lined with tissue paper. The lid of each box was provided with a square window covered with muslin cloth for proper aeration. After hatching, neonates were allowed to feed on castor leaves for 3 days

and then larvae were provided with small rectangular pieces of a semi-synthetic diet. The containers were cleaned and supplied with fresh semi-synthetic diet pieces every day until the larvae reached the pupal stage. The pupae (5pairs) were transferred carefully to sterilised plastic containers. After the emergence of adults, moths were placed inside an oviposition chamber for mating and provided with cotton soaked in honey and vitamin E solution. Paper strips were provided for oviposition. After three days, egg masses were collected and placed in containers along with fresh castor leaves. After hatching of eggs, neonates were transferred to rearing boxes and fed with castor leaves.

3.1.4.1.3 Mass rearing of *Spodoptera litura* on castor leaves

Egg masses of *S. litura* collected from 3.1.4.1.2 were placed in plastic boxes (45 cm in diameter and 5 cm in height) lined with tissue paper (Plate 5B). The lid of each box was provided with a square window covered with muslin cloth for proper aeration (Plate 5C). The containers were cleaned and provided with fresh castor leaves every day until the larvae reached pupal stage. Pupae were collected and placed in a plastic container for adult emergence as in 3.1.4.1.2. The larvae thus obtained were used for further studies.

3.1.4.2 Preparation of spore suspension of *Beauveria bassiana*

Beauveria bassiana (NBAIR-Bb-5a) was cultured on PDA plates under sterile conditions and incubated for 14 days in BOD at 28°C. The stock spore suspension was prepared under sterile conditions by adding sterile distilled water containing Tween 80 (0.01% v/v) to a 14-day-old sporulating culture in Petri plates and gently scraping the surface with a sterile spatula (Moraga *et al.,* 2009; Lopez and Sword, 2015). The resulting mycelia and spores were then filtered through three layers of sterile muslin cloth into a sterile beaker containing sterile distilled water along with Tween 80 (0.01% v/v) (Saruhan, 2018). The suspension was then homogenised with a vortex mixer for three min to get a uniform spore suspension. Spore count was assessed under the microscope using an improved Neubauer hemocytometer (Kiruthiga *et al.,* 2022).

A. Potato dextrose broth

B. Czapek Dox broth

Plate 1. *Beauveria bassiana* culture in growth medium after14th day of incubation

A. PDB

B. CDB

Plate 2. *Beauveria bassiana* culture filtrate

Plate 3. Extraction of solvent phase using separatory funnel

Plate 4. Vacuum rotary evaporator

$$\text{Number of conidia mL}^{-1} = \frac{X \times 400 \times 10 \times 1000 \times D}{Y}$$

Where,

X = Average number of conidia per square per small square

400 = Number of small squares counted

10 = Depth factor

1000 = Conversion factor from mm^3 to cm^3

D = Dilution factor

Y = Number of small squares checked

The final concentration was adjusted to 1×10^8 conidia mL^{-1} by adding sterile distilled water using the following formula.

$$\text{Final volume} = \frac{\text{Stock solution} \times \text{Stock concentration}}{1\times10^8}$$

3.1.4.3 Treatment of *Spodoptera litura* with spore suspension

Twenty, third-instar *S. litura* larvae were sprayed with 100 mL spore suspension of Bb-5a @ 1 x 10^8 spores mL^{-1} under aseptic conditions whereas the control group was sprayed with sterile water (Plate 6A and B). The treated larvae were kept in plastic boxes, where the lids of the boxes were replaced with muslin cloth and incubated at room temperature (28±2°C). Larval movement, feeding and mortality were observed up to 7 days after treatment.

Dead larvae were collected and surface-sterilised using 4 per cent sodium hypochlorite, followed by three rinses in distilled water. The larvae were then transferred to Petri plates lined with damp sterile tissue paper (humid chamber) and kept in a BOD incubator (28°C) to provide optimal conditions for fungal growth on the cadaver. The fungal growth was observed for up to 7 days, and microscopic observation of spores on the surface of the dead larvae was carried out to confirm the mortality caused by *B. bassiana* (Nithya *et al.,* 2019).

3.1.4.4 Extraction of metabolites from host insects

3.1.4.4.1 From infected *Spodoptera litura*

Twenty, Bb-5a infected *S. litura* larvae were homogenised in 150 mL of distilled water and centrifuged at 500 g for 5 min to remove insect debris (Plate 6C). The supernatant containing metabolites was collected, and the pH was adjusted to 2 with 37% w/v hydrochloric acid (Strasser *et al*., 2000).

Larval supernatant (150 mL) was extracted with an equal volume of ethyl acetate (150 mL) in the ratio of 1:1 (v/v) using a separatory funnel and shaken vigorously for 10 min, and the funnel was kept undisturbed for 10 min to separate the layers based on their relative solubilities. The ethyl acetate fraction (upper layer) and larval extract fraction (lower layer) were collected separately. The extraction process was repeated three times for accuracy and dried over Na_2SO_4 to remove water content from the ethyl acetate fraction.

The ethyl acetate fractions from both healthy and infected larvae were evaporated and concentrated separately under reduced pressure at vacuum of 168^{mbar} and temperature of $60^{o}C$ using a rotary vacuum evaporator (Heidolph®, Hei-VAP precision) (Nithya *et al.,* 2021). The crude toxin was recovered using 4 mL of HPLC-grade methanol and stored in the refrigerator at 4°C for further analysis.

3.1.4.4.2 From healthy *Spodoptera litura*

The extract from healthy larvae was collected as per 3.1.4.4.1 (Plate 6D).

3.2 Identification and characterization of secondary metabolites of *Beauveria bassiana*

The crude toxin extracted from *B. bassiana* cultured in two growth media (*i.e.,* PDB and CDB) and the larval extract of healthy and Bb-5a infected *S. litura* were analysed by chromatographic methods, *viz.,* HR-LCMS and HR-GCMS to identify the mycotoxins.

The analysis was carried out at the Sophisticated Analytical Instrument Facility (SAIF), IIT Mumbai.

A. Semi-synthetic media

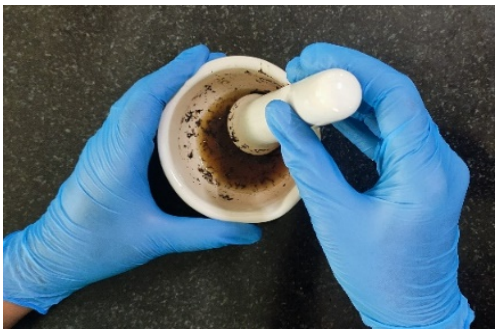
B. Rearing on castor leaves

C. Rearing setup

Plate 5. Mass rearing of *Spodoptera litura* on synthetic diet and castor leaves

A. Larvae treated with spore suspension of *Beauveria bassiana*

B. Larvae treated with sterile distilled water



C. From infected larvae

D. From healthy larvae

Plate 6. Treatment and homogenization of *Spodoptera* larvae for extracting crude larval extract

3.2.1 High Resolution-Liquid Chromatography-Mass Spectrometry (HR-LCMS)

3.2.1.1 Toxin from PDB

The crude toxin extracted from PDB, dissolved in methanol was subjected to HR-LCMS (G6550A system, Agilent Technologies, USA) analysis at IIT Bombay, to identify the secondary metabolites produced by *B. bassiana*. The sample was run isocratically for 30 min using acetonitrile (95%) as solvent. The MS analysis was carried out using ESI positive and negative ionisation modes. MS source conditions - capillary voltage 3500 V, gas temperature 250°C, drying gas flow 13 $Lmin^{-1}$, sheath gas temperature 300°C, sheath gas flow 11, nebulizing gas pressure 35 (psig), fragmentor 175 V, skimmer 65 V, Octopole RF Peak 750 V and mass range m/z 120-1200.

3.2.1.2 Toxin from CDB

The crude toxin from CDB was dissolved in methanol and subjected to HR-LCMS (G6550A system, Agilent Technologies, USA) analysis as per 3.2.1.1.

3.2.1.3 Toxin from *Beauveria* infected *Spodoptera litura*

The crude extract from *Beauveria* infected *S. litura* was dissolved in methanol and subjected to HR-LCMS (G6550A system, Agilent Technologies, USA) analysis as per 3.2.1.1.

3.2.1.4 Toxin from healthy *Spodoptera litura*

The crude extract from healthy *S. litura* was dissolved in methanol and subjected to HR-LCMS (G6550A system, Agilent Technologies, USA) analysis as per 3.2.1.1.

The structure of metabolites was ascertained based on high-resolution mass spectrometry and confirmed using the Metlin database.

3.2.2 High Resolution-Gas Chromatography Mass Spectrometry (HR-GCMS)

3.2.2.1 Toxin from PDB

Crude toxins extracted from PDB were analysed using HR-GCMS to detect volatile metabolites. It was carried out on a Perkin Elmer (Clarus 680) series GC-MS/MS (Marathon, USA) system equipped with a Clarus 600 (EI) auto-sampler coupled with an Elite-5 MS capillary column (30 m x 0.25 mm in dia and 0.250 μm) (PerkinElmer, Inc., USA). Helium was used as the carrier gas at a flow rate of 1 mL min., a split ratio of 10:1, a mass scan of 50-600 Da and an ionisation energy of 70. The oven temperature was programmed with the initial temperature at 60°C for 2 min, rising at 10°C min^{-1} up to 300°C and then being held isothermally at 300°C for 6 min, for a total run time of 32 min. The composition of the crude extract constituents was expressed as a percentage of the peak area. The chemical compounds were identified and characterized based on their retention time (RT). The mass spectral data retrieved were matched with those of standards available in the NIST database.

3.2.2.2 Toxin from CDB

The crude toxins extracted from CDB were analysed using HR-GCMS as per 3.2.2.1.

3.2.2.3 Toxin from *Beauveria* infected *Spodoptera litura*

The crude extract from *Beauveria* infected *S. litura* were analysed using HR-GCMS as per 3.2.2.1.

3.2.2.4 Toxin from healthy *Spodoptera litura*

The crude extract from healthy *S. litura* were analysed using HR-GCMS as per 3.2.2.1.

The analysis was carried out at the Sophisticated Analytical Instrument Facility (SAIF) at IIT Mumbai. The biological activity of metabolites was identified by mapping all the metabolites in the KEGG and PubChem databases.

3.3 Evaluation of Bioefficacy of the crude toxin on *Spodoptera litura*

A topical bioassay of crude toxin was performed on third-instar larvae of *S. litura* as per the procedure of Ragavendran *et al*. (2019). The bioefficacy of the crude toxin extracted from PDB was evaluated. Different doses were fixed based on the preliminary bioassay.

3.3.1 Preparation of samples for bioassay

3.3.1.1 Sample preparation for preliminary bioassay

Test concentrations were prepared by dissolving 10 mg of crude toxin in 1 mL of dimethyl sulfoxide (DMSO), which was then diluted using sterile distilled water to six different concentrations *viz.,* 5000 ppm, 1000 ppm, 500 ppm, 100 ppm, 50 ppm, and 10 ppm. Larvae treated with distilled water served as absolute control. The preliminary bioassay was performed with seven treatments (six doses plus one control) with one replication. Four numbers of third-instar larvae of *S. litura* were released per replication and sprayed using Potter's tower (Burkard Manufacturing Company, UK) (Plate 7A and B). Observations were recorded at 12, 24, 36, and 48 h up to mortality. Larvae were considered dead if they were unable to move in a coordinated way when prodded with a fine-haired brush.

3.3.1.2 Sample preparation for bioassay

Test concentrations were prepared by dissolving 100 mg of crude toxin in DMSO and then diluted using sterile distilled water to nine different concentrations *viz.,* 100 ppm, 90 ppm, 70 ppm, 50 ppm, 30 ppm, 10 ppm, and 9 ppm. DMSO dissolved in distilled water served as a negative control and distilled water served as an absolute control.

3.3.2 Bioassay

The experiment was performed with nine treatments (7 doses plus two controls) and six replications (Plate 8). Four numbers of third-instar larvae of *S. litura* were released for each replication and sprayed using Potter's tower. The mortality of test insects was recorded up to 96 h after treatment. Larvae were considered dead if

they were unable to move in a coordinated way when prodded with a fine-haired brush.

As the control treatments (both absolute control and negative control) did not record any mortality in the bioassay, the mortality data were directly subjected to probit analysis (Finney, 1971) by using the software PoloPlus (LeOra Software, 2002) for the calculation of median lethal concentration (LC_{50}) and median lethal time (LT_{50}).

3.4 *In silico* molecular docking studies of metabolites of *B. bassiana*

Docking is the virtual screening of compounds and predicting the strongest binders based on various scoring functions. Docking studies of the target protein (AChE) were done with secondary metabolites of *B. bassiana* (NBAIR-Bb-5a) to find the preferred orientation and binding affinity of the metabolites using scoring functions.

In silico docking studies were conducted to elucidate the mode of action of metabolites of *B. bassiana* (NBAIR-Bb-5a). *In silico* analysis was carried out at the Distributed Information Centre, the Centre for Plant Biotechnology and Molecular Research (CPBMB), College of Agriculture, Vellanikkara, Thrissur, Kerala.

3.4.1 Software

The software used for molecular docking was CB-Dock (cavity-detection guided blind docking) software, developed and distributed by Dr. Yang Liu, Sichuan University, China. The electrostatic potential calculation (number and length of H-bonds), model visualisation, and image generation were performed using the PyMOL software.

3.4.2 Ligands and protein targets

The different ligands were selected from the list of secondary metabolites in HR-LCMS data of *B. bassiana* (NBAIR-Bb-5a) from IIT, Mumbai. Metabolites that showed > 80 per cent best match in mzCloud results and metabolites reported to have biological activities, *viz.*, insecticidal, antimicrobial, *etc.*, were selected for *in silico*

A. Potter's tower

B. Injecting crude toxin

Plate 7. Potter's tower apparatus and injecting crude toxin to the reservoir

A. 100 ppm

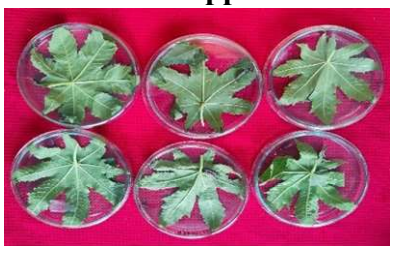
B. 90 ppm

C. 70 ppm

D. 50 ppm

E. 30 ppm

F. 10 ppm



G. 9 ppm

H. Absolute control

I. Negative control

Plate 8. Experimental setup for bioassay of crude toxin on third-instar larvae of *Spodoptera litura*

analysis. The 3D structures of secondary metabolites were downloaded from the PubChem database.

The X-ray crystallographic structure of the target protein acetylcholinesterase of *Drosophila melanogaster* (*Dm* AChE, PDB ID 1QON) was retrieved from the Protein Data Bank (PDB).

The ligands were filtered using the active sites of the target receptor and identified by the inhibitory properties of the amino acid residues present in the binding sites. Each ligand was docked with the target protein, AChE, and the interacting ligands were visualised and scored in PyMOL software.

3.4.3 Molecular docking

CB-Dock is the first cavity detection-guided blind docking tool designed with AutoDock Vina. This method automatically identifies the binding sites and improves the accuracy by predicting the binding sites of target proteins using a curvature-based cavity detection approach (CurPocket) and the binding poses of query ligands using AutoDock Vina (Liu *et al.*, 2020).

The steps followed for performing the work were as below:

Open CB-DOCK software

Upload target protein (AChE) file in the PDB format and ligand file in the SDF format

Submit to the CB-Dock server

While processing, a progress bar indicated the status of the docking. When the processing was complete (after approximately 2 min), the web page was updated with the results. The Table listed vina scores, cavity sizes, docking centres and sizes of predicted cavities. Once a ligand in the Table was selected, the structure in the interactive 3D graphics was visualized in PyMOL software.

3.5. AChE inhibitory activity in *Spodoptera litura*

Wet-lab studies were carried out to validate the AChE inhibitory activity of fungal metabolites of *B. bassiana* (NBAIR-Bb-5a) revealed through *in silico* molecular docking studies.

3.5.1 Sample Preparation

Twenty larvae of *S. litura* (third-instar) were treated with the crude toxin of *B. bassiana* (NBAIR-Bb-5a) at the concentration of 1000 ppm, as per the procedure of Ragavendran *et al*. (2019). The treated larvae were kept in Petri dishes and provided with fresh castor leaves. Larvae of *S. litura* treated with sterile distilled water as control. Observations were recorded till mortality (7 days).

Twenty healthy and dead larvae were weighed and rinsed with acetone to remove surface residues. The whole larval homogenate was prepared by grinding twenty larvae of *S. litura* in an ice-cold 0.1 M sodium phosphate buffer (pH 7.2). The homogenate was centrifuged at 10,000 rpm for 20 min at 4ºC. The supernatant was stored at -20˚C and used as an enzyme source.

3.5.2 Estimation of total protein

The total protein content of twenty healthy and dead *S. litura* was estimated as per the procedure given by Lowry *et al.* (1951).

3.5.2.1 Preparation of standard Bovine Serum Albumin solution

A stock Bovine Serum Albumin (BSA) was prepared by dissolving 50 mg of BSA in 50 mL of distilled water in a volumetric flask. The working standard solution was prepared by pipetting out 10 mL from the stock solution and making up to 50 mL with distilled water in a volumetric flask so that 1 mL of the solution contained 200 µg of protein. Different aliquots of 200 µL, 400 µL, 600 µL, 800 µL, and 1000 µL were pipetted into different test tubes from the working standard and made up to 1 mL using distilled water. A test tube with distilled water alone was served as blank. The reagents used are given in Table 1.

Table 1. Reagents for protein estimation

Reagents	Particulars
Reagent A	2% Sodium carbonate in 0.1 N NaOH
Reagent B	0.5% Copper sulphate solution in 1 % of sodium potassium tartrate solution
Reagent C	50 mL of reagent A and 1mL of reagent B, prepared just prior to the use
Reagent D	Folin- ciocalteu reagent (FCR) diluted in 1:1 ratio with distilled water before use

Five millilitres of reagent C were added to all test tubes, including blank. The contents in the test tubes were mixed well and allowed to set for 10 min. Afterwards, reagent D (0.5 mL) was added, mixed thoroughly, and incubated at room temperature in the dark condition for 30 min. The absorbance of the developed blue colour was recorded at 660 nm using spectrophotometer (Agilent Cary 60 UV-VIS®). A standard graph was drawn using the measured absorbance value and the corresponding concentrations of BSA (Fig 1).

3.5.2.2. Total protein estimation

Fifty microlitres of supernatant/enzyme extract prepared (3.4.1) were taken in a test tube, and 2.5 mL of reagent C was added. After an incubation period of 10 min, 250 µL of reagent D was added. The reaction mixture was kept in dark for 30 min at room temperature (28±2°C). Absorbance was recorded at 660 nm in spectrophotometer (Agilent Cary 60 UV-Vis®).

The protein content was calculated from the standard graph and expressed in mg mL^{-1}. The facility available at the Pesticide Residue Analysis Laboratory, Dept. of Agricultural Entomology, College of Agriculture, Vellanikkara, was utilised for the study.

3.5.3. Assay of Acetylcholine Esterase (AChE) enzyme

Acetylcholine esterase activity in healthy and dead larvae was assayed as per the procedure of Kranthi (2005) using acetylthiocholine iodide as a substrate.

3.5.3.1 Preparation of stock solutions for AChE assay

- Acetylthiocholine iodide (0.1M) was prepared by dissolving 0.28g acetylthiocholine iodide in 10 mL of 0.1M sodium phosphate buffer (pH 8).
- DTNB (0.01M) was prepared by dissolving 0.039g of 5, 5' – dithiobis- (2-nitrobenzoic acid) (DTNB) and 0.15g of sodium carbonate (1.5%) in 10 mL of 0.1M sodium phosphate buffer (pH 8).
- Sodium phosphate buffer (0.1M) (pH 8) was prepared by taking 50 mL of 0.2M sodium phosphate buffer (pH 8), made up to 100 mL using distilled water.

3.5.3.2 AChE assay

100 microliters of enzyme stock + 2.96 mL of 0.1M sodium phosphate buffer (pH 8.0)

Mixed thoroughly and incubated for 5 min at room temperature (28±2°C)

Added 10 µL of DTNB solution and 30 µL of acetylthiocholine iodide

Incubated the reaction mixture for 30 min at room temperature (28±2°C)

Change in absorbance was measured at 412 nm for 30 min against blank using spectrophotometer (Agilent Cary 60 UV-Vis®).

Blank was prepared by adding 10 µL of DTNB solution and 30 µL of acetylthiocholine-iodide to 2.96 mL of 0.1 M phosphate buffer (pH 8.0) and it was incubated at room temperature for 30 min.

The AChE activity was expressed as µ moles of acetylcholine hydrolysed min^{-1} mg^{-1} protein.

$$\text{AChE activity} = \frac{\text{Change in absorbance } (\Delta A) \text{ x volume of the reaction mixture x } 1000}{\varepsilon \text{ x } 0.10 \text{ x protein (mg)}}$$

Where,

- $\Delta A = \frac{\text{Final Absorbance value – Initial Absorbance value}}{\text{Total time}}$
- ε is the molar extinction coefficient of the chromophore at 412 nm = 1.36 x 10^4 Mm^{-1} cm^{-1}
- 0.1 is the volume of enzyme source.

AChE activity inhibition was calculated according to the following formula (Maazoan *et al.,* 2017).

$$\text{AChE activity inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \text{ x } 100$$

Where,

- $A_{control}$ = the absorbance of the control
- A_{sample} = the absorbance of the sample

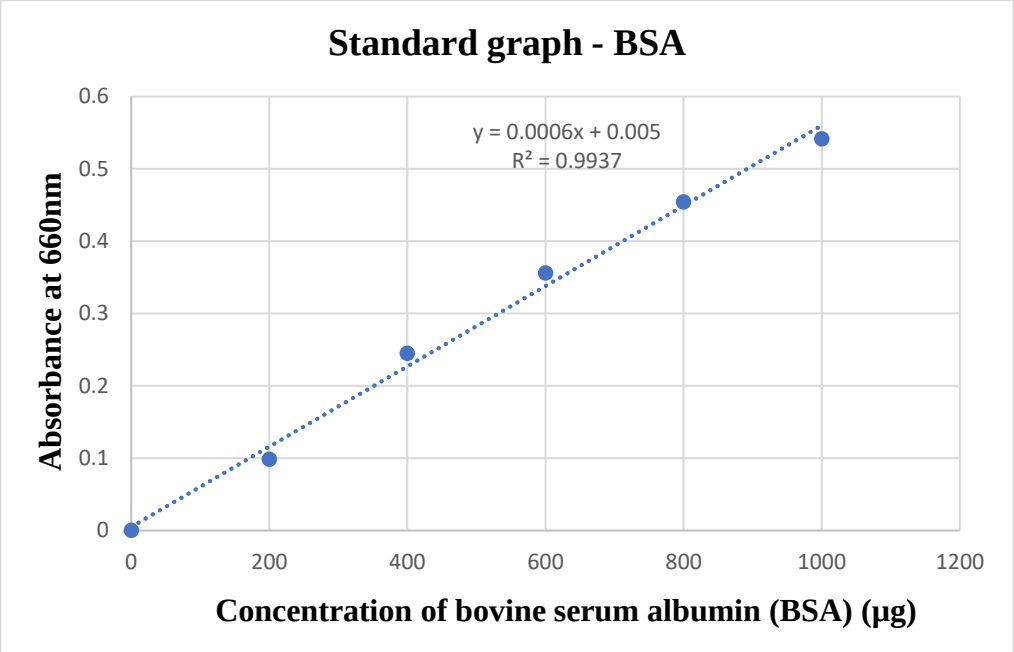


Fig. 1. Standard curve of bovine serum albumin (BSA)

RESULTS

4. RESULTS

The results of the study on "Bioactive metabolites of *Beauveria bassiana* (Balsamo) Vuillemin and its efficacy on tobacco caterpillar, *Spodoptera litura* (Fab.)" conducted at the Department of Agricultural Entomology, College of Agriculture, Vellanikkara are presented in this chapter.

4.1 Extraction and identification of secondary metabolites

4.1.1 Extraction of secondary metabolites from *Beauveria bassiana* (Bb-5a) - PDB

Beauveria bassiana cultured in Potato Dextrose Broth (PDB) was found to support high production of secondary metabolites. Dark brown pigmentation was observed in the growth medium after 14 days of incubation at room temperature (28±2°C) as against light brown colour in uninoculated medium. The vacuum evaporation of ethyl acetate fraction yielded 0.28 g of crude toxin per litre of culture filtrate (Plate 9A).

4.1.1.1 Identification and characterisation of secondary metabolites of *Beauveria bassiana* – PDB

The crude toxin from ethyl acetate fraction of culture filtrate from PDB was subjected to chromatographic methods *viz.*, HR-LCMS and HR-GCMS.

4.1.1.1.1 Metabolites detected through High Resolution-Liquid Chromatography Mass Spectrophotometry (HR-LCMS)

The crude toxin isolated from the culture filtrate of *B. bassiana* grown in PDB medium subjected to HR-LCMS, revealed the presence of 45 secondary metabolites and the chromatogram is depicted in Fig 2. The biological activities of selected metabolites were identified through PubChem and KEGG databases. The identified metabolites with molecular formula, molecular weight, and best match are depicted in the Annexure II.1.

The compounds such as beauvericin, 1,2-dipalmitin, á-D-glucopyranose, 1,6-anhydro, 2-palmitoylglycerol, ethylenediamine-N, N'-dipropionic acid, harmine, cyclohexylmethyl undecyl ester, cordycepin, piperidinine, hexadecanoic acid, *etc.*

detected through HR-LCMS of the crude toxin of *B. bassiana* possess insecticidal activity based on PubChem and KEGG databases. The molecular weight, molecular formula, and best match of insecticidal compounds are depicted in Table 2.

Table 2. Insecticidal metabolites of *Beauveria bassiana* detected by HR-LCMS – PDB

Sl. No.	Name of the compound	Molecular formula	Molecular weight	Best match	Reference
1	Beauvericin	$C_{45}H_{57}N_3O_9$	800.43	87.10	Wang and Xu, (2012)
2	5-Oxotetrahydrofuran-2-carboxylic acid	$C_5H_6O_4$	130.09	88.30	Oller-Lopez *et al.* (2005)
3	á-D-Glucopyranose, 1,6-anhydro-	$C_6H_{10}O_5$	162.14	83.50	Syed *et al.* (2018)
4	1,2-Dipalmitin	$C_{35}H_{68}O_5$	568.92	85.12	Ragavendran *et al.* (2017)
5	2-Palmitoylglycerol	$C_{19}H_{38}O_4$	330.50	89.60	Nagalakshmi and Murthy (2015)
6	Dihydrothiophenone	$C_4H_4O_S$	102.15	86.10	Champagne *et al.* (1986)
7	Cyclohexylmethyl undecyl ester	$C_{18}H_{36}O_3S$	332.54	87.67	Domon *et al.* (2018)
8	Ethylenediamine-N, N'-dipropionic acid	$C_8H_{16}N_2O_4$	204.22	85.59	Paulraj *et al.* (2011)
9	Piperidone	C_5H_9NO	99.06	89.20	Shonouda *et al.* (2008)
10	Cordycepin	$C_{10}H_{13}N_5O_3$	251.10	80.20	Kim *et al.* (2002)
11	Picolinic acid	$C_6\ H_5\ NO_2$	123.03	88.90	Bacon *et al.* (1996)

4.1.1.1.2 Metabolites detected through High Resolution-Gas Chromatography Tandem Mass Spectrometry (HR-GCMS)

The crude toxins extracted from PDB were analysed using HR-GCMS to detect volatile metabolites, revealed the presence of 37 compounds. The volatile compounds present in the crude toxin were identified using NIST database and

enlisted with the molecular formula, molecular weight, and retention values in the Annexure I.2. The biological activities of selected metabolites were identified through PubChem and KEGG databases. The compounds such as 4-dodecylbenzenesulfonic acid, pimelic acid, tetraacetylethylenediamine, ethylmalonic acid, 4-undecylbenzenesulfonic acid, harmine, hexadecenoic acid, 2,6-pyridine dicarboxylic acid, *etc.,* were detected in HR-GCMS chromatogram (Fig. 3). The important insecticidal compounds with their molecular formula and molecular weight are presented in Table 3.

Table 3. Insecticidal metabolites of *Beauveria bassiana* detected by HR-GCMS - PDB

Sl. No.	Name of thecompound	Molecular formula	Molecular weight	Reference
1	Harmine	$C_{13}H_{12}N_2O$	212.25	Zeng *et al.* (2010)
2	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.24	Rahuman *et al.* (2000)
3	2,6-pyridine dicarboxylic acid	$C_7H_5NO_4$	167.12	Tanvir *et al.* (2018)

4.1.2 Extraction of secondary metabolites from *Beauveria bassiana* (Bb-5a) - CDB

Beauveria bassiana cultured in Czapek Dox Broth (CDB) supported the production of secondary metabolites. Light-yellow pigmentation was observed in the growth medium after 14 days of incubation at room temperature (28±2°C). The ethyl acetate fraction of 1L of culture filtrate under vacuum evaporation yielded 0.23 g of crude toxin (Plate 9B).

4.1.2.1 Identification and characterization of secondary metabolites of *Beauveria bassiana* - CDB

4.1.2.1.1 Metabolites detected through High Resolution-Liquid Chromatography Tandem Mass Spectrometry (HR-LCMS)

HR-LCMS spectra of the crude toxin extracted from CDB culture filtrate is depicted in Fig.4. It revealed the presence of 44 secondary metabolites whose biological activities were identified through PubChem and KEGG databases. The

identified metabolites such as, beauvericin, 5-oxotetrahydrofuran-2-carboxylic acid, 1,2-dipalmitin, 2-palmitoylglycerol, piperidone, picolinic acid, imidazole-2-methanol, 1- methyl-, phenobarbital, pentobarbital, sphinganine and 2-undecyl-4(1H)-quinolinone N-oxide *etc.*, with molecular weight, molecular formula and best match are listed in the Annexure III.1. The compounds with insecticidal activity are presented in Table 4.

Table 4. Insecticidal metabolites of *Beauveria bassiana* detected by HR-LCMS – CDB

Sl. No.	Name of the compound	Molecular formula	Molecular weight	Best match	Reference
1	Beauvericin	$C_{45}H_{57}N_3O_9$	800.43	87.10	Wang and Xu, (2012)
2	5-Oxotetrahydrofuran-2-carboxylic acid	$C_5H_6O_4$	130.09	88.30	Oller-Lopez *et al.* (2005)
3	1,2-Dipalmitin	$C_{35}H_{68}O_5$	568.92	85.12	Ragavendran *et al.* (2017)
4	2-Palmitoylglycerol	$C_{19}H_{38}O_4$	330.50	89.60	Nagalakshmi and Murthy (2015)
5	Piperidone	C_5H_9NO	99.06	89.20	Shonouda *et al.* (2008)
6	Picolinic acid	$C_6 H_5 NO_2$	123.03	88.90	Bacon *et al.* (1996)

4.1.2.1.2 Metabolites detected through High Resolution-Gas Chromatography - Tandem Mass Spectrometry (HR-GCMS)

The crude toxins extracted from CDB was analysed using HR-GCMS to detect volatile metabolites and the chromatogram is presented below (Fig. 5). The analysis detected the presence of 33 volatile compounds. The biological activities of selected metabolites were identified through PubChem and KEGG databases and insecticidal compounds are presented in Table 5. The compounds like 2,6-pyridinecarboxylic acid, 1,3-phenylenediamine, 3,5-dichloro-2,6- dimethoxybenzohydrazide, alpha-aminoisobutyronitrile, 12-bromolauric acid, and hexadecanoic acid, *etc*. are represented along with the molecular weight, molecular formula, and retention time in the Annexure III.2.

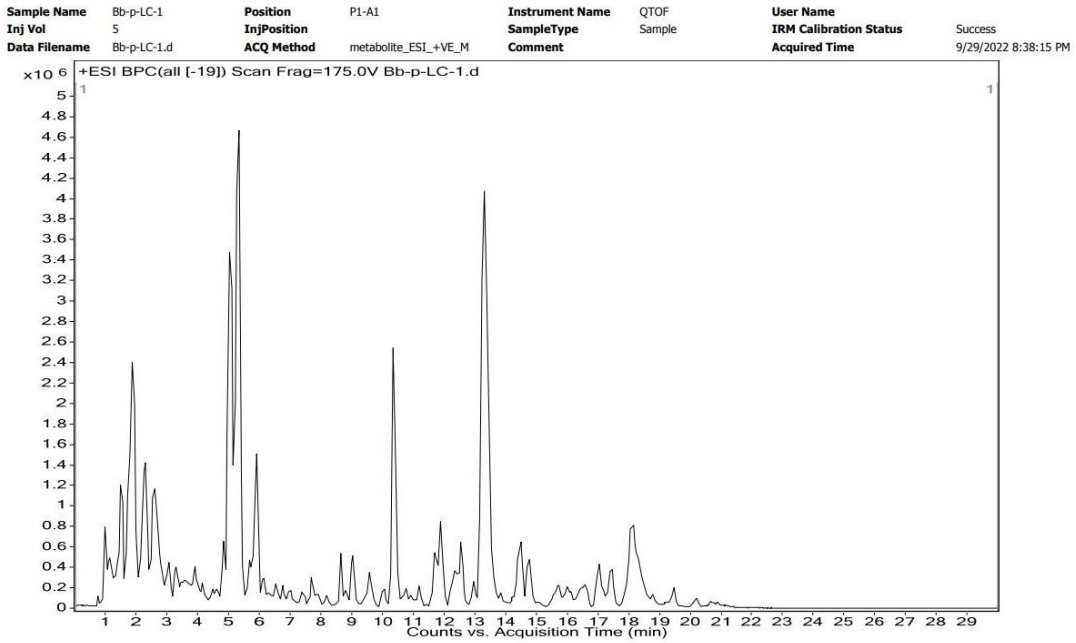


Fig. 2. HR-LCMS spectra of crude toxin of *Beauveria bassiana* extracted from PDB medium

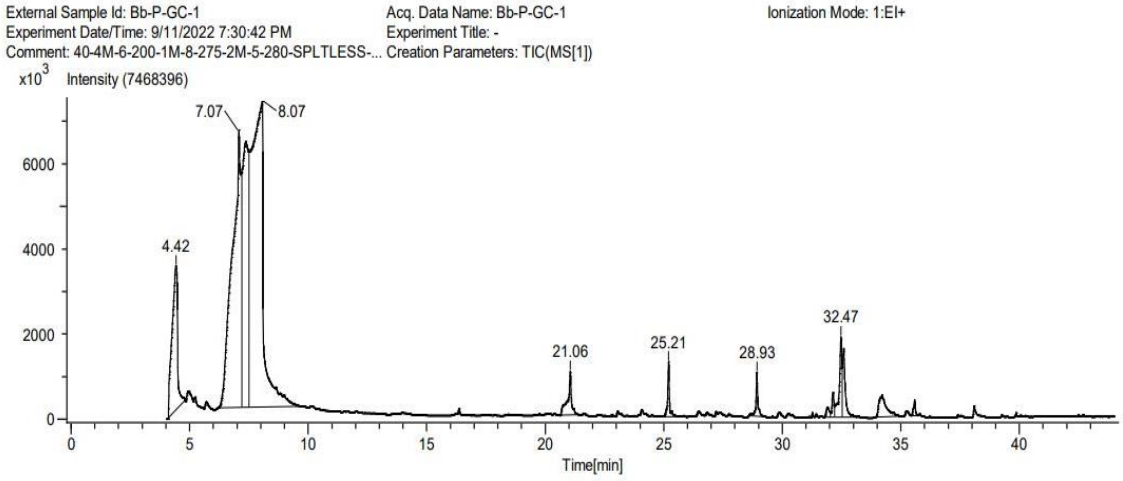


Fig. 3. HR-GCMS spectra of crude toxin of *Beauveria bassiana* extracted from PDB medium

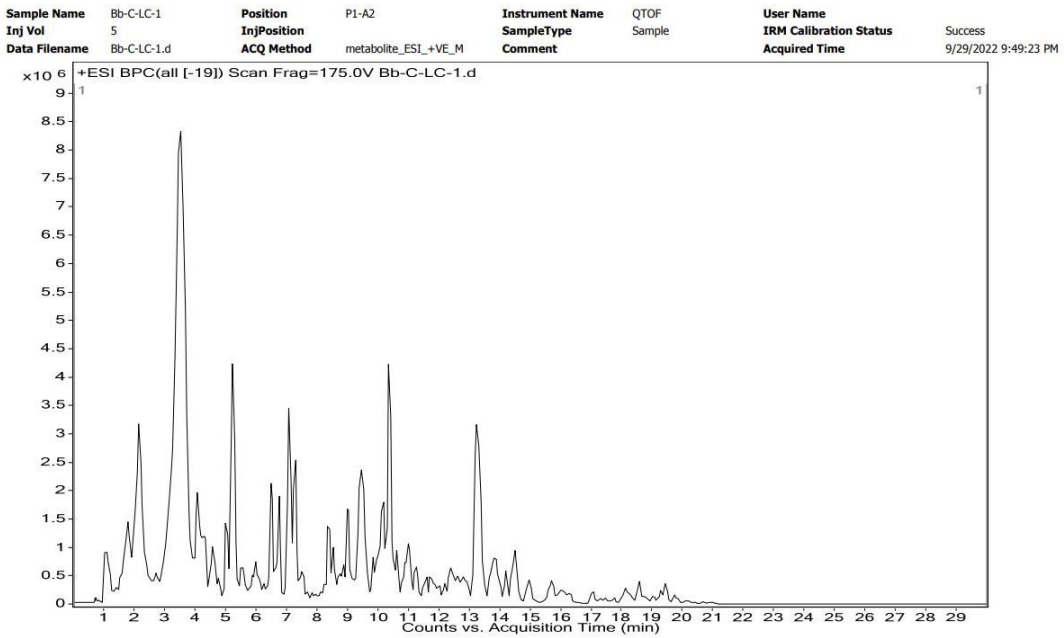


Fig. 4. HR-LCMS spectra of crude toxin of *Beauveria bassiana* extracted from CDB medium

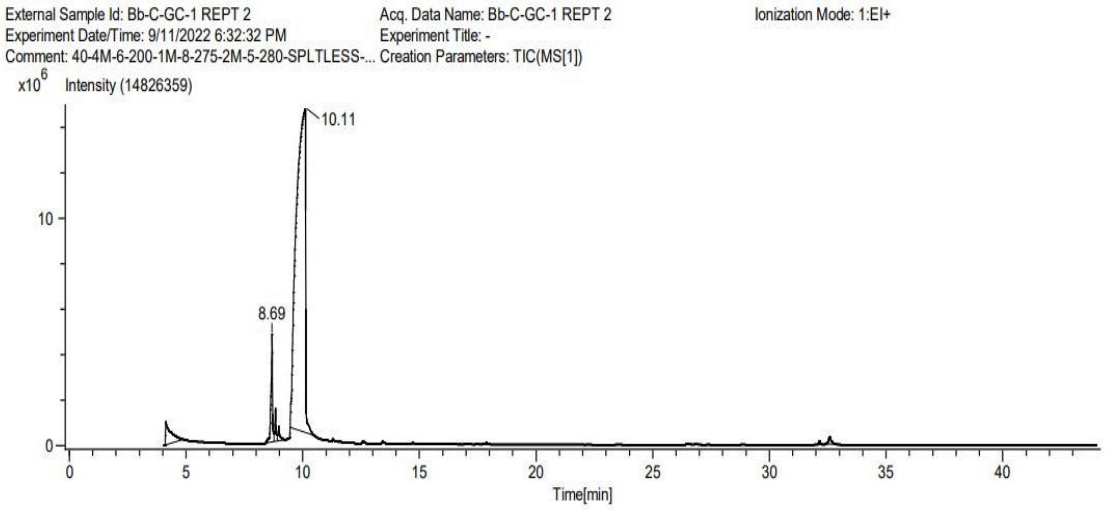


Fig. 5. HR-GCMS spectra of crude toxin of *Beauveria bassiana* extracted from CDB medium

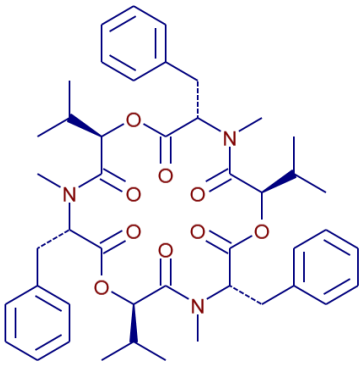
Table 5. Insecticidal metabolites of *Beauveria bassiana* detected by HR-GCMS - CDB

Sl. No.	Name of thecompound	Molecular formula	Molecular weight	Reference
1	α-Hydroxyhippuric acid	$C_9H_9NO_4$	195.05	Merkler *et al.* (2008)
2	4,7-Difluoro-1-indanone	$C_9H_6F_2O$	168.00	Hamilton, 2018
3	Hexadecanoic acid	$C_{16}H_{32}O_2$	256.24	Rahuman *et al.* (2000)
4	Fomepizole	$C_4H_6N_2$	82.05	Ansari *et al.* (2017)

4.1.2.2 Important insecticidal compounds of *Beauveria bassiana* from the culture medium

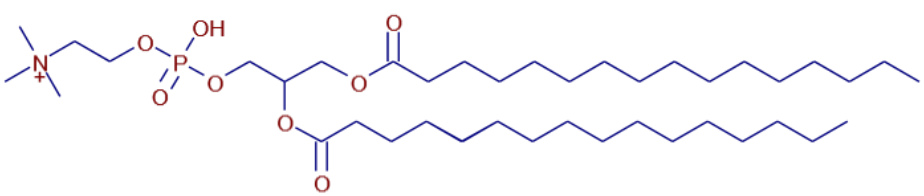
4.1.2.2.1 Beauvericin

It is a despipeptide with molecular formula, $C_{45}H_{57}N_3O_9$ and molecular weight, 800.43.



4.1.2.2.2 1,2-Dipalmitin

It is a diacylglycerol with a molecular formula $C_{35}H_{68}O_5$.

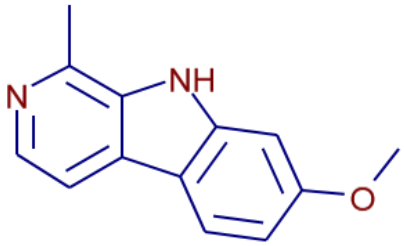


4.1.2.2.3 1-Dodecanol

It is a fatty acid with a molecular formula of $C_{12}H_{26}O$ and molecular weight of 186.

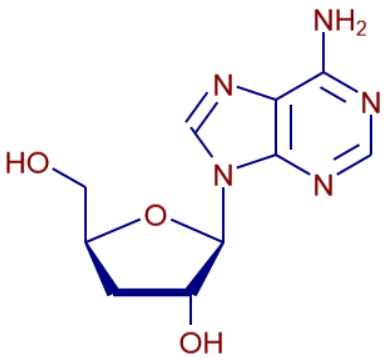
4.1.2.2.4 Harmine

It is a volatile alkaloid with a molecular formula $C_{13}H_{12}N_2O$.



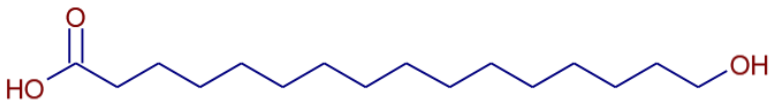
4.1.2.2.5 Cordycepin

It is a nucleoside analogue with a molecular formula $C_{10}H_{13}N_5O_3$ and molecular weight of 251.10.



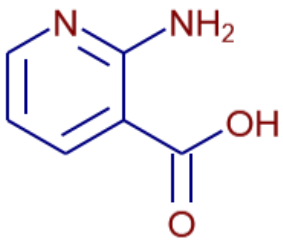
4.1.2.2.6 Hexadecanoic acid

It is a fatty acid with molecular formula of $C_{16}H_{32}O_2$



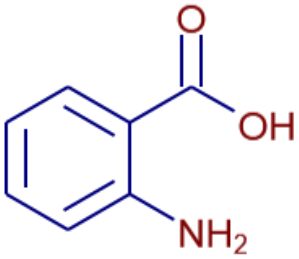
4.1.2.2.7 Nicotinic acid

Alkaloid with a molecular formula of $C_6H_5NO_2$ and a mass of 123.03.



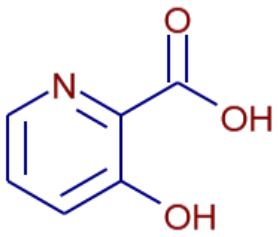
4.1.2.2.8 Anthranilic acid

An organic acid with a molecular formula of C_7H_7NO and a molecular weight of 137.04



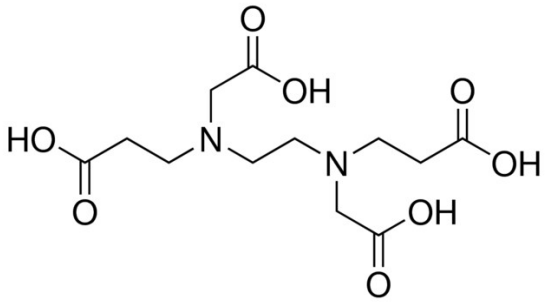
4.1.2.2.9 Picolinic acid

It is an organic acid with a molecular formula of $C_6H_5NO_2$ and a molecularweight of 123.03



4.1.2.2.10 Ethylenediamine-N, N'-dipropionic acid

It is an organic acid with a molecular formula of $C_8H_{16}N_2O_4$ and a molecular weight of 204.22.



4.1.3 Bioactive metabolites of *Beauveria bassiana* (NBAIR-Bb-5a)

HR-LCMS analysis of the crude toxin from PDB and CDB, revealed many secondary metabolites, many of which were common in both media. Biological properties of metabolites detected through HR-LCMS and HR-GCMS analysis were ascertained from general chemical databases *viz.*, PubChem and KEGG databases. Their nematicidal and antimicrobial activities of metabolites of *B. bassiana* are presented below.

4.1.3.1 Antimicrobial metabolites

HR-LCMS analysis of crude toxin from two growth media identified many compounds with antimicrobial properties. Those known to be antimicrobial were coumarin-6-carboxaldehyde, dioctyl phthalate, 6-hydroxy nicotinic acid from PDB media and N-nitroso-2,4,4- trimethyloxazolidine and vanillic acid from CDB. Cyclohexanone, 2-(2-butynyl)-, hydroxydocosahexaenoic acid, erucamide, and harmine were the antibacterial metabolites identified through analysis. The compounds with antimicrobial activity are listed along with molecular formula, molecular weight, and best match (Table 6).

4.1.3.2 Nematicidal metabolites

HR-LCMS analysis of *B. bassiana* in two nutrient media (PDB and CDB) revealed the presence of a few metabolites with nematicidal properties. HR-GCMS analysis of PDB revealed the presence of dihydrothiophenone, nicotinic acid, octadecanoic acid, dihydrothiophenone, 3-hydroxyanthranillic acid, 2,6-pyridine dicarboxylic acid and sphinganine, linoleic acid from CDB, which are known to be nematicidal. The list of nematicidal metabolites with molecular formula, molecular weight and best match, along with references are presented in Table 7.

4.1.3.3 Other bioactivity of metabolites of *Beauveria bassiana*

HR-LCMS analysis of *B. bassiana* in two nutrient media (PDB and CDB) revealed the presence of metabolites with different bioactivities. The compounds such as octadecanoic acid and 9,12-Octadecadienoic acid (Z, Z)- are reported to be involved in fatty acid metabolism, whereas 1,3-propanediol and 2- methyl-2-propyl- are involved in lipid metabolism. Anthranilic acid and dibutyl phthalate are reported to function as insect repellents. The metabolites with different bioactivity are tabulated along with their molecular weight and molecular formula (Table 8).

Table 6. Antimicrobial metabolites of *Beauveria bassiana* (NBAIR-Bb-5a)

Sl. No.	Name of the compound	Molecular formula	Molecular weight (g)	Best match	Reference
1	Beauvericin	$C_{45}H_{57}N_3O_9$	800.43	87.10	Wang and Xu, (2012)
2	Coumarin-6-carboxaldehyde	$C_{10}H_6O_3$	174.15	85.30	Al-Majedy *et al.* (2017)
3	Cyclohexanone, 2-(2-butynyl)-	$C_{10}H_{14}O$	150.22	88.80	Liu *et al*. (2009)
4	Hydroxydocosahexaenoic acid	$C_{22}H_{32}O_3$	344.50	87.30	Mil-Homens *et al.* (2012)
5	N-Nitroso-2,4,4-trimethyloxazolidine	$C_6H_{12}N_2O_2$	144.17	87.10	Kim and Hur (2018)
6	Harmine	$C_{13}H_{12}N_2O$	212.25	88.60	Zeng *et al.* (2010)
7	Vanillic acid	$C_8H_8O_4$	168.04226	86.7	Srivastava *et al.* (2013)
8	Erucamide	$C_{22}H_{43}NO$	337.600	85.9	Tanvir *et al*. (2018)

Table 7. Nematicidal metabolites of *Beauveria bassiana* (NBAIR-Bb-5a)

Sl. No.	Name of the compound	Molecular formula	Molecular weight	Best match	Reference
1	Nicotinic acid	$C_6H_5NO_2$	123.03	89.60	Aissani (2013)
2	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	88.80	Zhang *et al.* (2012)
3	Dihydrothiophenone	C_5H_6OS	102.15	83.90	Hudson and Towers (1991)
4	2,6-pyridine dicarboxylicacid	$C_7H_5NO_4$	167.12	90.80	Al-Majedy *et al.* (2017)
5	3-hydroxyanthranillic acid	$C_{16}H_{31}NO_3Si_3$	369.68	92.80	Zhang *et al.* (2022)

Table 8. Bioactivity of metabolites of *Beauveria bassiana* (NBAIR-Bb-5a)

Sl. No.	Name of the compound	Molecular formula	Molecular weight	Biological function	Reference
1	Anthranilic acid	C_7H_7NO	137.14	Insect repellant	Zhao *et al.* (2019)
2	Phenobarbital	$C_{12}H_{12}N_2O_3$	232.08	Xenobiotic metabolism (Cytochrome p-450, GST enzyme inducer)	Willoughby *et al.* (2006)
3	Pentobarbital	$C_{11}H_{18}N_2O_3$	226.13	GABA-A receptor Anticonvulsant activity	Mehta *et al.* (1990)
4	Octadecanoic acid	$C_{18}H_{36}O_2$	284.48	Fatty acid metabolism, nematicidal	Zhang *et al.* (2012)
5	9,12-Octadecadienoic acid (Z, Z)-	$C_{18}H_{32}O_2$	280.40	Fatty acid metabolism	Zhang *et al.* (2012)
6	1,3-Propanediol, 2-methyl-2-propyl-	$C_7H_{16}O_2$	132.20	Lipid metabolism	Liu *et al.* (2015)
6	Nicotinamide	$C_6H_6N_2O$	122.04	Inhibitor of vascular endothelial growth factor receptor-2 tyrosine kinase	Lin *et al.* (2010)
7	Undecane	$C_{11}H_{24}$	184.37	Mild sex attractant of moths	Hölldobler and Wilson (1990)
8	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.15	Insect repellent	Wu *et al.* (2022)
9	Ochrolifuanine A	$C_{29}H_{34}N_4$	438.27	Antiplasmodial activity	Mahindra *et al.* (2015)

4.1.4 Extraction of secondary metabolites from host insect

4.1.4.1 From healthy *Spodoptera litura*

Third-instar larvae of *S. litura* sprayed with sterile distilled water did not exhibit any mortality till the end of 168 h of treatment. The ethyl acetate fraction (900 mL) of healthy *S. litura* larval extract yielded 0.20 g of crude extract under vacuum evaporation (Plate 10A) and the spectra of HR-LCMS and HR-GCMS are depicted in Fig. 6 and 7. The metabolites identified are listed with molecular weight, molecular formula and best match in Appendix IV.1 and IV.2 .

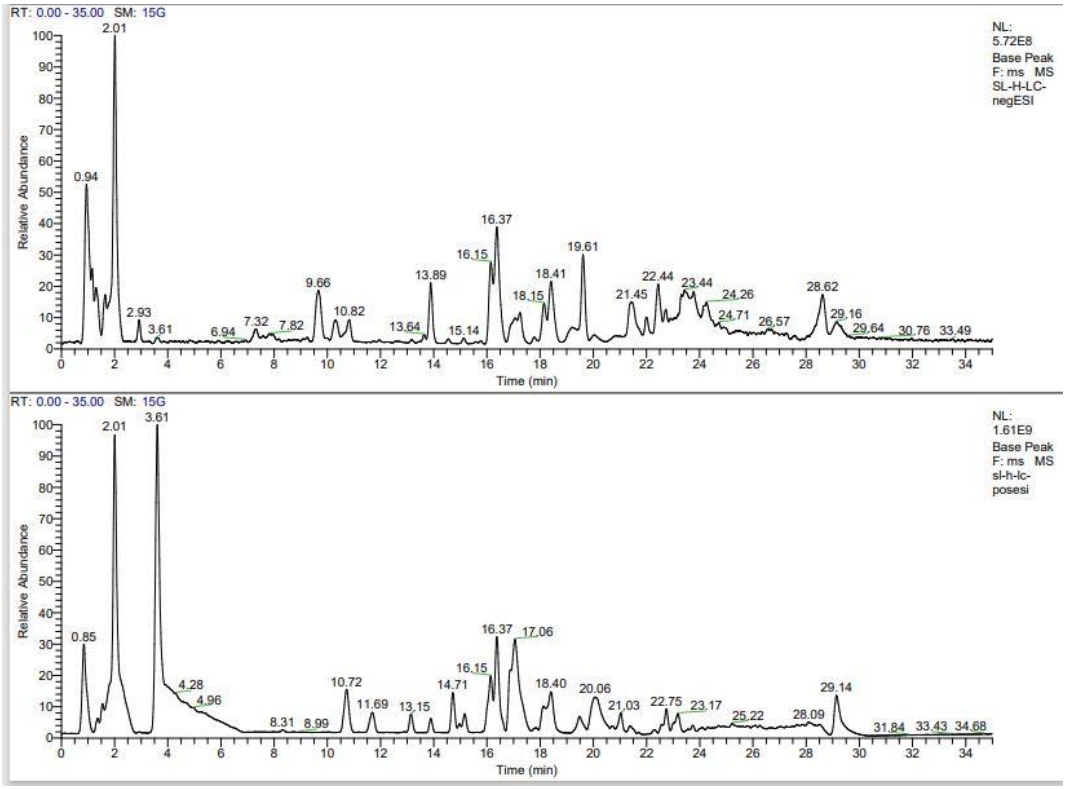


Fig. 6. HR-LCMS spectra of crude extract from healthy *Spodoptera litura* larvae

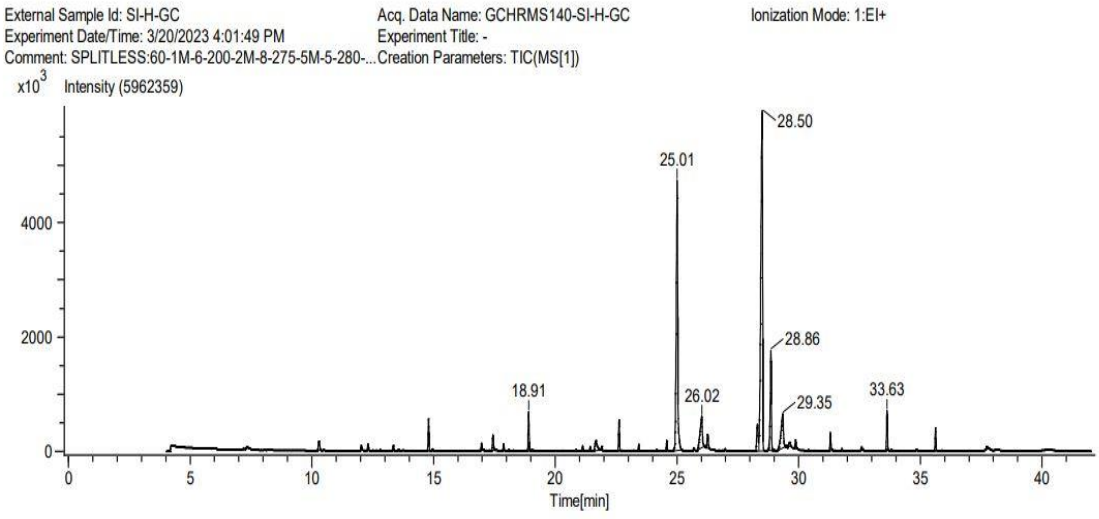


Fig. 7. HR-GCMS spectra of crude extract from healthy *Spodoptera litura* larvae

4.1.4.2 From infected *Spodoptera litura*

Third-instar larvae of *S. litura* treated with spore suspension of Bb-5a @ 1×10^8 spores mL^{-1}, exhibited behavioral changes in movement and feeding. At 24 h after treatment, normal feeding behaviour was observed in all the treated larvae. However, after 48 h of treatment, five larvae out of 20 stopped feeding on castor leaves and became inactive. At 72 h after treatment, larvae were unable to move in a coordinated way when prodded with a fine-haired brush. A mortality of 30 per cent was observed in the larvae after 72 h of treatment. A gradual increase in the mortality was observed from 96 h after treatment, and the mortality recorded was 45, 60, 85, and 100 per cent at 96, 120,144, and 168 h after treatment, respectively.

Fungal growth was observed on cadavers obtained from the Bb-5a-treated larvae kept in BOD incubator. The growth of white mycelium was evident after the fourth day of incubation and the cadavers were completely covered with white cottony mass after the seventh day of incubation (Plate 11A and B). The *B. bassiana* infection was confirmed by microscopic observation of the mycosed larvae. The microscopic examination revealed the characteristics of *B. bassiana i.e.,* the presence of septate hyphae with round or oval shaped conidia on flask-shaped phialides.

Larval extract/supernatant of the *B. bassiana* infected *S. litura* was extracted with ethyl acetate. The ethyl acetate fraction (900 mL) yielded 0.18 g of crude extract under vacuum evaporation and recovered using methanol for analysis (Plate 10B).

4.1.4.2.1 Metabolites detected through High Resolution-Liquid Chromatography – Tandem Mass Spectrometry (HR-LCMS)

The crude toxin isolated from infected *S. litura* was subjected to HR-LCMS, (Fig. 8) showed the presence of 43 secondary metabolites, the biological activities of which were identified using the PubChem and KEGG databases (Table 9). The secondary metabolites exclusively present in the infected *S. litura* with the molecular formula, molecular weight, and best match is presented in Annexure V.1

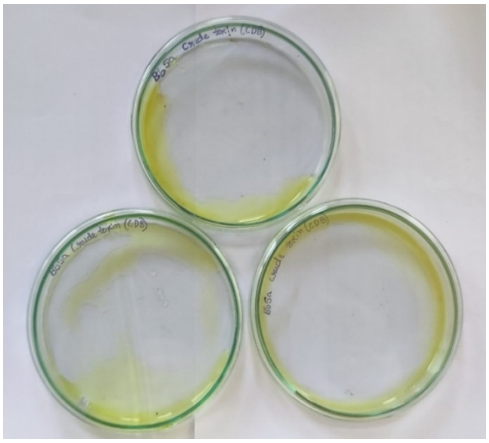
Table 9. Bioactivity of metabolites of *Beauveria bassiana* extracted from infected *Spodoptera litura* detected by HR-LCMS

Sl. No.	Name of the compound	Molecular formula	Molecular weight (g)	Biological function	Reference
1	Monobutyl phthalate	$C_{12}H_{14}O_4$	222.24	Insecticidal	Vivekanandhan *et al.* (2022)
2	12-Oxo phytodienoic acid	$C_{18}H_{28}O_3$	292.40	Source of pyrethrolone moiety in pyrenthrin II	Matsui *et al.* (2020)
3	16-Hydroxyhexadecanoic acid	$C_{16}H_{32}O_3$	272.42	Anticancerous and cytotoxic activity	Mustafa *et al.* (2004)
4	2,5-Dihydroxybenzaldehyde	$C_7H_6O_3$	138.12	Antibacterial	Friedman *et al.* (2003)
5	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	294.40	Peroxisome proliferator-activated receptor α (PPARα) lipid metabolism	Cho *et al.* (2011)
6	(+)-Aphidicolin	$C_{20}H_{34}O_4$	338.50	Antiviral and antitumor activity	-
7	Mesaconic acid	$C_5H_6O_4$	130.10	Antifungal activity	Castaldi *et al.* (2021)
8	Hydroxyquebrachamine	$C_{19}H_{26}N_2O$	298.43	Insecticidal	Kadhim *et al.* (2016)

4.1.4.2.2 Metabolites detected through High Resolution-Gas Chromatography – Tandem Mass Spectrometry (HR-GCMS)

The crude extract from infected *S. litura* were analysed through HR-GCMS to detect volatile metabolites and chromatogram is depicted in Fig. 9. The 33 metabolites detected are listed in Annexure V.2 along with their molecular weight, molecular formula, and retention time. The biological activities of selected metabolites were identified through PubChem and KEGG databases (Table 10).

A. Crude toxin from *Beauveria bassiana* in PDB



B. Crude toxin from *Beauveria bassiana* in CDB

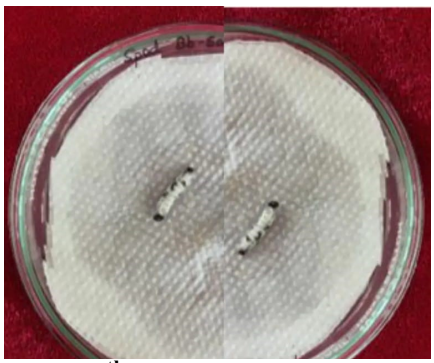
Plate 9. Crude toxin of *Beauveria bassiana* extracted from culture media

A. Healthy larvae

B. Infected larvae

Plate 10. Larval extract of *Spodoptera litura*

A. 4th day after incubation



B. 7th day after incubation

Plate 11. Mycosis in *Spodoptera litura* infected with *Beauveria bassiana*

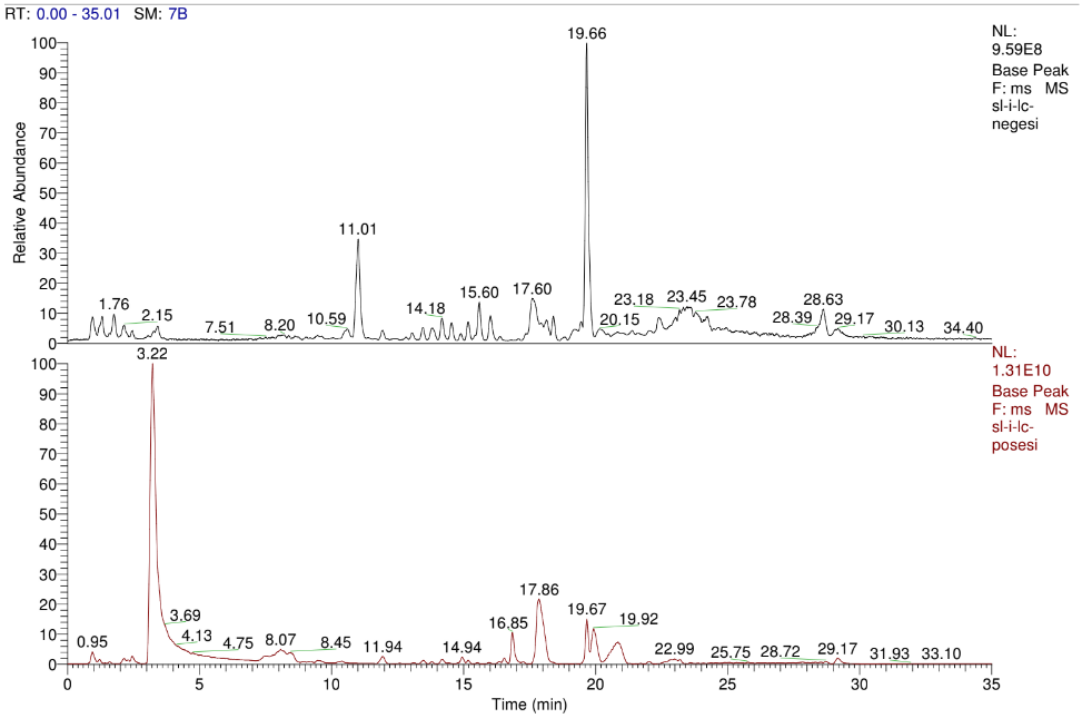


Fig. 8. HR-LCMS spectra of crude extract from *Beauveria* infected *Spodoptera litura* larvae

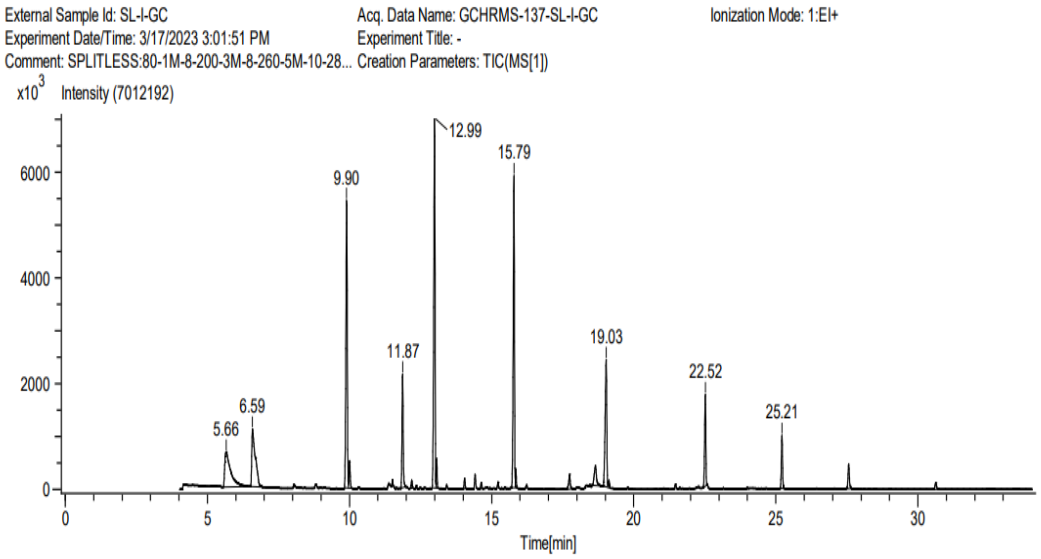


Fig. 9. HR-GCMS spectra of crude extract from infected *Spodoptera litura* larvae

Table 10. Bioactivity of metabolites of *Beauveria bassiana* extracted from infected *Spodoptera litura* detected by HR-GCMS

Sl. No.	Name of the compound	Molecular formula	Molecular weight (g)	Biological function	Reference
1	1-Undecene	$C_{11}H_{22}$	154.00	Antifungal	Tagele *et al.* (2019)
2	Cyclopropane, octyl-	$C_{11}H_{22}$	154.00	Antibacterial	Ehsani *et al.* (2019)
3	9-Octadecene, (E)-	$C_{18}H_{36}$	252.00	Antimicrobial and antioxidant activity	Photolo *et al.* (2020)
4	7-Hexadecene, (Z)-	$C_{16}H_{32}$	224.00	Antimicrobial, antioxidant, and cytotoxic activit y	Syame *et al.* (2022)
5	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284.00	Antimicrobial and Anti-Inflammatory Activity	Kaur *et al.* (2022)
6	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390.56	Insecticidal	Wakil *et al.* (2018)
7	Heptaoxacycloheni cosan-2-one	$C_{14}H_{26}O_8$	322.35	Pheromonal action	Bhutia *et al.* (2010)
8	Cyclopenta(phenan threne-17-yl) propanoic acid	$C_{27}H_{42}O_4$	430.62	Tyrosine phosphatase inhibitor	Urbanek *et al.* (2001)

4.1.5 Comparison of metabolites of *Beauveria bassiana* crude toxin from growth media and host insects

The metabolites identified from the two-growth media *i.e.*, PDB and CDB were compared with the metabolites identified from host insects for their insecticidal properties. The metabolites like beauvericin, á-D-glucopyranose, 1,6-anhydro-, cyclohexylmethyl undecyl ester, piperidone, cordycepin, hexadecanoic acid, and harmine were found in crude toxin extracted from two growth media and host insects. Some metabolites like monobutyl phthalate, picolinic acid, 2-palmitoylglycerol and ethylenediamine-N, N'-dipropionic acid were common in crude toxin extracted from PDB growth medium and host insects. Metabolites such as hydroxyquebrachamine

and dihydrothiophenone were present in the crude toxin extracted from CDB growth medium and host insects. Most of the metabolites extracted from PDB, CDB, and infected host insects were identical and exhibited insecticidal properties. The list of metabolites having insecticidal properties are compared and presented in Table 11.

4.1.6 Comparison of metabolites – From infected and healthy larvae

The metabolites produced exclusively by the infected host insects were analyzed through HR-LCMS. The bioactivity of those metabolites was identified using PubChem and KEGG databases. The metabolites detected by HR-LCMS, which are biologically active, are depicted below with molecular weight and molecular formula along with reference (Table 12). The hydroxyquebrachamine and monobutyl phthalate were identified to be insecticidal. 12-oxo phytodienoic acid and 9-oxo-10(E),12(E)-octadecadienoic acid were identified to be a source of pyrethrolone moiety in pyrenthrin II and involved in the peroxisome proliferator-activated receptor-α (PPARα) lipid metabolism, respectively. Other metabolites like 2,5-dihydroxybenzaldehyde, mesaconic acid, and (+)-aphidicolin are involved in antibacterial, antifungal, and antiviral activities, respectively.

The bioactive metabolites detected by HR-GCMS with molecular weight, molecular formula, and their specific biological functions are listed below in Table 13. Diisooctyl phthalate is known to be insecticidal whereas other metabolites like 9-octadecene, (E)-, 7-hexadecene, (Z)- and heptadecanoic acid, methyl ester possess antimicrobial activities. Cyclopropane, octyl- is detected to have antibacterial activity and cyclopenta(phenanthrene-17-yl) propanoic acid is involved in the inhibition of tyrosine phosphatase.

Table 11. Insecticidal metabolite profile of *Beauveria bassiana* crude toxin from PDB, CDB, and infected *Spodoptera litura* larvae

SL. No	Name of the compound	PDB	CDB	Infected *Spodoptera litura* larvae
1	Beauvericin	+	+	+
2	Monobutyl phthalate	+	-	+
3	Picolinic acid	+	-	+
4	Hydroxyquebrachamine	-	+	+
5	5-Oxotetrahydrofuran-2-carboxylic acid	+	+	-
6	á-D-Glucopyranose, 1,6-anhydro-	+	+	+
7	1,2-Dipalmitin	+	+	-
8	2-Palmitoylglycerol	+	-	+
9	Dihydrothiophenone	-	+	+
10	Cyclohexylmethyl undecylester	+	+	-
11	Ethylenediamine-N, N'-dipropionic acid	+	-	-
12	Piperidone	+	+	+
13	Cordycepin	+	+	+
14	Harmine	+	-	+
15	Hexadecanoic acid	+	+	+
16	2,6-pyridine dicarboxylic acid	+	+	-

‘+’ - present

‘-’ – absent

Table 12. HR-LCMS based metabolite profiles of healthy and *Beauveria bassiana*-infected larvae of *Spodoptera litura*

Sl. No.	Metabolites	Healthy larvae	Infected larvae	Molecular formula	Molecular weight (g)	Biological functions
1	Monobutyl phthalate	-	+	$C_{12}H_{14}O_4$	222.08	Insecticidal
2	2,5-Dihydroxybenzaldehyde	-	+	$C_7H_6O_3$	138.12	Antibacterial
3	12-Oxo phytodienoic acid	-	+	$C_{18}H_{28}O_3$	292.40	Source of pyrethrolone moiety in pyrenthrin II
4	16-Hydroxyhexadecanoic acid	-	+	$C_{16}H_{32}O_3$	272.42	Anticancerous and cytotoxic activity
5	Citraconic acid	-	+	$C_5H_6O_4$	130.02	BCCA (branched chain ami no acid) metabolism
6	9-Oxo-10(E),12(E)-octadecadienoic acid	-	+	$C_{18}H_{30}O_3$	294.40	Peroxisome proliferator-activated receptor α (PPARα) lipid metabolism
7	(+)-Aphidicolin	-	+	$C_{20}H_{34}O_4$	338.50	Antiviral and antitumor activity
8	Mesaconic acid	-	+	$C_5H_6O_4$	130.10	Antifungal activity
9	Hydroxyquebrachamine	-	+	$C_{19}H_{26}N_2O$	298.43	Insecticidal

'+' - present

'-' – absent

Table 13. HR-GCMS based metabolite profiles of healthy and *Beauveria bassiana* -infected larvae of *Spodoptera litura*

Sl. No.	Metabolites	Healthy larvae	Infected larvae	Molecular formula	Molecular weight (g)	Biological functions
1	1-Undecene	-	+	$C_{11}H_{22}$	154.29	Antifungal
2	Cyclopropane, octyl- (ring chain isomers)	-	+	$C_{11}H_{22}$	154.29	Antibacterial
3	Chromone	+	-	$C_{14}H_{16}O_6$	280.27	Insect sensory cues
4	Olean-12-ene-hexol	+	-	$C_{30}H_{50}O_6$	506.71	Lipid metabolism
5	9-Octadecene, (E)-	-	+	$C_{18}H_{36}$	252.00	Antimicrobial and antioxidant activity
6	7-Hexadecene, (Z)-	-	+	$C_{16}H_{32}$	224.00	Antimicrobial, antioxidant, and cytotoxic activity
7	Heptadecanoic acid, methyl ester	-	+	$C_{18}H_{36}O_2$	284.00	Antimicrobial and Anti-Inflammatory Activity
8	Thiophene	+	-	$C_8H_{12}S$	140.24	Faecal matter component
9	Diisooctyl phthalate	-	+	$C_{24}H_{38}O_4$	390.56	Insecticidal
10	Heptaoxacyclohenicosan-2-one	-	+	$C_{14}H_{26}O_8$	322.35	Pheromonal action
11	Decane	+	-	$C_{10}H_{22}$	142.28	Fatty acid biosynthesis
12	1-Docosene	+	-	$C_{22}H_{44}$	308.59	Insect derived plant regulator
13	Cyclopenta(phenanthrene-17-yl) propanoic acid	-	+	$C_{27}H_{42}O_4$	430.62	Tyrosine phosphatase inhibitor

'+' - Present

'-' – Absent

4.2 Evaluation of bioefficacy of crude toxin of *Beauveria bassiana*

Crude toxin of *B. bassiana* extracted from PDB growth media was subjected to bioassay studies in the third-instar larvae of tobacco caterpillar, *S. litura*.

4.2.1 Preliminary bioassay

The preliminary bioassay was conducted with different concentrations in a broad range *viz.,* 5000 ppm, 1000 ppm, 500 ppm, 100 ppm, 50 ppm, 10 ppm, and control with one replication each. Observations recorded after 48 h, revealed 100 per cent mortality with higher concentration of 5000 and 1000 ppm, while those with 500 and 100 ppm ranked second (80 each), followed by 50 ppm (60%). A lower mortality rate was observed with 10 ppm (40 %). No mortality was recorded in the control group. Based on the mortality observed in the preliminary bioassay, the different doses for bioassay were fixed. Thus, the test concentrations of 100, 90, 70, 50, 10 and 9 ppm were selected for carrying out the bioassay experiment.

4.2.2 Bioefficacy of crude toxin

The *B. bassiana* crude toxin extracted from PDB was evaluated for its bioefficacy against third-instar larvae of *S. litura* through a topical bioassay using Potter's Tower. Test concentrations of nine different concentrations *viz.,* 100 ppm, 90 ppm, 70 ppm, 50 ppm, 30 ppm, 10 ppm, 9 ppm, absolute control and negative control were evaluated for the bioefficacy studies. Mortality of third-instar larvae (%) was calculated and are depicted in Table 14.

After 12 h of treatment, 100 ppm crude toxin recorded the highest mortality of 20.83 per cent while the mortality with 90 and 70 ppm was negligible (4.16% each) and were on par with each other. However, no mortality was recorded at the lowest concentration of 70, 50, 30, 10, and 9 ppm. The larvae treated with a higher concentration of 100 ppm were inactive, unable to move in a coordinated way when prodded with a fine-haired brush, and they had stopped feeding on castor leaves. Whereas normal feeding and activity were observed in all other test concentrations, including absolute and negative control.

After 24 h of treatment, crude toxin at 100 ppm resulted in 50 per cent mortality of third-instar larvae, while it was significantly lower with 90 ppm (25%), followed by 16.67, 12.5 and 12.5 per cent mortality with 70, 50 and 30 ppm, respectively. After 24 h of treatment, 10 and 9 ppm continued to show zero mortality.

After 36 h of treatment, crude toxin at 100 ppm recorded the highest mortality of 62.5 per cent and was on par with 54.16 per cent mortality with 90 ppm concentration followed by 70 ppm (37.50%). The mortality rate noted with 50, 30 and 10 ppm were 25, 20.83, and 12.50 per cent, respectively, which were statistically on par. The lowest mortality was recorded with 9 ppm (8.83%).

A similar trend was observed at 48h of treatment. At all concentrations, mortality differed significantly among each other. The highest mortality of 91.67 and 83.30 per cent was recorded with 100 and 90 ppm, respectively. The mortality rate recorded with 70, 50, 30, and 10 ppm were 62.5, 45.83, 37.50, and 20.83 per cent respectively, which were statistically lower than the former but higher than the mortality recorded with 9 ppm (12.5%).

After 60 h of treatment, the highest per cent mortality was observed with 100 ppm crude toxin (100%), which was statistically on par with mortality at 90 ppm (91.67%). The mortality recorded at different lower concentrations differed significantly from each other. It was 79.16, 62.50, 54.16 and 37.50 per cent with 70, 50, 30, and 10 ppm concentrations, respectively. The mortality recorded with 9 ppm was the lowest at 20.83 per cent.

After 72 h of treatment, mortality observed with 70 ppm (87.5%) was significantly on par with 50 ppm (75%), followed by 70.83 per cent with 30 ppm, 58.33 per cent with 10 ppm and 41.67 per cent with 9 ppm.

After 84 h of treatment, there was 100 per cent mortality with crude toxin at higher concentrations of 100, 90 and 70 ppm, which were statistically on par with the mortality recorded at 50 ppm (91.67%). When the concentrations were lowered to 30 and 10 ppm, the mortality recorded was on par with each other (79.16 and 70.83%), whereas significantly lower mortality was observed with 9 ppm (58.63%).

Even at the end of the experimental period (96 h after treatment), highest mortality was recorded with 100, 90, 70, and 50 ppm (100% each), while it was significantly higher than the mortality recorded with 30, 10 and 9 ppm (95.83, 87.50 and 75%, respectively)

Table 14. Bioefficacy of crude toxin of *Beauveria bassiana* on third-instar larvae of *Spodoptera litura*

Concentration (ppm)	*Mean cumulative mortality of larvae at 12 h interval (%)							
	12	24	36	48	60	72	84	96
9	0.00^{b}	0.00^{c}	8.83^{c}	12.50^{e}	20.83^{e}	41.67^{c}	58.33^{b}	75.00^{b}
10	0.00^{b}	0.00^{c}	12.50^{bc}	20.83^{de}	37.50^{de}	58.33^{bc}	70.83^{ab}	87.50^{ab}
30	0.00^{b}	12.50^{bc}	20.83^{bc}	37.50^{cde}	54.16^{cd}	70.83^{abc}	79.16^{ab}	95.83^{ab}
50	0.00^{b}	12.50^{bc}	25.00^{bc}	45.83^{cd}	62.50^{bcd}	75.00^{ab}	91.67^{a}	100.00^{a}
70	4.16^{b}	16.67^{bc}	37.50^{ab}	62.50^{bc}	79.16^{abc}	87.50^{ab}	100.00^{a}	100.00^{a}
90	4.16^{b}	25.00^{b}	54.16^{a}	83.30^{ab}	91.67^{ab}	100.00^{a}	100.00^{a}	100.00^{a}
100	20.83^{a}	50.00^{a}	62.50^{a}	91.67^{a}	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}
SEM	2.728	5.546	6.339	6.18	7.487	7.487	5.85	4.98
CD (0.05)	3.858	7.843	8.964	8.74	10.588	10.588	8.274	7.043

*Each value is mean of six replications

The p-value in ANOVA table is < 0.05.

Values sharing same alphabets in superscript are statistically on par based on LSD.

4.2.2.1 Dose mortality (LC_{50}) of crude toxin

As the control treatment *viz.*, absolute, and negative control did not record any mortality in bioassay, the mortality data was directly subjected to probit analysis by using the software PoloPlus (LeOra Software, 2002) for the calculation of median lethal concentration (LC_{50}) and are depicted in Table 15.

The dose mortality (LC_{50}) is the parameter used to indicate the compound's acute toxicity and quantify the results of different treatments. Crude toxin at 109.76 ppm effectively killed 50 per cent of test insects within 24 h after treatment. With the

advancement of exposure time, the concentration of the crude toxin required to kill the 50 per cent of the test insects decreased.

The crude toxin at 87.51, 37.90, and 20.41 ppm concentrations were required to kill half of the test insects, third-instar larvae of *S. litura*, with an exposure time of 36, 48 and 60h, respectively.

Table 15. Dose mortality response of crude toxin of *Beauveria bassiana* on *Spodoptera litura*

SL. No.	Exposure time (h)	LC_{50} (ppm)	Fiducial limit (95% CI)		χ^2	df	Slope (± SE)
			Lower	Upper			
1.	24	**109.76**	90.87	227.70	1.261	2	3.85 (±1.327)
2.	36	**87.51**	64.29	152.95	2.415	4	1.70 (±0.405)
3.	48	**37.90**	24.47	57.03	7.026	5	2.01 (±0.295)
4.	60	**20.41**	9.76	30.01	2.920	3	1.48 (±0.367)

SE - standard error, χ^2 - Chi-square, df – degree of freedom

4.2.2.2 Time mortality (LT_{50}) of crude toxin

The mortality data was directly subjected to probit analysis using the software PoloPlus (LeOra Software, 2002) to calculate median lethal time (LT_{50}). The LT_{50} values calculated for third-instar larvae of *S. litura* treated with the crude toxin of *B. bassiana* are represented in Table 16.

Median Lethal Time (LT_{50}) is the time required to kill 50 per cent of test insects after exposure to different crude toxin concentrations. As the concentration of the crude toxin decreased, the time required to kill 50 per cent of test insects increased. The crude toxin at 100 ppm concentration required a time of 22.77 h to kill half of the test insects. Similarly, 90, 70, 50, 30, and 10 ppm concentrations of crude toxin required a time of 32.40, 38.66, 43.20, 45.20, and 50.60 h, respectively to bring out 50 per cent mortality.

Table 16. Time mortality response of crude toxin of *Beauveria bassiana* on *Spodoptera litura*

SL. No.	Concentration (ppm)	LT_{50}	Fiducial limit		χ^2	df	Slope (± SEM)
			Lower	Upper			
1.	100	**22.77**	10.60	33.19	5.181	3	3.65 (±0.602)
2.	90	**32.40**	28.57	35.90	2.762	4	6.74 (±1.036)
3.	70	**38.66**	34.30	42.91	3.385	4	5.95 (±0.898)
4.	50	**43.20**	33.70	53.58	7.312	4	6.26 (±0.952)
5.	30	**45.20**	32.65	61.17	10.378	4	6.15 (±0.947)
6.	10	**50.60**	46.10	59.64	5.615	4	11.50(±1.864)

SE - standard error, χ^2 - Chi-square, df – degree of freedom

4.3 *In silico* molecular docking studies

Bioefficacy studies of *Beauveria* crude toxin on third-instar larvae of *S. litura* revealed 100 per cent mortality of test insects within 60 h after treatment @ 100 ppm concentration. This can be attributed to the presence of insecticidal metabolites in the crude toxin acting as nerve poison. *In silico* molecular docking studies were conducted with 20 selected metabolites as ligands and AChE of *Drosophila melanogaster* as target protein using CB-Dock software to confirm the AChE inhibitory activity.

Docking of 20 metabolites of *B. bassiana* (NBAIR-Bb-5a) revealed that 19 of them interacted with AChE at specific sites (Table 16). The interacting sites were the esteratic sites of the catalytic ennead, *viz.*, tyrosine, aspartic acid, threonine, leucine, tryptophan, histidine, serine, glycine, and asparagine. The binding sites of AChE (Dm AChE, PDB code IQON) were Tyr (71, 73, 324 and 370), Asp 375, Thr 154, Leu 328, Trp 472, His 480, Ser 238, Gly 151 and Asn 136. Metabolites with their docking scores such as binding energy, hydrogen bond length and interacting residues are presented in Table 17.

Among the 19 metabolites, N-[2-(2-benzoxazolylthio)-ethyl]benzenesulfonamide showed the highest interaction with acetylcholinesterase with a binding energy of -8.4 kcal mol^{-1}, followed by hydroxydocosahexaenoic acid and coumarin-6-carboxaldehyde (-7.5 kcal mol^{-1}), 9,12-octadecadienoic acid (Z,Z) and cyclohexylmethyl undecyl ester (-7.4 kcal mol^{-1}), phenobarbital and octadecanoic acid (-7.1 kcal mol^{-1}), 2-palmitoylglycerol (-6.9 kcal mol^{-1}), pentobarbital (-6.2 kcal mol^{-1}), N-nitroso-2,4,4- trimethyloxazolidine (-6.1 kcal mol^{-1}), beauvericin (-5.9 kcal mol^{-1}), ethylenediamine-N, N'-dipropionic acid(-5.8 kcal mol^{-1}), nicotinamide(-5.7 kcal mol^{-1}), 5-xotetrahydrofuran-2-carboxylic acid (-5.6 kcal mol^{-1}), á-D-glucopyranose, 1,6-anhydro-(-5.5 kcal mol^{-1}), 1,3-propanediol, 2-methyl-2-propyl- (-5.3 kcal mol^{-1}) and cyclohexanone, 2-(2-butynyl)-(-5.0 kcal mol^{-1}). The lowest binding energy was exhibited by dihydrothiophenone (-3.9 kcal mol^{-1}).

Metabolites exhibiting binding energy between -5 to -10 kcal mol^{-1} are considered to have the best interaction with the active sites of AChE, indicating the acetylcholinesterase inhibitory activity of metabolites of the *B. bassiana* (NBAIR-Bb-5a).

Metabolites with the best binding affinities were visualised and analysed using PyMol software. The 3D interactions of a ligand with a higher binding energy with the binding sites of AChE are shown below (Fig 10, 11 and 12).

Table 17. Docking scores of metabolites of *Beauveria bassiana* with protein target acetylcholinesterase

Sl. No.	Ligand	Binding energy (Kcal mol^{-1})	No. of Hydrogen bond	Hydrogen bond length	Interacting residue
1	Beauvericin	-5.9	3	3.2 3.4 2.7	ASN136 ASN136 ASN136
2	Phenobarbital	-7.1	2	3.2 2.0	THR154 THR154
3	Pentobarbital	-6.2	3	2.7 3.1 3.1	TYR71 TYR73 ASP375
4	5-Oxotetrahydrofuran-2-carboxylic acid	-5.6	3	3.1 3.2 2.8	ASP375 LEU328 TYR324
5	á-D-Glucopyranose, 1,6-anhydro-	-5.5	5	3.1 3.3 2.9 3.0 3.0	TYR370 HIS480 HIS480 SER238 THR154
6	Cyclohexanone, 2-(2-butynyl)-	-5.0	1	3.0	TRP472
7	2-Palmitoylglycerol	-6.9	4	3.1 3.0 2.9 2.9	TYR71 SER238 TYR370 HIS480
8	Octadecanoic acid	-7.1	2	3.1 3.2	HIS480 SER238
9	Dihydrothiophenone	-3.9	1	3.1	TYR71
10	Coumarin-6-carboxaldehyde	-7.5	1	3.1	TYR71
11	Imidazole-2-methanol, 1-methyl-	-4.7	2	2.9 3.0	TYR324 TYR324
12	9,12-Octadecadienoic acid (Z, Z)-	-7.4	3	3.3 3.3 2.8	HIS480 GLY151 SER238
13	1,3-Propanediol, 2-methyl-2-propyl-	-5.3	4	2.7 2.7 3.2 2.5	TYR324 TYR324 LEU328 ASP375
14	Hydroxydocosahexaenoic acid	-7.5	2	3.1 3.2	TYR370 THR154
15	Cyclohexylmethyl undecyl ester	-7.4	2	3.0 3.3	TYR73 TYR73

16	Nicotinamide	-5.7	2	3.0 2.9	TYR71 TYR324
17	N-Nitroso-2,4,4-trimethyloxazolidine	-6.1	3	3.0 3.1 3.2	TYR71 TYR71 TYR324
18	Ethylenediamine-N, N'-dipropionic acid	-5.8	5	2.1 2.4 3.1 3.2 3.1	ASP375 TYR324 TYR324 TYR73 TYR71
19	N-[2-(2-Benzoxazolylthio) ethyl] benzenesulfonamide	-8.5	3	3.1 3.3 3.0	TYR73 TYR73 TYR324

4.4 AChE assay

In silico molecular docking studies revealed the AChE inhibitory activity of metabolites of *B. bassiana*. The inhibitory activity of bioactive metabolites on acetylcholinesterase enzyme was determined in the infected and healthy *S. litura* under *in vitro* condition to validate the results of molecular docking studies.

4.4.1 Estimation of total protein

Total protein content in the healthy as well as in the *Beauveria* infected *S. litura* was determined by Lowry's method (Table 18) and are expressed in mg mL^{-1}.

Total protein content of infected and healthy larvae was significantly different as per t-test at 1 and 5 per cent level of significance. Among the two, the *Beauveria* infected larvae recorded higher protein content of 0.0795 mg mL^{-1}. Whereas, healthy larvae recorded low protein content of 0.0559 mg mL^{-1}.

Table 18. Total protein (mg mL^{-1}) in healthy and *Beauveria*-infected larvae

Sl. No.	Samples	Protein content (mg mL^{-1})
1	Healthy larvae	0.0559**
2	*Beauveria* infected larvae	0.0795**

**Samples are significantly different at both 1% and 5% level of significance

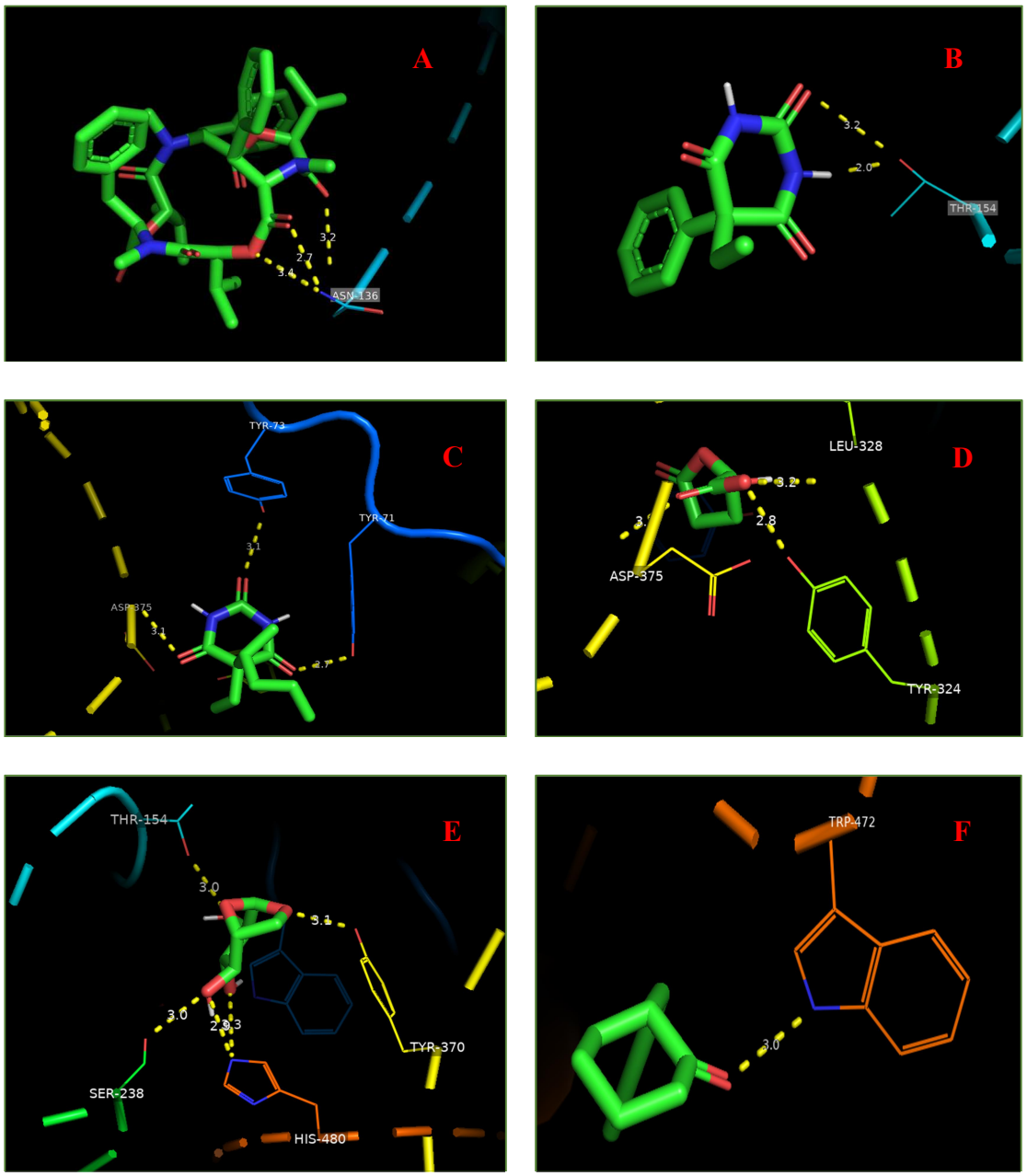


Fig 10. Three-dimensional representation of AChE-ligand complex

A. AChE with Beauvericin
B. AChE with Phenobarbital
C. AChE with Pentobarbital
D. AChE with 5-Oxotetrahydrofuran-2-carboxylic acid
E. AChE with á-D-Glucopyranose, 1,6-anhydro-
F. AChE with Cyclohexanone, 2-(2-butynyl)-

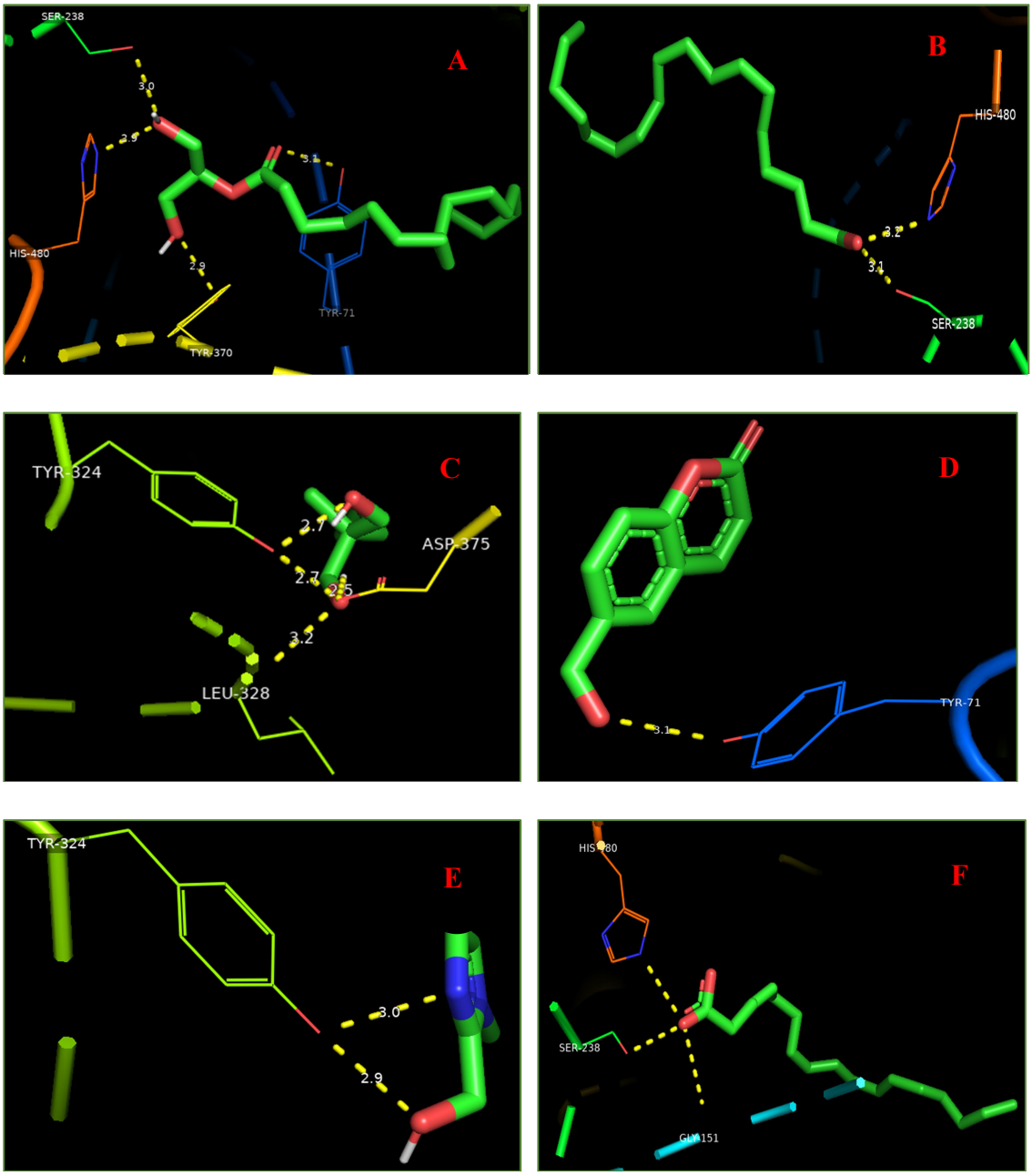


Fig 11. Three-dimensional representation of AChE-ligand complex

A. AChE with 2-Palmitoylglycerol
B. AChE with Octadecanoic acid
C. AChE with 1,3-Propanediol, 2-methyl-2-propyl-
D. AChE with Coumarin-6-carboxaldehyde
E. AChE with Imidazole-2-methanol, 1-methyl-
F. AChE with 9,12-Octadecadienoic acid (Z, Z)-

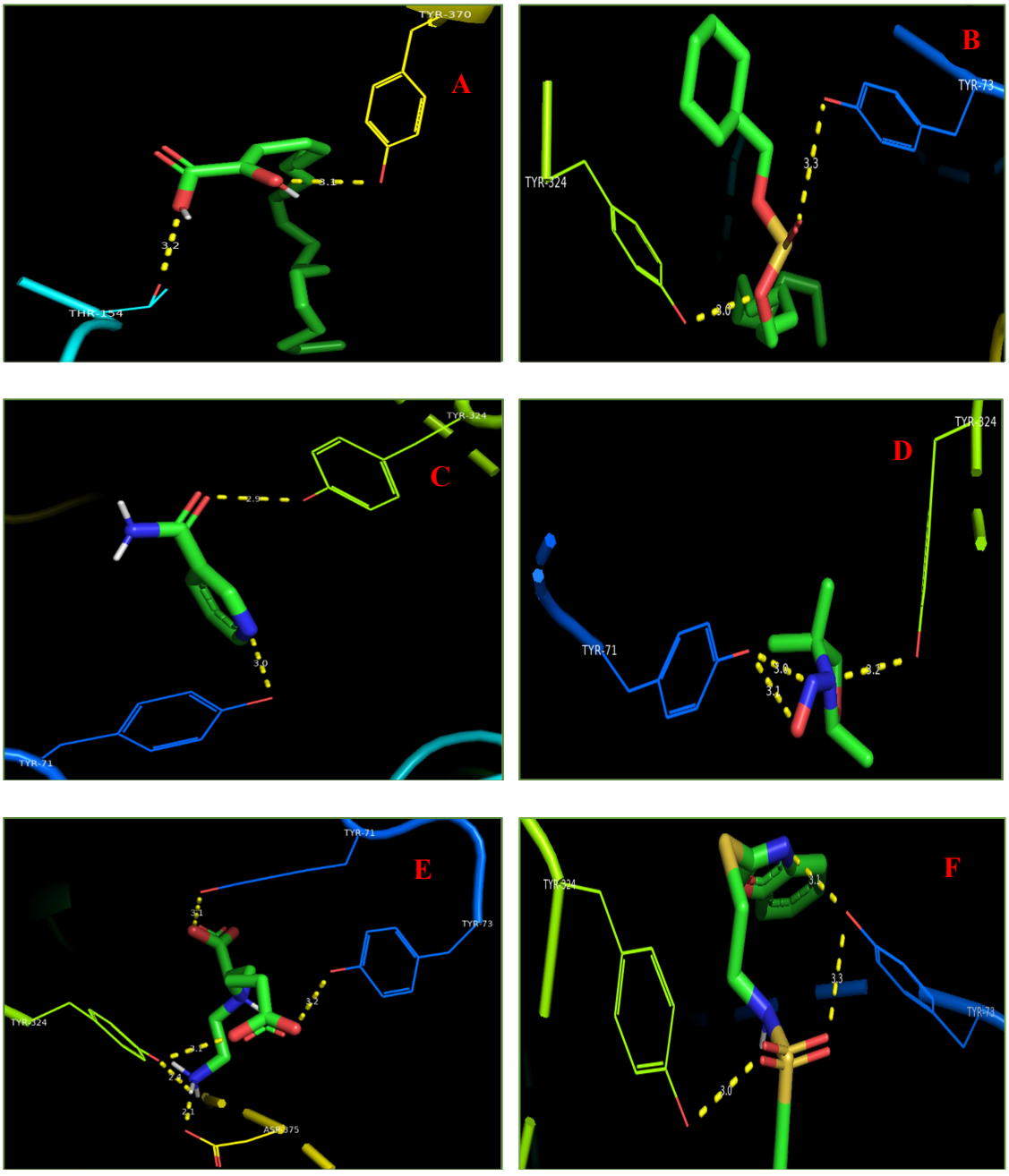


Fig 12. Three-dimensional representation of AChE-ligand complex

A. AChE with Hydroxydocosahexaenoic acid
B. AChE with Cyclohexylmethyl undecyl ester
C. AChE with Nicotinamide
D. AChE with N-Nitroso-2,4,4- trimethyloxazolidine
E. AChE with Ethylenediamine-N, N'-dipropionic acid
F. AChE with N-[2-(2-Benzoxazolylthio) ethyl] benzenesulfonamide

4.4.2 Estimation of AChE activity

The specific activity of acetylcholinesterase in healthy and *Beauveria* infected larvae were determined as per the procedure of Kranthi, (2005) and expressed in µMol min^{-1} mg^{-1} protein (Table 19).

Among two samples, the *Beauveria* infected larvae recorded significantly lower specific activity of acetylcholinesterase (0.0445 µMol min^{-1} mg^{-1} protein) compared to the specific activity of acetylcholinesterase in healthy larvae (0.0912 µMol min^{-1} mg^{-1} protein) after 96 h of treatment.

The inhibitory effect of secondary metabolites on acetylcholine esterase was calculated in both infected and healthy larvae. The result revealed a significant difference in the inhibitory activity between both groups. The highest inhibition was recorded in *B. bassiana* infected larvae to the tune of 69.57 per cent as against 11.30 per cent in healthy larvae.

Table 19. The specific activity of acetylcholinesterase in healthy and *Beauveria* infected larvae

Sl. No.	Samples	AChE activity (µM min^{-1} mg^{-1})	AChE inhibition (%)
1	Healthy larvae	0.0912**	11.30
2	*Beauveria* infected larvae	0.0445**	69.57

**Samples are significantly different at both 1% and 5% level of significance

DISCUSSION

5. DISCUSSION

Entomopathogenic fungi are being studied for their potential role in the biocontrol of insect pests. Unlike other groups, such as bacteria and viruses, entomopathogenic fungi invade host insects by entering through the cuticle. Infectivity is a complicated and multi-factorial phenomenon that includes mechanical pressure, cuticle degrading enzymes and secondary metabolites such as toxins, pigments, *etc*. The current study focuses on the mycotoxins of *B. bassiana*, NBAII-Bb-5a, potent strain that was isolated from coffee berry borer in coffee plantations of Kodagu district, Karnataka (Haraprasad *et al.,* 2001), which is currently being widely used in Kerala.

5.1 Extraction and identification of secondary metabolites from *Beauveria bassiana*

Beauveria bassiana plays a very important role in the ecofriendly pest management. It is successfully employed to manage *Helicoverpa armigera* (Prasad and Syed, 2010) and *H. hampei* (Uma *et al.,* 2013; Hollingsworth *et al.,* 2020) as an ecosafe strategy in integrated pest management. Moreover, it is known as an effective pathogen against invasive spotted lanternfly, *Lycorma delicatula*, *S. litura, B. tabaci* and *Trialeurodes vaporariorum* (Clifton *et al.,* 2022; Islam *et al.,* 2023; Jang *et al.,* 2023).

Beauveria bassiana is widely recognised for producing a wide range of biologically active metabolites (Amobonye *et al.,* 2020). In this study, *B. bassiana* (NBAIR-Bb-5a) cultured on two growth media (PDB and CDB) produced a wide array of secondary metabolites.

The investigation of crude toxins isolated from PDB using HR-LCMS and HR-GCMS revealed diverse compounds. The growth and incubation conditions are vital factors that influence the production of secondary metabolites by entomopathogenic fungi.

The important bioactive metabolites detected through HR-LCMS and HR-GCMS analysis were beauvericin, 1,2-dipalmitin, á-D-glucopyranose, 1,6-anhydro, 2-palmitoylglycerol, ethylenediamine-N, N'-dipropionic acid, harmine,

cyclohexylmethyl undecyl ester, cordycepin, coumarin-6-carboxaldehyde, dioctyl phthalate, dihydrothiophenone, nicotinic acid, 6-hydroxy nicotinic acid piperidinine, anthranilic acid, phenobarbital, pentobarbital, hexadecanoic acid, *etc.*

Diversity in the production of metabolites in different culture media is attributed to many factors, *viz.*, differences in the type of culture medium, temperature, pH, and incubation period. The perfect combination of these variables will result in an optimal environment for fungal growth and synthesis of secondary metabolites. A similar observation was made by Molen *et al.* (2013), who assessed the utility and dependability of several media producing secondary metabolites of *Glomerella acutata*.

Further, the production of diverse metabolites in different media is regulated by nutrient factors. The presence and concentration of various nutrients in the growth medium can influence the metabolic pathways activated by the fungi. Different nutrients serve as precursors for specific metabolites, and their availability can lead to the upregulation or downregulation of genes involved in the synthesis of metabolites. Production of different metabolites by *B. bassiana* in different media has already been established by previous studies. Potato dextrose medium supports the production of secondary metabolites from different fungi. Oosporein was produced in potato dextrose broth by *Chaetomium cupreum* at 150 rpm at 25 ° C and kept for 4 days (Mao *et al.,* 2010) as well as from *Cochliobolus kusanoi* (Alurappa *et al.,* 2014). Additionally, it has been reported from *L. aphanocladii* kept at 30 °C for 7 days in dark conditions (Costa Souza *et al.,* 2016). The static liquid cultivation of entomopathogenic fungi with an optimum temperature of 25°C was favourable for producing secondary metabolites using dextrose (PDB) as a carbon source.

Serrano-Carreón *et al.* (2002) reported the production of dioctyl phthalate by *Trichoderma harzianum* in PDB at 29°C. The production of beauvericin by *Isaria* sp. and *Fusarium* sp. has been reported when cultured in PDB and incubated at 25°C (Luangsa-Ard *et al.,* 2009; Xu *et al.,* 2010). *Aspergillus tamarii* and *Trichoderma* sp cultured in PDB supported the production of hexadecenoic acid (Yahaya *et al.,* 2017; Malonzo *et al.,* 2018).

The metabolites like N-nitroso-2,4,4- trimethyloxazolidine, vanillic acid, 3-hydroxyhexobarbital, 3,3-dimethyl-1-phenyltriazene, sphinganine, phalaenopsine, 3,5-dimethylpyrazole, 5-methyl-3-pyrazolamine and 2-hexyl-3,5-dipentylpyridine, *etc*. were produced by *B. bassiana* cultured in CDB growth medium. The chemical composition of CDB includes sucrose, sodium nitrate, dipotassium phosphate, magnesium sulphate, potassium chloride and ferrous sulphate, which accounts for the difference in the synthesis of metabolites with that of PDB. Vanillic acid was reported to be produced by the fungus *Sporotrichum pulverulentum*, *S. thermophile,* and *Aspergillus niger* (Ander *et al.,* 1980; Topakas *et al.,* 2003; Lubbers *et al.,* 2021).

Many studies reported similar metabolites identified in the present study from *B. bassiana*. Liu *et al.* (2015) identified the sphinganine produced by *B. bassiana* through HPLC-MS analysis and detected its bioactivity through KEGG database. Nithya *et al.* (2019) identified the N-nitroso-2,4,4- trimethyloxazolidine metabolites from BR strain of *B. bassiana* cultured in potato dextrose broth through GC-MS analysis.

The analysis of crude toxin extracted from *Beauveria* infected *S. litura* revealed the presence of bioactive metabolites from the infected host insects, suggesting that the toxins are involved in pathogenesis.

The metabolite profiling of both infected and healthy host insects revealed the presence of bioactive metabolites, which were not detected in healthy larvae. Monobutyl phthalate, 12-oxo phytodienoic acid, 16-hydroxyhexadecanoic acid, 2,5-dihydroxybenzaldehyde, 9-oxo-10(E),12(E)-octadecadienoic acid, (+)-aphidicolin, mesaconic acid, hydroxyquebrachamine, *etc*. were the bioactive metabolites i.dentified from the infected host insect through HR-LCMS analysis. Whereas the volatile metabolites detected from HR-GCMS were 1-undecene, cyclopropane, octyl-, 9-octadecene, (E)-, 7-hexadecene, (Z)-, heptadecanoic acid, methyl ester, diisooctyl phthalate, heptaoxacyclohenicosan-2-one, cyclopenta (phenanthrene-17-yl) propanoic acid, *etc*.

The diversity of secondary metabolites produced by a particular fungal strain is highly dependent on the host insect. Skrobek *et al.* (2008) reported that the production of destruxin A by *M. anisopliae* was more strongly supported by *Galleria*

mellonella than *Tenebrio molitor*. Abdullah (2019) identified 9,12(E)-octadecadienoic acid through GC-MS analysis as insecticidal secondary compounds from the extract of *B. bassiana* and *Trichoderma harzianum*. Vivekanandhan *et al.* (2022) detected monobutyl phthalate as the important metabolite of *M. anisopliae*, and the metabolite profiles of *B. bassiana* isolates (BHT021 and BHT030) revealed the presence of aphidicolin through LC-MS analysis (Zhang *et al.*, 2022).

Elevated levels of secondary metabolites in *B. bassiana* (NBAIR-Bb-5a) discovered during the early phase of incubation suggested its pathogenicity and virulence. These mycotoxins may interfere with the pathogen's early infection process to combat the host immune system, clearing the way for the action of additional insecticidal metabolites generated during succeeding stages of pathogenesis.

The metabolites *viz.*, 1,2-dipalmitin, á-D-glucopyranose, 1,6-anhydro, 2-palmitoylglycerol, ethylenediamine-N, N'-dipropionic acid, cyclohexylmethyl undecyl ester, cordycepin, octadecanoic acid, anthranilic acid, dibutyl phthalate, *etc.* were common in both the growth media (PDB and CDB). Further, metabolites such as hydroxyquebrachamine and dihydrothiophenone were present in the crude toxin extracted from CDB growth medium and host insects. Some metabolites like monobutyl phthalate, picolinic acid, were common in crude toxins extracted from PDB growth medium and host insects. Beauvericin, 2-palmitoylglycerol and ethylenediamine-N, N'-dipropionic acid were common in both growth media (PDB and CDB) and in infected host insects.

The study revealed the production of the same metabolites in different growth media. This might be due to the ability of the fungus to produce metabolites in different growth media, which utilize a wide array of nutrients for synthesizing metabolites. However, the variation in metabolites is due to different culture conditions in terms of nutrients, pH, and temperature supporting the production of different metabolites by the same species. Understanding the role of nutritive media in regulating metabolite production in entomopathogenic fungi is essential for optimizing their use in various applications, including biocontrol and the development of biopesticides.

5.2 Bioactivity of fungal metabolites

The bioactivities of the wide array of metabolites produced by *B. bassiana* Bb5a were determined using the PubChem and KEGG databases. Among the metabolites discovered, 18 were insecticidal, 16 were antimicrobial, and 6 were nematicidal, with others demonstrating various bioactivities such as enzyme inhibition, lipid and fat metabolism, and pheromonal actions.

Entomopathogenic fungi are known to be pathogenic to insects, nematodes, mites, and even microbes. These fungi have evolved various strategies to aid in their pathogenicity and ensure the successful infection of their hosts. One of the crucial mechanisms they employ is the production of insecticidal, nematicidal and antimicrobial metabolites. These metabolites play important roles in the fungi's pathogenicity against their respective hosts.

5.2.1 Insecticidal metabolites

During fungus-host interaction, the entomopathogenic fungi release toxic secondary metabolites into the host. These metabolites suppress the insect immune system by arresting the host detoxifying mechanism or by inducing apoptosis in host haemocytes (Podsiadlowski *et al.*, 1998; Gillespie *et al.*, 2000). These metabolites have been studied for their insecticidal activity to manage insect pests as an eco-friendly measure.

Beauvericin was the most important insecticidal metabolite produced in PDB, CDB and infected host insects. Many researchers had already described the insecticidal, antimicrobial, nematicidal and cytotoxic activity of beauvericin. Grove and Pople (1980) reported the insecticidal activity of beauvericin against larvae of *A. aegypti* at a concentration of 10 and 20 μg mL^{-1}. Shin *et al.* (2009) reported beauvericin as the most effective inhibitor of HIV-1 integrase with the IC_{50} 1.9 μM, which confirmed the antiviral activity of beauvericin.

The metabolites like hexadecenoic acid, octadecanoic acid, harmine, piperidinine, picolinic acid, anthranilic acid, cordycepin, dibutyl phthalate, *etc.* were identified in the crude toxin of *Beauveria* extracted from PDB and CDB. According to

literature analysis, most metabolites in *B. bassiana* exhibited strong insecticidal activity (Mil-Homens *et al.*, 2012; Paulraj *et al.*, 2016; Al-Majedy *et al.*, 2017).

The results are in conformity with the findings of Rahuman *et al.* (2000), who identified the insecticidal activity of hexadecanoic acid derived from *Feronia limonia* against fourth-instar larvae of *Aedes aegypti* and *C. quinquefasciatus.* Kim *et al.* (2002) observed that cordycepin produced by *B. bassiana* has insecticidal action against *P. xylostella.* Additionally, Vivekanandhan *et al.* (2018) reported bioactivity of cordycepin retrieved from *B. bassiana* against *C. quinquefasciatus* larvae. Furthermore, Sreeja and Rani (2019) reported the insecticidal activity of hexadecanoic and octadecanoic acids in *L. saksenae*.

The compounds identified in this study *viz.*, harmine and piperidine, had previously been reported in many plant species. Shonouda *et al.* (2008) observed piperidinine as the primary metabolite in the plant *Peganum harmala* that exhibited insecticidal action against *S. littoralis.* Bouayad *et al.* (2012) observed the negative effect of harmine on metamorphosis in the Indian meal moth *Plodia interpunctella* (Hüb.) by reducing larval weight and delaying pupation and adult emergence.

Picolinic acid detected in our study was first reported from *Fusarium* spp. (Bacon *et al*., 1996) and it was reported for its insecticidal activity against cockroach, *Periplanata americana* and further, detected from *L. saksenae* (Sreeja, 2020) through HR-LCMS analysis.

Entomopathogenic fungi produce insecticidal compounds, such as toxins or enzymes, that target and disrupt the physiology and defense mechanisms of their insect hosts. These compounds overcome the insect's natural defense, penetrate its body and eventually cause its death.

5.2.2 Nematicidal metabolites

The principal nematicidal toxins uncovered *via* spectrometric investigation were nicotinic acid, hexadecanoic acid, 3-hydroxyanthranillic acid, octadecanoic acid, linoleic acid, sphinganine, and harmine. In addition to insects, certain entomopathogenic fungi can infect and kill nematodes. This may account for the

synthesis of nematicidal compounds by *B. bassiana* to target and eliminate nematodes in addition to insects.

Nematicidal compounds identified in this study were reported from nematophagous fungi. Linoleic acid detected in our study, has already been identified in nematode-trapping fungi *viz., Arthrobotrys conoides* and *A. oligospora* and reported its activity against free living *Caenorhabditis elegans* (Stadler *et al.,* 1993). The presence of volatile metabolites in *B. bassiana*, such as hexadecaneic acid, octadecanoic acid, harmine and others, confirmed their nematicidal activity, as these compounds are responsible for causing death in *M. incognita* (Rajeswari *et al.*, 2012).

Similar metabolites were recorded by Aissani (2013) in the fruit pulp of *Melia azedarach* Linn, which exhibited nematicidal properties and was effective against *M. incognita*. Gao *et al.* (2016) studied the nematicidal activity of sphinganine and sphingosine against *M. incognita.* The metabolites hexadecaneic acid and octadecanoic acid produced by *B. amyloliquefaciens* were reported to be effective against root-knot nematode (Tadigiri *et al.*, 2020)

5.2.3 Antimicrobial metabolites

Beauveria bassiana has antimicrobial properties as evidenced by the presence of metabolites such beauvericin, coumarin-6-carboxaldehyde, dioctyl phthalate, 6-hydroxy nicotinic acid, N-nitroso-2,4,4- trimethyloxazolidine, vanillic acid, cyclohexanone, 2-(2- butynyl)-, hydroxydocosahexaenoic acid, and pyrrolo (1,2) pyrazine 1,4-dinonehexahydro were identified through HR-GCMS analysis and vanillic acid, erucamide, *etc.* detected by HR-LCMS analysis.

In a competitive environment, once entomopathogenic fungi infect an insect or a nematode, they have to face the challenge of other microorganisms such as bacteria and other fungi, that could potentially interfere with their successful colonization. Production of antimicrobial metabolites will enable fungi to suppress the growth and proliferation of competing microorganisms, thus increasing their chances of successful infection and host colonization. Hence the multitudes of antimicrobial metabolites produced by *B. bassiana* may protect the insect cadaver from microbial infection. This is supported by Fan *et al.* (2017), who reported that oosporein

produced by *B. bassiana* protected the host cadaver from bacterial infection and helped the fungus utilise the host nutrients to complete its life cycle.

The antibacterial activity of dioctyl phthalate produced by *Phoma herbarum* and *Schizophyllum commune* was reported against gram-positive bacteria (Bhimba *et al.,* 2012; Joel and Bhimba, 2013). Erucamide identified in our study, has already been detected from *Trichoderma* spp. KACC 40557 and reported its antimicrobial activity (Bae *et al.,* 2017; Tanvir *et al.,* 2018).

Beauvericin, produced by *B. bassiana* is a potent antimicrobial agent, which has been exploited by the pharmaceutical industries. Zhang *et al.* (2016) studied the antibacterial activity against *Staphylococcus aureus* and Olleik *et al.* (2019) reported the antimicrobial activity and found that beauvericin, mainly containing iso-propyl or phenylmethyl groups, acts by inhibiting the synthesis of protein as tetracycline do. Yousaf *et al.* (2023) also confirmed the antifungal activity of vanillic acid against *Sclerotium rolfsi.*

5.3 Bioefficacy

The present study established the potential of secondary metabolites of *B. bassiana* against *S. litura*. Bioefficacy studies carried out with the crude toxin of *B. bassiana* revealed 91.67 per cent mortality in third-instar larvae, *S. litura* at 48 h after treatment. While concentrations *viz.,* 90, 70, 50, 30 and 10 ppm caused mortality of 83.30, 62.50, 45.83, 37.5, 25 and 12.5 per cent respectively, at 48 h after treatment. The corresponding mortality at 84 h after treatment, was 100 per cent with 100, 90, and 70 ppm concentration and 50, 30, 10 and 9 ppm yielded mortality of 91.67, 79.16, 70.83 and 58.33 per cent respectively (Fig 13).

The study revealed the potential of toxic metabolites of *B. bassiana* (Bb-5a) against third-instar larvae of *S. litura*. The results of the present study agree with that of Gurulingappa *et al.* (2011), who reported that methanolic fractions of *L. lecanii* and *B. bassiana* induced mortality ranging from 24 to 82 per cent and 45 to 97.5 per cent, respectively, at 0.25 to 2.0 per cent concentrations. Khan *et al.* (2016) reported the intra-haemocelic injection of Bb70p, an insect-toxic protein isolated from *B. bassiana* 70, producing substantial mortality in *G. mellonella*, with an LD_{50} of 334.4 g g^{-1} body

weight. The toxicity of bassianolide, an important secondary metabolite of *B. bassiana* against third-instar larvae of *P. xylostella* was pointed out by Keppanan *et al.* (2018). A higher dosage of 0.5 mg mL^{-1} had significantly higher mortality at 120 h after inoculation.

Soesanto *et al.* (2020) investigated the efficiency of raw secondary metabolites of *B. bassiana* B16 and *B. bassiana* B10, revealing higher mortality of 77.82 and 71.47 per cent respectively in *B. tabaci* Genn. On the contrary, the mortality caused by the Bb-5a strain of *B. bassiana* was 100 per cent at the highest concentration of 100 ppm after 60 h of treatment and 75 per cent mortality at the lowest concentration of 9 ppm after 96 h of treatment. The higher mortality is attributed to the presence of insecticidal compounds in the crude toxin. However, the reduced mortality with lower concentrations is due to the low dose of toxic compounds in the treatments.

In our studies, we found that the colour of the dead insect treated with mycotoxins turned to black after 6 days. Altimira *et al.* (2022), also reported colour change in *G. mellonella* larvae from yellow-brown to purple-black after 7 days of treatment with *B. pseudobassiana* strain RGM 2184.

Probit analysis revealed that crude toxins were effective to third-instar larvae of *S. litura* with LC_{50} of 109.76, 87.51, 37.90 and 20.41 ppm for 24, 36, 48 and 60 h respectively. The LT_{50} values for 100, 90, 70, 50, 30, and 10 ppm were 22.77, 32.40, 38.66, 43.20, 45.20 and 50.60 h, respectively. The lower LC_{50} of 20.41 ppm and LT_{50} of 22.77 h recorded at 60 h and 100 ppm concentration, respectively, might be attributed to the higher concentration of insecticidal molecules in the crude toxin.

Crude extracts from entomopathogenic fungi are reported to have insecticidal effect on different insects. The crude toxin (toxinV3450 and toxinVp28) extracted from *L. lecanii* recorded LC_{50} values of 111 mg L^{-1} and 216 mg L^{-1}, respectively, for nymphs of sweet potato whitefly, *B. tabaci* (Wang *et al.,* 2007). Wang *et al.* (2017) tested the contact toxicity of *L. attenuatum* against third-instar nymphs of pea aphid, *Acyrthosiphon pisum*, and recorded the LC_{50} values of 251.34±49.54 mg L^{-1} after 6 days of exposure. These studies are in parallel to our results, where the *B. bassiana* crude toxin caused complete mortality in the higher concentrations of 100, 90, 70 and 50 ppm after 96 h after treatment.

5.4 *In silico* molecular docking studies

An insight into the insecticidal compounds produced by entomopathogenic fungi and their active site of interaction with the host cell will eventually lead to the identification of hypervirulent strains and bioactive compounds. Molecular docking was carried out to determine the mode of action of the metabolites identified in our study and promising results were observed.

In silico molecular docking of the compounds detected from *B. bassiana* revealed that 19 of them interacted with acetylcholinesterase (AChE) at the esteratic site of the catalytic ennead *viz.*, tyrosine, aspartic acid, threonine, leucine, tryptophan, histidine, serine, glycine and asparagine. Among the 19 metabolites, N-[2-(2-Benzoxazolylthio) ethyl] benzenesulfonamide showed the highest interaction with acetylcholine esterase with the binding energy of -8.4 Kcal mol^{-1}.

Hydroxydocosahexaenoic acid, coumarin-6-carboxaldehyde exhibited the binding energy -7.5 Kcal mol^{-1}. 9,12-octadecadienoic acid (Z, Z)- and cyclohexylmethyl undecyl ester showed a moderate interaction with acetylcholine esterase with the binding energy of -7.4 Kcal mol^{-1}. The binding sites of AChE (Dm AChE, PDB code IQON) were Tyr (71, 73, 324, and 370), Asp 375, Thr 154, Leu 328, Trp 472, His 480, Ser 238, Gly 151 and Asn 136. This conformed to the interaction of acetylcholinesterase with selected secondary metabolites of *B. bassiana*. Likewise, according to Harel *et al.* (2000), the conformation of acetylcholinesterase of *Drosophila melanogaster* was changed due to the insertion of two inhibitors *i.e.*, 1,2,3,4-tetrahydro-N-(phenylmethyl)-9-acridinamine and 1,2,3,4- tetrahydro-N-(3-iodophenyl-methyl)-9-acridinamine. The inhibitors were found to interact with the amino acid residues *viz.*, Tyr 71, Trp 83, Tyr 324, Phe 330, Tyr 370, Phe 371, Tyr 374, Trp 472 and His 480.

Studies on the docking of fungal metabolites with acetylcholinesterase supports our findings. Raghavendran *et al.* (2019) investigated the acetylcholinesterase inhibitory activity of a crude toxin that comprises various secondary metabolites isolated from *Pencillium sp*. in fourth-instar larvae of *Aedes aegypti*. The activity was attributed to AChE-inhibitory monoterpenoid fractions in crude extracts. It was also shown that *Pencillium* secondary metabolites such as 1-

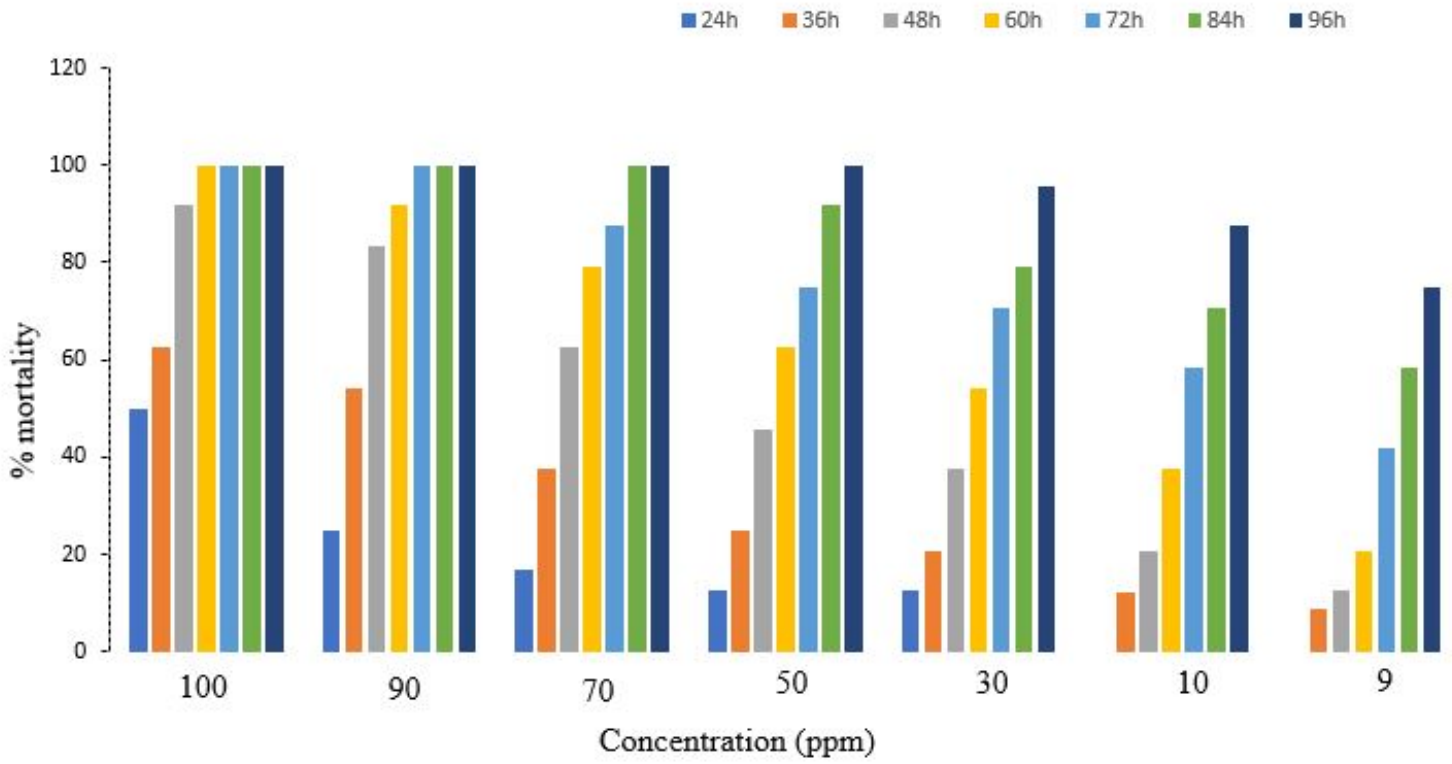


Fig 13. Effect of crude toxin of *Beauveria bassiana* on third-instar larvae of *Spodoptera litura*

octadecene, 1-nonadecene, and 9-octadecenoic acid reduced acetylcholinesterase activity in fourth-instar larvae of *C. quinquefasciatus*.

Hydroxydocosahexaenoic acid, coumarin-6-carboxaldehyde 9,12-octadecadienoic acid (Z, Z)- and cyclohexylmethyl undecyl ester showed the equal binding affinity as the aspergillol B compound (− 7.74 kcal mol^{-1}) towards the acetylcholinesterase enzyme, while evaluating the biological potential of the secondary metabolites of *Aspergillus* sp. Against *A. aegypti* vectors (Araújo *et al.*, 2022).

The binding affinities of octadecanoic acid and phenobarbital observed in our studies are in line with the findings of Félix *et al.* (2022), who reported that the 2,5,5,8a-tetramethyloctahydro-2 H-chromene metabolite extracted from *A. flavus*, showed the highest binding energy (–7.3 kcal mol^{-1}) in the formation of molecular complexes with acetylcholinesterase. The compounds interacting with the inhibitory sites of AChE are considered to have nerve action. The secondary metabolites of *B. bassiana* showed high affinities towards the active sites of AChE.

5.5 AchE assay

AChE is a key enzyme that terminates nerve impulses by catalyzing the hydrolysis of the neurotransmitter, acetylcholine (ACh), in the nervous system in various organisms (Wang *et al.*, 2004). AChE is of interest because it is the target site for organophosphate and carbamate insecticides in the central nervous system, and its role in cholinergic synapses is essential for insects (Nathan *et al.*, 2008).

AChE activity in *S. litura* larvae treated with crude toxin was 0.0445 µM min^{-1} mg^{-1} protein at the concentration of 1000 ppm, while activity in the nontreated larvae was 0.0912 µM min^{-1} mg^{-1} protein after 72 h of treatment. Inhibition of AChE causes accumulation of ACh at the synapses, so that the post-synaptic membrane remains in a state of permanent stimulation, which causes paralysis, ataxia, general lack of coordination in the neuromuscular system, and eventual death. The death of treated larvae after a short period of 72 h indicated the strong AChE activity of secondary metabolites. Many studies have been conducted to support the potentiality of

secondary metabolites to reduce the activity of AChE (Jukic *et al.,* 2007; Lopez-Hernandez *et al.,* 2009; Yu *et al.,* 2010).

In our study, treatment of third-instar larvae of *S. litura* by the crude toxin extracted from the entomopathogenic fungus, *B. bassiana* (NBAIR-Bb-5a), inhibited 69.57 per cent activity of acetylcholinesterase enzyme compared to 11.30 per cent inhibition in healthy *S. litura* after 72 h of treatment. This could show its inhibitory effect of secondary metabolites on AChE in synapses. Our results followed the finding of Maazoun *et al*. (2017), who reported AChE inhibition activity to the tune of 73.37 ± 0.34 and 81.23 ± 0.58 per cent for *Urginea* extract and galanthamine, respectively, at a concentration of 1000 μg mL^{-1}, in comparison to the control.

Prior research on the role of secondary metabolites in inhibiting the acetylcholinesterase enzyme in insects supported the findings of this study. Zibaee *et al.* (2009) reported significantly decreased activity of AChE (0.5129 mOD min^{-1}) on all 5 days after treatment except on the first day (0.230 mOD min^{-1}) while activity in the control group was regular over the entire 5 days after treatment (0.841 mOD min^{-1}). The inhibition in acetylcholinesterase activity can be attributed to some of the metabolites in the crude toxin which interfere with the insect's nervous system. Due to continuous stimuli transmission across membrane, the insect might have experienced paralysis and death. This supports the insecticidal activity of selected metabolites against target host insect, *S. litura*.

The crude toxin consists of an array of secondary metabolites. The secondary metabolites with acetylcholinesterase activity in the crude toxin might have inhibited the AChE activity in the *S. litura* larvae. Zhang *et al.* (2022) reported a significant reduction of AChE activity in *Megalurothrips usitatus* on treatment with *B. bassiana* and revealed that bassianolide, a secondary metabolite produced by *B. bassiana* affected the acetylcholine receptors of insect muscles by reducing the production of AChE.

The present study reveals that *B. bassiana* is a treasure trove of secondary metabolites that may be used in pesticide chemistry to develop insecticidal compounds for pest management. The several additional metabolites identified in the

chromatographic study of crude toxin must be further characterised and studied for various bioactivities.

SUMMARY

6. SUMMARY

Beauveria bassiana (Balsamo) Vuillemin is one of the most important entomopathogenic fungus (EPF) and has a key role in eco-friendly pest management. Among different strains of *B. bassiana*, NBAIR-Bb-5a is a promising strain currently being used against a wide range of insect pests in diverse agroecosystems of Kerala. The pathogenic process in EPF is mainly mediated by an array of bioactive metabolites that provide valuable insights into the host-pathogen interaction. Hence, the study entitled "Bioactive metabolites of *Beauveria bassiana* (Balsamo) Vuillemin and its efficacy on tobacco caterpillar, *Spodoptera litura* (Fab.)" was carried out at the Department of Agricultural Entomology, College of Agriculture, Vellanikkara, during 2020–2022 to identify and assess the effectiveness of metabolites from *B. bassiana* against *S. litura*.

The salient findings of our study are summarized below:

- ✓ *Beauveria bassiana* grown in PDB and Czapek Dox broth produced as many as 45 and 44 secondary metabolites respectively, when subjected to HR-LCMS analysis.
- ✓ Screening of metabolites using PubChem and KEGG databases unveiled the insecticidal, nematicidal and antimicrobial properties of the metabolites identified in the crude toxin.
- ✓ Beauvericin, 1,2-dipalmitin, á-D-glucopyranose, 1,6-anhydro, 2-palmitoylglycerol, ethylenediamine-N, N'-dipropionic acid, harmine, cyclohexylmethyl undecyl ester, cordycepin, piperidinine and hexadecanoic acid, *etc.* were detected through HR-LCMS and were common in both growth media and showed insecticidal activity.
- ✓ Hexadecenoic acid and 2,6-pyridine dicarboxylic acid were identified as insecticidal.
- ✓ Antimicrobial compounds such as coumarin-6-carboxaldehyde, dioctyl phthalate, 6-hydroxy nicotinic acid were detected from PDB media and N-nitroso-2,4,4- trimethyloxazolidine and vanillic acid from CDB.
- ✓ Antibacterial metabolites identified from both PDB and CDB were cyclohexanone, 2-(2-butynyl)-, hydroxydocosahexaenoic acid and pyrrolo (1,2) pyrazine 1,4-dinonehexahydro.

- ✓ Nematicidal compounds detected from PDB through chromatographic methods were dihydrothiophenone and nicotinic acid, whereas octadecanoic acid and linoleic acid were detected from CDB.
- ✓ Among the two nutritive media, PDB media supported the production of more secondary metabolites than CDB.
- ✓ Hydroxyquebrachamine, 12-oxo phytodienoic acid, 9-oxo-10(E),12(E)-octadecadienoic acid, (+)-aphidicolin, diisooctyl phthalate, 1-octadecene, (E)-, 7-hexadecene, (Z)- and heptadecanoic acid, methyl ester were specific to crude toxin extracted from infected *Spodoptera.*
- ✓ Metabolites like beauvericin, monobutyl phthalate, picolinic acid, 2-palmitoylglycerol, and ethylenediamine-N, N'-dipropionic acid were common in crude toxin extracted from PDB growth medium and host insects.
- ✓ Metabolites such as hydroxyquebrachamine and dihydrothiophenone were present in the crude toxin extracted from CDB growth medium and host insects. Most of the metabolites extracted from PDB, CDB, and growth media were identical.
- ✓ Bioefficacy study revealed the toxicity of crude toxin. Crude toxin of 100 ppm caused 100 per cent mortality at 60 h after treatment. The concentration of crude toxin *i.e.,* 90, 70, and 50 ppm caused 100 per cent mortality at 72, 84, and 96 h after treatment, respectively. The highest mortality was due to bioactive insecticidal compounds in crude toxin.
- ✓ LC_{50} of crude toxin was 109.76 ppm at 24 h after treatment.
- ✓ The crude toxin at 100 ppm concentration required a lethal time (LT_{50}) of only 22.77 h to kill half of the test insects.
- ✓ *In silico* molecular docking revealed the interaction of metabolites with AChE. Interaction exhibited with binding energy from -5 to -10 kcal mol^{-1} were considered to have the best interaction with the active sites of AChE.
- ✓ The metabolite, N-[2-(2-benzoxazolylthio)-ethyl]benzenesulfonamide exhibited highest interaction (-8.5 kcal mol^{-1}).
- ✓ The study revealed the insecticidal potential of metabolites of the *B. bassiana* (NBAIR-Bb-5a) metabolites.
- ✓ The *Beauveria* infected larvae recorded significantly higher protein content (0.0795 mg mL^{-1}) than healthy larvae (0.0559 mg mL^{-1}).

- ✓ The *Beauveria* infected larvae recorded significantly lower specific activity of acetylcholinesterase (0.0445 µMol min^{-1} mg^{-1} protein) compared to the specific activity of acetylcholinesterase in healthy larvae (0.0445 µMol min^{-1} mg^{-1} protein) after 96 h after treatment.
- ✓ Acetylcholinesterase enzyme inhibition was highest in *Beauveria* infected larvae to the tune of 69.57 per cent compared to the inhibition of AChE in healthy larvae (11.30%) and were statistically significant.

The study indicated the bioefficiency of the crude toxin of *B. bassiana* against third-instar larvae of *S. litura*. The metabolites with a high binding affinity towards AChE could serve as a source for potent insecticidal compounds. Further, research on the characterization of individual metabolites may ease the way for the development of effective pesticides with potent insecticidal activity.

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APPENDICES

APPENDIX I

1. Composition of potato dextrose agar (PDA) media

Ingredient	Quantity
Potato infusion	200g
Dextrose	20g
Agar	20g
Distilled water	1L

Final pH (at 25°C) 5.6±0.2

2. Composition of Czapex-Dox agar (CZA) media

Ingredients	Quantity
Sucrose	30g
Sodium nitrate	2g
Dipotassium phosphate	1g
Magnesium sulfate	0.5g
Potassium chloride	0.5g
Ferrous sulfate	0.01g
Agar	15g
Distilled water	1L

Final pH (at 25°C) 7.3±0.2

3. Composition of semi-synthetic media for *Spodoptera litura*

Ingredients	Quantity
Kidney bean	65 g
Wheat bran	65 g
Yeast extract powder	25 g
Casein	3 g
Ascorbic acid	4 g
Sorbic acid	0.9 g
Methyl para hydroxy benzoate	0.4 g
Cholesterol	0.3 g
Streptomycin sulphate	0.1 g
Multivitamin	1 No.
Formaldehyde	2 mL
Agar	12 g
Distilled water	600 mL

APPENDIX II

1. List of metabolites from PDB – HRLC-MS

SL. No	Name of the compound	Molecular formula	Best match	Molecular weight
1	Beauvericin	$C_{45}H_{57}N_3O_9$	87.10	800.43
2	5-Oxotetrahydrofuran-2-carboxylic acid	$C_5H_6O_4$	88.30	130.09
3	á-D-Glucopyranose, 1,6-anhydro-	$C_6H_{10}O_5$	83.50	162.14
4	1,2-Dipalmitin	$C_{35}H_{68}O_5$	85.12	568.92
5	2-Palmitoylglycerol	$C_{19}H_{38}O_4$	89.60	330.50
6	D-Sorbitol	$C_6H_{14}O_6$	91.36	182.07
7	Dihydrothiophenone	$C_4H_4O_S$	86.10	102.15
8	Cyclohexylmethyl undecyl ester	$C_{18}H_{36}O_3S$	87.67	332.54
9	Prolylhydroxyproline	$C_{10}H_{16}N_2O_4$	85.37	228.10
10	Valinopine	$C_{10}H_{17}NO_6$	91.24	247.10
11	Miserotoxin	$C_9H_{17}NO_8$	96.92	267.09
12	2,3-Pyridinedicarboxylic acid	$C_7H_5NO_4$	96.41	167.02
13	4-Nitrobenzoic acid	$C_7H_5NO_4$	98.78	167.02
14	Gentianadine	$C_8H_7NO_2$	85.59	149.04
15	2-Propenyl 2-aminobenzoate	$C_{10}H_{11}NO_2$	87.67	177.07
16	N-Undecylbenzenesulfonic acid	$C_{17}H_{28}O_3S$	96.43	312.17
17	Imidazole-2-methanol, 1-methyl-	$C_5H_8N_2O$	86.10	112.06
18	5-Methyl-3-pyrazolamine	$C_4H_7N_3$	83.50	97.06
19	3-Phenylpyrazole-4-carbaldehyde	$C_{10}H_8N_2O$	89.60	172.06
20	2-Hydrazino-1H-benzimidazole	$C_7H_8N_4$	90.40	148.07

21	2,6-Pyridinecarboxylicacid	$C_7H_5NO_4$	83.30	167.02
22	3'-Hydroxyhexobarbital	$C_{12}H_{16}N_2O_4$	94.69	252.10
23	Choline	$C_5H_{13}NO$	96.70	103.09
24	3,3-Dimethyl-1-phenyltriazene	$C_8H_{11}N_3$	88.20	149.09
25	Dibutyl phthalate	$C_{16}H_{22}O_4$	84.50	278.15
26	3-(6'-Methylthio) hexylmalic acid	$C_{11}H_{20}O_5S$	86.62	264.10
27	1-Amino-1-deoxy-scyllo-inositol	$C_6H_{13}NO_5$	82.13	179.07
28	2-Propenyl 2-aminobenzoate	$C_{10}H_{11}NO_2$	87.67	177.07
29	2-Descarboxy-cyclo-dopa	$C_8H_9NO_2$	81.08	151.06
30	N-Methyl-N,4-dinitrosoaniline	$C_7H_7N_3O_2$	46.82	165.05
31	Gentianadine	$C_8H_7NO_2$	85.59	149.04
32	Ethylenediamine-N, N'-dipropionic acid	$C_8H_{16}N_2O_4$	85.59	204.22
33	Piperidone	C_5H_9NO	89.20	99.06
34	Cordycepin	$C_{10}H_{13}N_5O_3$	80.20	251.10
35	Picolinic acid	$C_6\ H_5\ NO_2$	88.90	123.03
36	Nicotinic acid	$C_6H_5NO_2$	89.60	123.03
37	Octadecanoic acid	$C_{18}H_{36}O_2$	88.80	284.48
38	Monobutyl phthalate	$C_{12}H_{14}O_4$	98.90	222.08
39	5-Acetyl-2,3-dihydro-1,4-thiazine	C_6H_9NOS	97.98	143.04
40	Isopentenyl adenosine	$C_{15}H_{21}N_5O_4$	88.13	335.15
41	Indoleacrylic acid	$C_{11}H_9NO_2$	82.31	187.06
42	Harmaline	$C_{13}H_{14}N_2O$	94.26	214.10
43	Perlolyrine	$C_{16}H_{12}N_2O_2$	84.41	264.09
44	L, L-Cyclo(leucylprolyl)	$C_{11}H_{18}N_2O_2$	97.12	210.13
45	N-[2-(2-benzoxazolylthio)-ethyl]benzenesulfonamide	$C_{15}H_{14}N_2O_3S_2$	85.61	334.40

2. List of metabolites from PDB – HRGC-MS

SL. NO.	Name of the compound	Molecularformula	Retention time (min)	Molecular weight
1	N-[2-(2-Benzoxazolylthio) ethyl]benzenesulfonamide	$C_{15}H_{14}N_2O_3S_2$	1.47	334.04
2	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	15.43	326.19
3	N-Acetyl-D-alloisoleucine	$C_8H_{15}NO_3$	3.85	173.10
4	Picolinic acid	$C_6H_5NO_2$	1.48	123.03
5	Quinolinic acid	$C_7H_5NO_4$	1.88	167.02
6	(4-Chlorophenyl) [5-hydroxy-3- methyl-5-(trifluoromethyl)-4,5- dihydro-1H-pyrazol-1-yl] methanone	$C_{12}H_{10}Cl\ F_3N_2O_2$	4.14	306.03
7	Hexadecanoic acid	C16H32O2	20.59	256.24
8	4,4'-Difluorobenzophenone	$C_{13}H_8F_2O$	0.99	218.05
9	Flufenamic Acid	$C_{14}H_{10}F_3\ NO_2$	3.90	281.06
10	2,6-Dichlorotoluene	$C_7H_6Cl_2$	0.81	159.98
11	Ethylmalonic acid	$C_5H_8O_4$	27.9	132.04
12	4-Undecylbenzenesulfonic acid	$C_{17}H_{28}O_3S$	13.86	312.17
13	L-(+)-Tartaric acid	$C_4H_6O_6$	1.11	150.01
14	Dimethyl itaconate	$C_7H_{10}O_4$	28.05	158.05
15	Ethylmalonic acid	$C_5H_8O_4$	27.45	132.04
16	Adipic acid	$C_6H_{10}O_4$	27.43	146.05
17	6-(Chloromethyl)-N-(2,5-dimethoxyphenyl)-1,3,5-triazine-2,4-diamine	$C_{12}H_{14}Cl\ N_5O_2$	4.28	295.08
18	2-({2-[(2-Fluorophenyl) amino]-2-oxoethyl} sulfanyl) benzoic acid	$C_{15}H_{12}FN\ O_3S$	3.94	305.05

19	5-Methyl-2-nitro-1,3-cyclohexanediyldiacetate	$C_{11}H_{17}NO_6$	4.24	259.10
20	5-bromobenzofuran	C_8H_5BrO	1.13	195.95
21	2,2-Bis(hydroxymethyl)propionic acid	$C_5H_{10}O_4$	28.10	134.05
22	Pimelic acid	$C_7H_{12}O_4$	27.91	160.07
23	2,6-Piperidinedicarboxylic acid	$C_7H_{11}NO_4$	27.73	173.06
24	Harmine	C13H12N2O	25.21	212.25
25	2,6-pyridine dicarboxylic acid	C7H5NO4	31.90	167.12
26	1,3,4,5-Tetrahydroxycyclohexanecarboxylicacid	$C_7H_{12}O_6$	27.75	192.06
27	Ethane-1,2-diylbis(dichloromethylsilane)	$C_4H_{10}Cl_4Si_2$	0.77	253.90
28	2-Deoxyhexopyranose	$C_6H_{12}O_5$	28.03	164.06
29	3-Acetyl-2,5-dichlorothiophene	$C_6H_4Cl_2O$ S	28.83	193.93
30	1,1'-hexane-1,6-diylbis(1H-pyrrole-2,5-dione)	$C_{14}H_{16}N_2O_4$	3.85	276.11
31	Quizalofop-methyl	$C_{18}H_{15}Cl\ N_2O_4$	5.72	358.07
32	2,2-Bis(hydroxymethyl)propionic acid	$C_5H_{10}O_4$	27.49	134.05
33	3-(Methoxycarbonyl)-2-(2-methyl-2-propanyl)-5-oxo-1,3-oxazolidine-4- carboxylic acid	$C_{10}H_{15}NO_6$	3.77	245.08
34	4-Bromo-N-(chloroacetyl)-1H-pyrazole-1-carboxamide	$C_6H_5BrClN_3O_2$	1.32	264.92
35	Tetraacetylethylenediamine	$C_{10}H_{16}N_2O_4$	3.31	228.11
36	2-Oxo-2-[(tetrahydro-2-furanylmethyl)amino] ethyl acetate	$C_9H_{15}NO_4$	3.96	201.09
37	1-(tert-butoxycarbonyl) proline	$C_{10}H_{17}NO_4$	3.75	215.11

APPENDIX III

1. List of metabolites from CDB – HRLC-MS

SL. No	Name of the compound	Molecular formula	Best match	Molecular weight
1	Beauvericin	$C_{45}H_{57}N_3O_9$	87.10	800.43
2	N, N-di-(n-Undecyl) heptafluorobutanamide	$C_{26}H_{46}F_7NO$	83.80	521.34
3	Choline	$C_5H_{13}NO$	96.70	103.09
4	Neuraminic acid	$C_9H_{17}NO_8$	93.54	267.09
5	Alpha-N-Phenylacetyl-L-glutamine	$C_{13}H_{16}N_2O_4$	96.88	264.11
6	Gentianadine	$C_8H_7NO_2$	94.56	149.04
7	Imidazole-2-methanol, 1-methyl-	$C_5H_8N_2O$	86.10	112.06
8	Diamino-pimelic acid	$C_7H_{14}N_2O_4$	88.60	190.09
9	DL-Phenylalanine	$C_9H_{11}NO_2$	89.70	165.07
10	2,6-Pyridinecarboxylic acid	$C_7H_5NO_4$	86.80	167.02
11	Phenobarbital	$C_{12}H_{12}N_2O_3$	94.50	232.08
12	Pentobarbital	$C_{11}H_{18}N_2O_3$	88.50	226.13
13	Tetracyanoethylene	C_6N_4	94.10	128.01
14	3'-Hydroxyhexobarbital	$C_{12}H_{16}N_2O_4$	94.69	252.10
15	Alanyl-Tyrosine	$C_{12}H_{16}N_2O_4$	96.29	252.11
16	2-Hydroxyfelbamate	$C_{11}H_{14}N_2O_5$	97.25	254.08
17	2,6-Pyridinecarboxylic acid	$C_7H_5NO_4$	86.80	167.02
18	5,6,7,8-Tetrahydro-1,2,4-benzotriazin-3-amine	$C_7H_{10}N_4$	90.40	150.09
19	3,3-Dimethyl-1-phenyltriazene	$C_8H_{11}N_3$	86.40	149.09
20	Choline	$C_5H_{13}NO$	95.20	103.09

21	2E-Decenedioic acid	$C_{10}H_{16}O_4$	97.69	200.10
22	Diaziquone	$C_{18}H_{22}N_2O_6$	96.84	362.14
23	3,4-Dimethylbenzoic acid	$C_9H_{10}O_2$	98.24	150.06
24	Petasitenine	$C_{19}H_{27}NO_7$	88.50	381.17
25	Alpha-N-Phenylacetyl-L-glutamine	$C_{13}H_{16}N_2O_4$	96.88	264.11
26	Hydroxyprolyl-Valine	$C_{10}H_{18}N_2O_4$	82.70	230.12
27	Neuraminic acid	$C_9H_{17}NO_8$	93.54	267.09
28	Diamino-pimelic acid	$C_7H_{14}N_2O_4$	90.33	190.09
29	Gentianadine	$C_8H_7NO_2$	94.56	149.04
30	3'-Hydroxyhexobarbital	$C_{12}H_{16}N_2O_4$	94.69	252.10
31	Sphinganine	$C_{16}H_{35}NO_2$	95.08	273.26
32	Phalaenopsine T	$C_{20}H_{27}NO_5$	81.14	361.19
33	Ochrolifuanine A	$C_{29}H_{34}N_4$	93.56	438.27
34	5-Oxotetrahydrofuran-2-carboxylic acid	$C_5H_6O_4$	88.30	130.09
35	1,2-Dipalmitin	$C_{35}H_{68}O_5$	85.12	568.92
36	Piperidone	C_5H_9NO	89.20	99.06
37	N-Nitroso-2,4,4-trimethyloxazolidine	$C_6H_{12}N_2O_2$	87.10	144.17
38	Vanillic acid	$C_8H_8O_4$	86.70	168.04
39	Coumarin-6-carboxaldehyde	$C_{10}H_6O_3$	85.30	174.15
40	Cyclohexanone, 2-(2-butynyl)-	$C_{10}H_{14}O$	88.80	150.22
41	Hydroxydocosahexaenoic acid	$C_{22}H_{32}O_3$	87.30	344.50
42	Nicotinic acid	$C_6H_5NO_2$	89.60	123.03
43	Octadecanoic acid	$C_{18}H_{36}O_2$	88.80	284.48
44	Dihydrothiophenone	C_5H_6OS	83.90	102.15

2. List of metabolites from CDB – HRGC-MS

SL. No	Name of the compound	Molecular formula	Retention time (min)	Molecular weight
1	2,6-Pyridinecarboxylic acid	$C_7H_5NO_4$	1.45	167.02
2	α-Hydroxyhippuric acid	$C_9H_9NO_4$	8.66	195.05
3	2-Hexyl-3,5-dipentylpyridine	$C_{21}H_{37}N$	16.02	303.29
4	(1E)-1-Chloro-2-(propylsulfanyl)-1-propene	$C_6H_{11}ClS$	0.79	150.02
5	(E)-Chlorfenvinfos	$C_{12}H_{14}Cl_3O_4P$	18.96	357.97
6	4,7-Difluoro-1-indanone	$C_9H_6F_2O$	0.79	168.0
7	(5E)-5-[(1-Methyl-1H-pyrazol-4-yl)methylene]-2-thioxo-1,3-thiazolidin-4-one	$C_8H_7N_3OS_2$	28.83	225.00
8	25-Amino-22-hydroxy-22-oxido-16-oxo-17,21,23-trioxa-22lambda~5~-phosphapentacosan-19-yl (9Z,12Z)-9,12-octadecadienoate	$C_{39}H_{74}NO_8P$	21.42	715.51
9	2,5-Bis(5-tert-butyl-benzoxazol-2-yl) thiophene	$C_{26}H_{26}N_2O_2S$	23.12	430.17
10	2,3-Bis[(9E)-9-octadecenoyloxy] propyl-2-(trimethylammonio)ethylphosphate	$C_{44}H_{84}NO_8P$	28.72	785.59
11	4-Chlorophtalic anhydride	$C_8H_3ClO_3$	29.15	181.97
12	Fomepizole	$C_4H_6N_2$	28.99	82.05
13	1-(p-nitrophenyl)-2-amino-1,3-propanediol	$C_9H_{12}N_2O_4$	1.46	212.07
14	alpha-Aminoisobutyronitrile	$C_4H_8N_2$	25.38	84.06
15	N-(5-Chloro-2-methoxyphenyl)-2-iodobenzamide	$C_{14}H_{11}ClINO_2$	1.45	386.95
16	DL-Carbidopa	$C_{10}H_{14}N_2O_4$	5.37	226.09
17	Glyceric acid 2-phosphate	$C_3H_5O_7P$	28.83	183.97

18	Fomepizole	$C_4H_6N_2$	25.41	82.05
19	3,5-Dichloro-2,6-dimethoxybenzohydrazide	$C_9H_{10}C_{l2}N_2O_3$	0.79	264.00
20	Imidazole-2-methanol, 1-methyl-	$C_5H_8N_2O$	27.67	112.06
21	alpha-Aminoisobutyronitrile	$C_4H_8N_2$	27.74	84.06
22	Imidazole-2-methanol, 1-methyl-	$C_5H_8N_2O$	25.84	112.06
23	N-acetyl-dl-leucine	$C_8H_{15}NO_3$	5.13	173.10
24	3',5'-Difluoro-4' hydroxypropiophenone	$C_9H_8F_2O_2$	0.79	186.04
25	Hexadecanoic acid	$C_{16}H_{32}O_2$	20.59	256.24
26	2-[(9E)-9-Octadecenoyloxy]-3-(palmitoyloxy)propyl 2-(trimethylammonio)ethyl phosphate	$C_{42}H_{82}NO_8P$	28.71	759.57
27	3,5-Dimethylpyrazole	$C_5H_8N_2$	25.76	96.06
28	5-Methyl-3-pyrazolamine	$C_4H_7N_3$	25.84	97.06
29	N-Acetylvaline	$C_7H_{13}NO_3$	2.22	159.08
30	12-Bromolauric acid	$C_{12}H_{23}BrO_2$	7.43	278.08
31	Di-2-Thienylmethane	$C_9H_8S_2$	28.83	180.00
32	4-Azidobenzenesulfonyl chloride	$C_6H_4ClN_3O_2S$	28.82	216.97
33	1,3-Phenylenediamine	$C_6H_8N_2$	1.09	108.06

APPENDIX IV

1. List of metabolites from healthy *Spodoptera* HR-LCMS

Name of the compound	Molecular formula	Molecular weight	Best match
Glycolic acid	$C_2H_4O_3$	76.01	99.90
Citric acid	$C_6H_8O_7$	192.02	99.80
Acrylic acid	$C_3H_4O_2$	72.02	99.70
Catechol	$C_6H_6O_2$	110.03	99.40
Salicylic acid	$C_7H_6O_3$	138.03	99.20
4-Hydroxybenzoic acid	$C_7H_6O_3$	138.03	99.20
Azelaic acid	$C_9H_{16}O_4$	188.10	98.60
Gallic acid	$C_7H_6O_5$	170.02	98.60
Stearic Acid	$C_{18}H_{36}O_2$	284.27	98.50
Benzoic acid	$C_7H_6O_2$	122.03	98.50
Oleic Acid	$C_{18}H_{34}O_2$	282.25	98.40
Pyruvic acid	$C_3H_4O_3$	88.01	98.30
Ethyl myristate	$C_{16}H_{32}O_2$	256.24	98.30
Xanthurenic acid	$C_{10}H_7NO_4$	205.03	97.70
N-Acetyl-D- alloisoleucine	$C_8H_{15}NO_3$	173.10	81.50
Xanthine	$C_5H_4N_4O_2$	152.03	96.50
N-Acetyl-L- cysteine	$C_5H_9NO_3S$	163.03	96.00
2-Oxobutyric acid	$C_4H_6O_3$	102.03	95.40
Gentisic acid	$C_7H_6O_4$	154.02	95.30
Methylmalonic acid	$C_4H_6O_4$	118.02	95.30
Homogentisic acid	C8H8O4	168.04	95.00

Itaconic acid	C5H6O4	130.02	94.90
Ferulic acid	C10H10O4	194.05	93.80
4-Oxoproline	C5H7NO3	129.04	93.60
Indole-2-carboxylic acid	C9H7NO2	161.04	93.30

2. List of metabolites from healthy *Spodoptera* HR-GCMS

Name of the compound	**Molecular formula**	**Molecular weight**	**Retention time (min)**
Pyrimidine-4,6-diol,5-methyl-	$C_5H_6N_2O_2$	126	18.91
2,4,6-Trihydroxybenzoicacid	$C_7H_6O_5$	170	25.01
1,3,5-Benzenetriol	$C_6H_6O_3$	126	18.91
1-[2-Pyridyl]-2,2- dimethyl-2-piperidinoethanol	$C_{14}H_{22}N_2O$	234	22.62
1-Dodecene	$C_{12}H_{24}$	168	25.01
E-11,13-Tetradecadien-1-ol	$C_{14}H_{26}O$	210	28.86
Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206	26.02
Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	$C_{19}H_{30}O_3$	306	35.62
Ethyl 4-t- butylbenzoate	$C_{13}H_{18}O_2$	206	21.66
Methyl (Z)-5,11,14,17-eicosatetraenoate	$C_{21}H_{34}O_2$	318	28.86
Heneicosanoic acid,methyl ester	$C_{22}H_{44}O_2$	340	33.63
7-Octene-1,2-diol	$C_8H_{16}O_2$	144	14.79
Bicyclo[2.1.1]hexane-1-carboxylic acid, 5,5-dimethyl	$C_9H_{14}O_2$	154	18.91
1-Octanamine, N, N-dioctyl	$C_{24}H_{51}N$	353	26.02
Tris(2-ethylhexyl)amine	$C_{24}H_{51}N$	353	26.02

1-Hexanamine, 2-ethyl-N-(2-ethylhexyl)-N- methyl-	$C_{17}H_{37}N$	255	31.31
1-Methyl-5,6- diaminouracil	$C_5H_8N_4O_2$	156	29.35
3-Piperidinol, 1-(1,1-dimethylethyl)-4- methyl	$C_{10}H_{21}NO$	171	35.62
d-Ribitol, 1-deoxy-1-nonylamino	$C_{14}H_{31}NO_4$	277	28.50
10-Nonadecanamine	$C_{19}H_{41}N$	283	26.02
d-Glucitol, 1-deoxy-1-(nonylamino)	$C_{15}H_{33}NO_5$	307	14.78
Dinonylamine	$C_{18}H_{39}N$	269	21.65
2,4(1H,3H)- Pyrimidinedione, dihydro-1,3,5- trimethyl	$C_7H_{12}N_2O_2$	156	33.63
Pentadecanoic acid,14-methyl-, methylester	$C_{17}H_{34}O_2$	270	28.30
Tridecanoic acid,methyl ester	$C_{14}H_{28}O_2$	228	18.91

APPENDIX V

1. List of metabolites from *Beauveria* infected *Spodoptera* HR-LCMS

SL. No	Name of the compound	Molecular formula	Molecular weight	Best match
1	Monobutyl phthalate	$C_{12}H_{14}O_4$	222.08	98.90
2	12-Oxo phytodienoic acid	$C_{18}H_{28}O_3$	292.20	92.20
3	2-Hydroxycinnamic acid	$C_9H_8O_3$	164.04	98.20
4	Pyruvic acid	$C_3H_4O_3$	88.01	97.60
5	2-Oxoglutaric acid	$C_5H_6O_5$	146.02	92.70
6	Succinic semialdehyde	$C_4H_6O_3$	102.03	95.80
7	Oleoyl-L-α- lysophosphatidic acid	$C_{21}H_{41}O_7P$	436.25	98.00
8	16-Hydroxyhexadecanoicacid	$C_{16}H_{32}O_3$	272.23	96.70
9	Trans-Petroselinic acid	$C_{18}H_{34}O_2$	282.25	98.40
10	Ethyl myristate	$C_{16}H_{32}O_2$	256.24	98.30
11	Linoleic acid	$C_{18}H_{32}O_2$	280.24	98.20
12	5-Methoxysalicylic acid	$C_8H_8O_4$	168.04	98.40
13	2,5 Dihydroxybenzaldehyde	$C_7H_6O_3$	138.03	99.30
14	Glycolic acid	$C_2H_4O_3$	76.01	99.90
15	Kynurenic acid	$C_{10}H_7NO_3$	189.04	97.60
16	5-Methoxysalicylic acid	$C_8H_8O_4$	168.04	98.40
17	Oleoyl-L-α- lysophosphatidic acid	$C_{21}H_{41}O_7P$	436.25	98.00
18	Citraconic acid	$C_5H_6O_4$	130.02	92.10
19	Isoferulic acid	$C_{10}H_{10}O_4$	194.05	92.30

20	2,4-Dinitrophenol	$C_6H_4N_2O_5$	184.01	98.20
21	4-Hydroxybenzaldehyde	$C_7H_6O_2$	122.03	97.80
22	(2R)-2,3-Dihydroxypropanoic acid	$C_3H_6O_4$	106.02	82.70
23	Isoxanthopterin	$C_6H_5N_5O_2$	179.04	93.20
24	6-Hydroxycaproic acid	$C_6H_{12}O_3$	132.07	97.10
25	Citraconic acid	$C_5H_6O_4$	130.02	95.90
26	3-Hydroxyanthranilic acid	$C_7H_7NO_3$	153.04	96.80
27	Hexadecanedioic acid	$C_{16}H_{30}O_4$	286.21	97.50
28	Palmitoleic acid	$C_{16}H_{30}O_2$	254.22	97.60
29	2-Palmitoylglycerol	$C_{19}H_{38}O_4$	330.50	89.60
30	Mesaconic acid	C_5HO_4	130.02	86.70
31	Dodecanedioic acid	$C_{12}H_{22}O_4$	230.15	94.40
32	Ferulic acid	$C_{10}H_{10}O_4$	194.05	93.80
33	Xanthurenic acid	$C_{10}H_7NO_4$	205.03	97.70
34	3-Hydroxypicolinic acid	$C_6H_5NO_3$	139.02	91.40
35	2,6-Di-tert-butyl-1, 4-benzoquinone	$C_{14}H_{20}O_2$	220.14	80.30
36	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	294.21	81.20
37	Spectinomycin	$C_{14}H_{24}N_2O_7$	332.15	87.20
38	(+)-Aphidicolin	$C_{20}H_{34}O_4$	338.24	98.10
39	2-Hydroxybenzothiazole	C_7H_5NOS	151.00	98.00
40	Nicotinic acid	$C_6H_5NO_2$	123.03	97.40
41	4-Coumaric acid	$C_9H_8O_3$	164.04	84.80
42	6-Hydroxynicotinic acid	$C_6H_5NO_3$	139.02	91.30
43	9,10-dihydroxy- 12Z-octadecenoic acid	$C_{18}H_{34}O_4$	314.24	95.90

2. List of metabolites from *Beauveria* infected *Spodoptera* HR-GCMS

SL. No	Name of the compound	Molecular formula	Molecular weight	Retention time (min)
1	1-Dodecanol	$C_{12}H_{26}O$	186	18.91
2	1-Undecene	$C_{11}H_{22}$	154	29.35
3	3-Tetradecene, (Z)-	$C_{14}H_{28}$	196	21.66
4	Cyclopropane, octyl-	$C_{11}H_{22}$	154	14.78
5	E-11,13-Tetradecadien-1-ol	$C_{14}H_{26}O$	210	26.02
6	Cyclodecane, methyl-	$C_{11}H_{22}$	154	26.26
7	9-Octadecene, (E)-	$C_{18}H_{36}$	252	28.30
8	7-Hexadecene, (Z)-	$C_{16}H_{32}$	224	22.62
9	5-Tetradecene, (E)-	$C_{14}H_{28}$	196	18.91
10	1-Octanamine, N-methyl-N-octyl-	$C_{17}H_{37}N$	255	35.62
11	1-Hexanamine, 2- ethyl-N-(2- ethylhexyl)-N-methyl-	$C_{17}H_{37}N$	255	31.31
12	1-Methyl-5,6-diaminouracil	$C_5H_8N_4O_2$	156	29.35
13	3-Piperidinol, 1-(1,1-dimethylethyl)-4- methyl	$C_{10}H_{21}NO$	171	35.62
14	Cyclopenta(phenanthrene-17-yl) propanoic acid	$C_{27}H_{42}O_4$	430	28.50
15	10-Nonadecanamine	$C_{19}H_{41}N$	283	26.02
16	d-Glucitol, 1-deoxy-1-(nonylamino)-	$C_{15}H_{33}NO_5$	307	14.78
17	Heptaoxacyclohenicosan-2-one	$C_{14}H_{26}O_8$	322	21.65
18	2,4(1H,3H)-Pyrimidinedione, dihydro-1,3,5- trimethyl	$C_7H_{12}N_2O_2$	156	33.63
19	3-Tetradecene, (Z)-	$C_{14}H_{28}$	196	21.66

20	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270	28.30
21	Pentadecanoic acid,14-methyl-, methylester	$C_{17}H_{34}O_2$	270	28.30
22	Tridecanoic acid,methyl ester	$C_{14}H_{28}O_2$	228	18.91
23	Heptadecanoic acid, methyl ester	$C_{18}H_{36}O_2$	284	21.66
24	Heneicosanoic acid, methyl ester	$C_{22}H_{44}O_2$	340	35.62
25	6,10-Dimethyl-4-undecanol	$C_{13}H_{28}O$	200	22.62
26	Sulfurous acid, dodecyl 2-propyl ester	$C_{15}H_{32}O_3S$	292	22.02
27	3-Hexanol, 2,2-dimethyl	$C_8H_{18}O$	130	14.79
28	Oxalic acid, allyldodecyl ester	$C_{17}H_{30}O_4$	29	14.79
29	11,14-Eicosadienoic acid, methyl ester	$C_{21}H_{38}O_2$	322	28.86
30	9,12-Octadecadienoicacid, methyl ester, (E,E)-	$C_{19}H_{34}O_2$	294	26.26
31	9,12-Octadecadienoyl chloride, (Z, Z)-	$C_{18}H_{31}ClO$	298	25.01
32	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390	17.40
33	Bicyclo[2.1.1]hexane-1-carboxylic acid, 5,5-dimethyl	$C_9H_{14}O_2$	154	18.91

BIOACTIVE METABOLITES OF *Beauveria bassiana* (BALSAMO) VUILLEMIN AND ITS EFFICACY ON TOBACCO CATERPILLAR *Spodoptera litura* (FAB.)

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ABSTRACT OF THE THESIS

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ABSTRACT

Beauveria bassiana (Balsamo) Vuillemin is one of the most important entomopathogenic fungi (EPF) and has a key role in eco-friendly pest management. Among different strains of *B. bassiana*, NBAIR-Bb-5a is a promising strain currently being used against a wide range of insect pests in diverse agroecosystems of Kerala. The pathogenic process in EPF is mainly mediated by an array of bioactive metabolites that provide valuable insights into the host-pathogen interaction. Hence, the study entitled "Bioactive metabolites of *Beauveria bassiana* (Balsamo) Vuillemin and its efficacy on tobacco caterpillar, *Spodoptera litura* (Fab.)" was carried out at the Department of Agricultural Entomology, College of Agriculture, Vellanikkara, during 2020–2022 to identify and assess the effectiveness of metabolites from *B. bassiana* against *S. litura*

The secondary metabolites of *B. bassiana* (NBAIR-Bb-5a) were extracted from two different growth media *viz.*, potato dextrose broth (PDB) and Czapek -Dox broth (CDB) and from *B. bassiana* infected *S. litura*. The crude toxin was separated by solvent extraction of culture filtrate and concentrated through a rotary vacuum evaporator, yielding crude toxin of 0.28 and 0.23 g mL^{-1} of PDB and CDB culture filtrates, respectively. The third-instar larvae of *S. litura* infected with *B. bassiana* @ $1x10^8$ spores mL^{-1} yielded a crude toxin of 0.18 g mL^{-1} of larval extract. The spectral analysis of crude toxins extracted through standard procedures could detect an array of metabolites. HR-LCMS and HR-GCMS analysis identified 85 compounds in PDB, compared to 79 compounds each in CDB and infected *S. litura*

The bioactivity of metabolites produced by *B. bassiana* was identified through the PubChem and KEGG databases. Among the metabolites detected from three sources (PDB, CDB, and infected host), 18 were insecticidal, 16 of them were antimicrobial, and 6 were nematicidal compounds. HR-LCMS analysis detected the presence of predominant insecticidal compounds, *viz.,* beauvericin, 1,2-dipalmitin, á-D-glucopyranose, 1,6- anhydro, 2-palmitoylglycerol, ethylenediamine-N, N'-dipropionic acid, harmine, cyclohexylmethyl undecyl ester, cordycepin, piperidinine and hexadecanoic acid. The volatile insecticidal compounds detected through HR-GCMS were harmine, hexadecenoic acid, and 2,6-pyridine dicarboxylic acid in the

crude toxin extracted from both PDB and CDB. Coumarin-6-carboxaldehyde, dioctyl phthalate, 6-hydroxy nicotinic acid from PDB, and N-nitroso-2,4,4-trimethyloxazolidine and vanillic acid from CDB is found to have antimicrobial activity. Antibacterial metabolites detected from PDB and CDB were cyclohexanone, 2-(2-butynyl)-, hydroxydocosahexaenoic acid and pyrrolo (1,2) pyrazine 1,4-dinonehexahydro. HR-GCMS analysis revealed the presence of dihydrothiophenone and nicotinic acid in PDB and octadecanoic acid and harmine in CDB, which are known to be nematicidal in action.

The insecticidal metabolites identified in the infected *S. litura* through HR-LCMS were beauvericin, hydroxyquebrachamine, monobutyl phthalate, and diisooctyl phthalate was detected through HR-GCMS. Other metabolites like 2,5-dihydroxybenzaldehyde, mesaconic acid, and (+)-aphidicolin possess antibacterial, antifungal, and antiviral activities, respectively. The metabolites specifically detected in crude toxin extracted from infected *S. litura* were hydroxyquebrachamine, 12-oxo phytodienoic acid, 9-oxo-10(E),12(E)-octadecadienoic acid, (+)-aphidicolin, diisooctyl phthalate, 1-octadecene, (E)-, 7-hexadecene, (Z)- and heptadecanoic acid.

The bioefficacy of the crude toxin was evaluated against third-instar larvae of *S. litura* through topical bioassay using Potters Tower. Bioefficacy studies revealed dose-dependent mortality. The crude toxin at 100 ppm caused 100 per cent mortality at 60 h after treatment. The 90, 70 and 50 ppm concentrations caused 100 per cent mortality at 72, 84 and 96 h after treatment, respectively. The LC_{50} and LT_{50} were calculated using PoloPC software. Probit analysis revealed LC_{50} value of 109.76 ppm at 24 h whereas it was 87.51 ppm, 37.90 ppm and 20.41 ppm for an exposure time of 36, 48 and 60 h, respectively. LT_{50} values of crude toxin at 100 ppm was 22.77 h. At the same time, lower concentrations of 90 ppm, 70 ppm, 50 ppm, 30 ppm, and 10 ppm were 32.40, 38.66, 43.20, 45.20 and 50.60 h, respectively.

In silico molecular docking studies carried out with the 20 metabolites as ligands and AChE of as the target protein using CB-Dock software revealed the insecticidal potential of metabolites of *Beauveria bassiana* (NBAIR-Bb-5a). Among the 20 metabolites, 18 interacted with AChE at specific sites, *viz.*, serine, histidine, and glutamate. Interactions with binding energies ranging from -5 to -10 kcal mol^{-1} were considered to have the best interaction with the active sites of AChE. The

highest interaction was recorded by N-[2-(2-benzoxazolylthio)-ethyl]benzenesulfonamide with a binding energy of -8.5 kcal mol^{-1}, followed by hydroxydocosahexaenoic acid and coumarin-6-carboxaldehyde (-7.5 kcal mol^{-1}), 9,12-octadecadienoic acid (Z, Z) and cyclohexylmethyl undecyl ester (-7.4 kcal mol^{-1}), phenobarbital and octadecanoic acid (-7.1 kcal mol^{-1}). The lowest interaction was exhibited by hydrothiophenone with a binding energy of -3.9 kcal mol^{-1}.

Wet lab studies to validate the results of molecular docking of secondary metabolites of *Beauveria bassiana* revealed acetylcholinesterase activity of secondary metabolites. The *Beauveria*-infected larvae recorded a significantly high protein content of 0.0795 mg mL^{-1} with a lower specific activity of acetylcholinesterase of 0.0445 µMol min^{-1} mg^{-1} protein compared to the protein content of 0.0559 mg mL^{-1} and the specific activity of acetylcholinesterase of 0.0912 µMol min^{-1} mg^{-1} protein in healthy larvae. Acetylcholinesterase enzyme inhibition was 69.57 per cent in *Beauveria*-infected larvae compared to the inhibition of AChE in healthy larvae (11.30%) after 96 h of treatment.

The present study reveals that *Beauveria bassiana* (Bb5a) is a treasure trove of secondary metabolites that can be used as templates for the synthesis of novel insecticidal molecules for pest management. The wide array of metabolites identified in the crude toxin must be further characterised and studied for various bioactivities.