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EVALUATION OF POTENTIAL BIOACTIVITIES OF SECONDARY METABOLITES EXTRACTED FROM THE ENTOMOPATHOGENIC FUNGI

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ABSTRACT

Entomopathogenic fungi are a group of fungal organisms associated with insects and extensively used in various parts of the world as biopesticides against economic important insect pests. Various secondary metabolites produced by entomopathogenic fungi has a lot of potential biological activities such as antimicrobial and insecticidal activities. The present study is undertaken to evaluate the possible bioactive components in the ethyl acetate extract of entomopathogenic fungi *Beauveria bassiana* and *Nomuraea rileyi* and evaluation of potential biological activities such as anti microbial and insecticidal activities. Metabolites were extracted from culture free supernatant of the media after the extraction with ethyl acetate. Screening of possible components was carried out by GC-MS.GC-MS analysis of ethyl acetate extract of both the fungal organism was performed using a Perkin-Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS) Metabolites with different concentrations was used to study anti bacterial activity against human pathogenic bacteria *Escherichia coli, Salmonella typhi* and *Enterococci* species adopting dynamic growth curve inhibition assay and cytotoxicity against *Spodoptera frugiperda*-21 cell line (-21) adapting 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Both the fungal metabolites were found to cause distinct anti bacterial and cytotoxicity as dose dependent manner. The present study would suggest the possible utilisation of entomopathogenic fungal metabolites as an effective agent for controlling the economic important Lepidopteron insect pests.

KEYWORDS: Entomopathogenic fungi, Metabolites, cytotoxicity, Spodoptera *frugiperda*-21 cell line (Sf-21).

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INTRODUCTION

Herbivorous insects are a major threat in the continuous supply of food and fibres for human consumption. Additionally, parasitic insects and arthropod vectors of important diseases must be controlled. Synthetic insecticides play a major role in pest insect control, with chemical, environmental and toxicological properties having been improved considerably over the last six decades. In 2001, a total of 7.56 billion USD was spent to protect crops from damage by pest insect species[1, 2]. The world market for insecticides is still dominated by compounds irreversibly inhibiting acetylcholinesterases (AChE). Together, these AChE inhibitors (organophosphates [OPs] and carbamates) and the insecticides acting on the voltage-gated sodium channel (in particular the pyrethroids) account for approximately 70% of the world market. However, due to similarities in the nervous system of insects and vertebrates, these agents can exhibit considerable toxicity towards higher organisms and therefore, their non-selective mode of action may cause devastating environmental problems. The extensive use of such compounds has caused the development of resistance to these agents in many pest insects. In the end, high resistance levels lead to the fact that effective concentrations must exceed the legally recommended concentrations, thus making the compounds useless. Therefore, the need to search for novel insecticides with a better efficacy or a new mode of action is obvious and involves a race against time. An intense search for alternatives less harmful to the environment has been initiated in laboratories around the world. Since then, there is a steady progression towards the development of narrow-spectrum insecticides that act on insect specific targets. Microbial natural products remain the most promising source of novel secondary metabolites. The impact of microbial biodiversity favours the chance of isolating new antibiotics and development of new molecules to help in fighting many pathogens[3]. Fungi produce a wide range of secondary metabolites with high therapeutic value as antibiotics, cytotoxic substance and insecticide compounds that promote or inhibit growth, attractors, repellent and others[4]. These metabolites are being exploited in different fields of medicine and industries[5] Fungi produces various secondary metabolites which cause the entomopathogenic activity. Among the different group of organism, fungi were the first organisms to be used for the biological control of pests.90 genera of fungi are known to cause disease in insects associated with deuteromycetes and entomophthorales.Potential bioactive compounds have been isolated from entomopathogenic fungi. Insecticidal activity of ibotenic acid isolated from entomopathogenic fungi *Amanita muscaria, A. stabiliformis* and *A.pauturina*[6]. 4-(N-methyl N-phenylamino) butan-2-one and itaconic acid[3]. There are a certain number of key requirements such as intra and inter laboratory reproducibility, exact toxicity assessment, type of compounds to be tested, simplicity, low cost and benefit ratio that need thorough consideration before developing an alternative cell based testing procedure. Established insect cell lines fulfil these criteria and therefore should be useful tools for screening with enough homogeneous materials. Furthermore, tests employing cell cultures can be readily automated. Additionally, cell based assays can be developed that enable the elucidation of new modes of action for insecticide candidates. Insect cell cultures that have retained their arthropod specific metabolic pathways or hormonal regulation will also allow the development of screening procedures using insect specific targets. In the present study an attempt has been made to evaluate the possible bioactive components from ethyl acetate extract of entomopathogenic fungi *Beauveria bassiana* and *Nomuraea rileyi* against anti bacterial activity against human pathogenic bacteria and cytotoxic effect of against *Spodoptera frugiperda*-21 cell line (Sf-21).

MATERIALS AND METHODS

Entomopathogenic fungal culture

Beauveria bassiana, and *Nomuraea rileyi* were obtained from the microbial type culture

collection (MTCC, Chandigarh, India) and all strains were maintained on potato dextrose agar slants.

Crude Extraction of Metabolites Media

Savoured maltose yeast extract broth (4% maltose, 1% peptone, 0.5% yeast extract and pH 6.0) was selected for the crude extraction of metabolites[7].

Inoculum Preparation

The fungal inoculum was prepared from 15 days slant culture of respective fungal strains by scrapping off with a sterilized glass rod. A homogenous conidial suspension was prepared in sterile distilled water by adding a few drops of the wetting agent Tween 80 (0.01%). The conidial concentration of the suspension was determined using an improved Neubauer haemocytometer (Germany). Serial dilutions were made from the stock solution to obtain the spore concentrations as 10^8 spores/mL

Crude Extraction

SMYB media were prepared in one litre of distilled water with components as mentioned earlier and sterilized by autoclaving. After sterilization, 2.5 mL of respective fungal spore suspensions was inoculated and incubated at 25ºC for 14 days under shaking conditions. After incubation period the cultured broth was filtered through cheese cloth and the collected filtrate was extracted with double the volume of ethyl acetate. The resulting organic layer was evaporated under rotary evaporator and concentrated material was collected in a sterile screw cap vial and used for further studies.

GC –MS analysis

GC-MS analysis of ethyl acetate extract of both the fungal organism was performed using a Perkin-Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS).

Cytotoxicity studies Chemicals reagents

The following chemicals were obtained from Sigma Aldrich: DMSO, Grace insect media, penicillin-streptomycin. All compounds were analytical grade quality with a minimum purity 96.5%. Fetal calf serum and 3-(4,5-dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) were obtained Himedia, Mumbai, India.

Cell line

Sf-21 (*Spodoptera frugiperda*-21 cell lines) derived from the primary explants from pupal tissues of *Spodoptera frugiperda*, were obtained from national centre for cell sciences (NCCS), pune, India. The culture was routinely maintained at 27°C using Grace's TNM-FH medium supplemented with 10% FCS and 1% penicillin–streptomycin solution[8].

Cell bioassay

Cells were collected six days after the sub culturing and diluted with fresh medium to a density of $7.5x10^4$ cells/ml. Each well of a 96-well microtiter culture plate was loaded with 100 µL of cell solution containing 2 µL of the fungal metabolite, prepared in DMSO. The final concentration of fungal metabolites was 3.95, 7.8, 15.6, 31.2, 62.5, 125, 250µg/mL. Each concentration tested consisted of four replicates and the test was repeated two times after 72 hours of exposure[9]. The test medium was replaced with 20 µL of 2 mg/ml MTT, overnight staining at 27 ºC, the staining solution was carefully removed and 150 µL/well DMSO was added to solubilize the purple formazan crystals produced within the cell. The absorbance of each well was measured at 540 nm using a microplate reader[10]. The cell growth was expressed as a percentage of the absorbance ratio; absorbance in wells with fungal metabolites to control well (representing cells treated with 0 concentration of fungal metabolite). The inhibition concentration (**IC**) was calculated as follows

IC = (1.123-At / 1.123) x 100
At: absorbance value of tested wells.

RESULTS AND DISCUSSION

Biodiversity is a wonderful instrument used by fungal species to produce an enormous number of natural compounds, differing in chemical structure, biological activity, and mechanism of action, specificity and environmental impact[11]. Entomopathogenic fungi are a group of fungal organisms know to

affect insects and can be extensively used as biocontrol agents against economic important insect pests in various parts of the world. This fungal organism produces various secondary metabolites with potential biological activities which act on other organisms sometimes causing inhibition of growth, disease and even death. Some entomopathogenic fungi may also produce metabolites which can affect other organisms and insects.[3]Fungi such as *Beauveria bassiana*, *Paecilomyces fumosoroseus* and *Fusarium moniliforme* also produce cyclodepsipeptides, including Beauvercin and the enniatin complex[12, 13].In the present study, anti bacterial activity and cytotoxicity effect of entomopathogenic fungal metabolites extracted from *Beauveria bassiana* and *Nomuraea rileyi* against Sf-21cell line. The possible use of those compounds as pharmaceuticals has been widely studied, but there have been limited efforts to evaluate and understand their potential use in plant protection. Bioactive principles of the culture filtrate of *Beauveria bassiana* and *Nomuraea rileyi* were extracted into organic solvent ethyl acetate at pH 5.6-6.5 and followed by chromatographic separations followed by chromatographic separations using silica (G_{60}) chromatography with final yield of 177.5 mg/litre and 80mg/litre of medium respectively. Possible chemical components of *Beauveria bassiana* metabolites with GC-MS was carried out using a Perkin-Elmer GC Clarus 500 system comprising an AOC-20i auto-sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS).This analysis revealed that the presence of 9-6-hydroxy amino-5-m-totyla 20-1-pyh 122193 10003 18-25 imdine-2, 4-dione. Benzyloxy-2-f-136891141523-21-9-22 luoro-beta-hydroxy-methoxy (2s, 6b)-1-Benzyl-2-methyl-6-penthyl 220371000367 22-4-14 piperidin-4-1(Figure-1)and the GC-MS of *Nomuraearileyi* showed ethanediamidepentanoicacid,ethylester1-pentananmine,N-methyl-1-propanamine,N,2-dimethyl-1-propanamine,N,2-dimethyl-butanoicacid,3amino-;N,N- Dimethyl acetamideGuanidine,N,N-dimethyl-N-(2-Aminoethyl)-N-methylethylenedlamine; 2,3-Pentadienoic acid-, ethyl ester 2-cyclohexen-1-one, 3-(hydroxymethyl)-6-(1-methylethyl)-Methylenecyclopropanecarboxylic acid ; 4H-Pyran-4one , 5-hydroxy-2-(hydroxymethyl)-4H-Pyran-4-one, 5-hydroxy-2(hydroxymethyl)-4H-Pyran-4-one, 5-hydroxy-2(hydroxymethyl).(Figure-2) Metabolites with different concentration showed distinct effect on dynamic growth curve of all the tested bacteria during all the tested time periods and more effect was observed in high concentration (Figure3-8). Increasing concentration of metabolites progressively inhibited the growth of all the tested bacteria the lag phase of all the tested bacteria in both the metabolites treatment was found to be more prolonged than the control. The invitro cytotoxicity of *Beauveria bassiana* and *Nomuraea riley* were screened against *Spodoptera frugiperda*-21 cell line (Sf-21) by means of MTT assay. A serial tenfold dilution of respective metabolites was prepared. Sf-21 cells were incubated in 96 wells plate and viability in respective concentration was done by MTT assay, which reveals that the viability of the cells was highly influenced by the concentration of the metabolite used. Effective high cytotoxic effect was recorded in *Nomuraea rileyi* metabolite treatment. Percentage of viability at respective concentration of 250 , 125, 62.5, 31.2, 15.6 , 7.8 , 3.95 µg/mL was 26.09 , 31.07 , 34.81 , 48.17 , 60.81 , 65.80 , 77.20 respectively (Figure-9).Microscopic examination of *Nomuraea rileyi* treated with Sf21 cells revealed complete disruption and fragmentation of cells. (Figure-10) In *Beauveria bassiana* metabolite treatment cytotoxicity was also influenced by dosage of the metabolites. Maximum cytotoxicity was recorded at 250µg followed by 125 µg with 27.60 % cell viability and 42.111 % cell viability respectively. (Figure-11).Microscopic examination also revealed distinct cytotoxic effect as in *Nomuraea rileyi* treatment which can be easily inferred. (Figure-12) Cytotoxicity of Beauvercin cyclodepsipeptide produced by entomopathogenic fungi *Beauveria bassiana* against Sf-21 and Sf-9 cell line has been reported.[14] Further studies will be helpful to identify and formulate the anti microbial and insecticidal metabolites extracted from *Beauveria bassiana* and *Nomuraea rileyi* as an effective pharmacological agents.

Figure 1
Gas chromatography mass spectrograph of Nomuraea rileyi metabolit

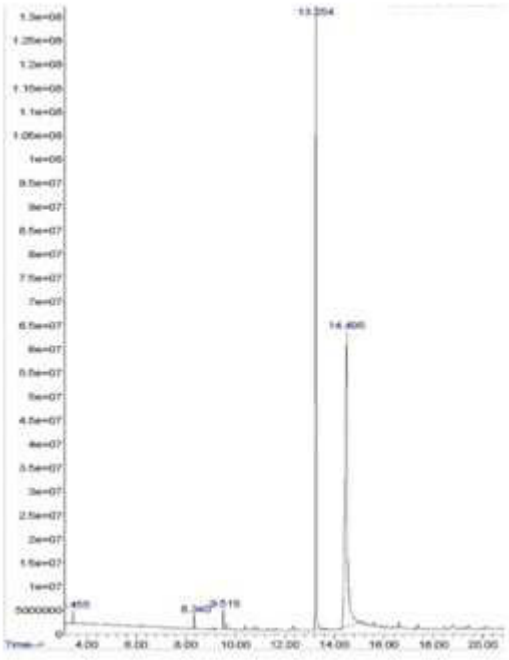


Figure 2
Gas chromatography mass spectrograph of Beauveria bassiana metabolite

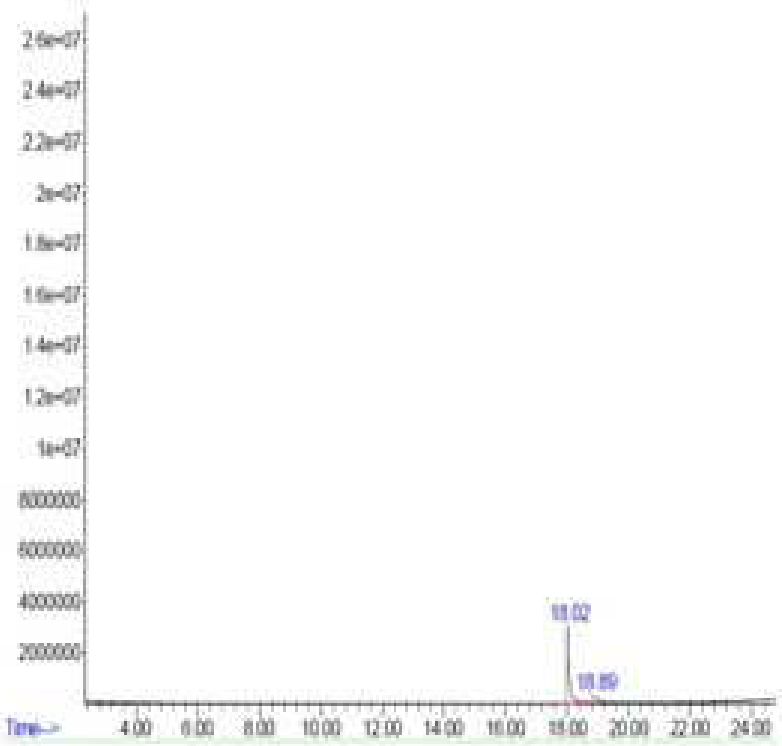


Figure 3
Optical density of E.coli grown in LB broth containing Different concentration of Nomuraea rileyi metabolites

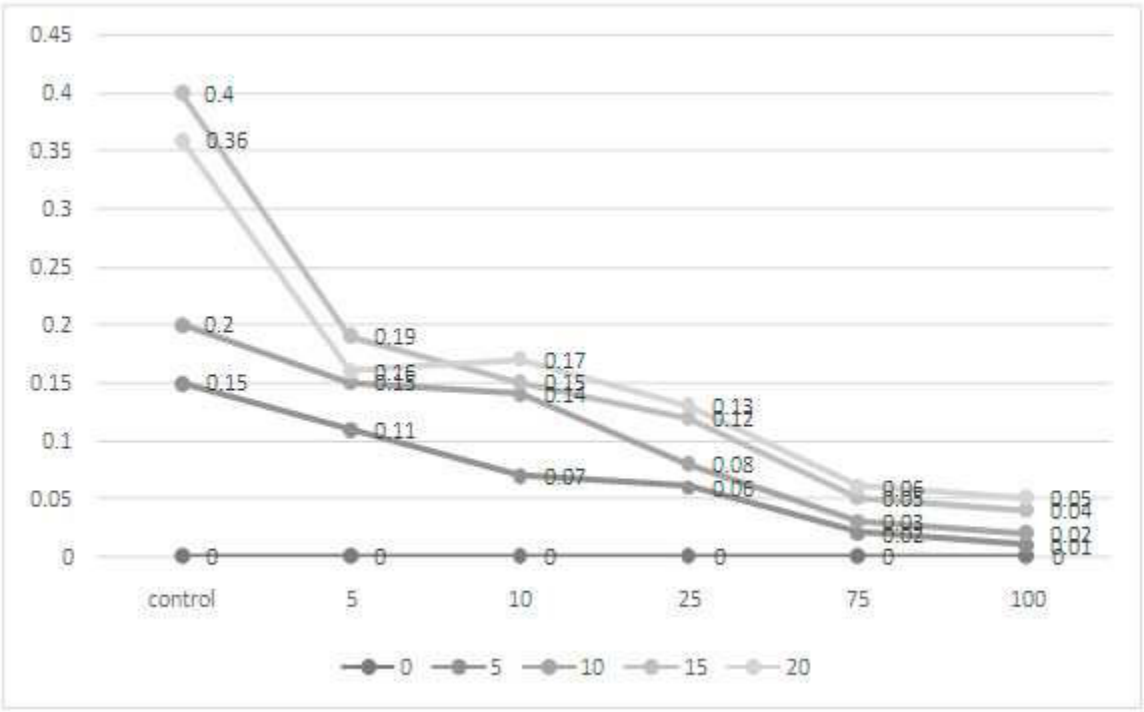


Figure 4
Optical density of Enterococci grown in LB broth containing different concentration of Nomuraea rileyi metabolites

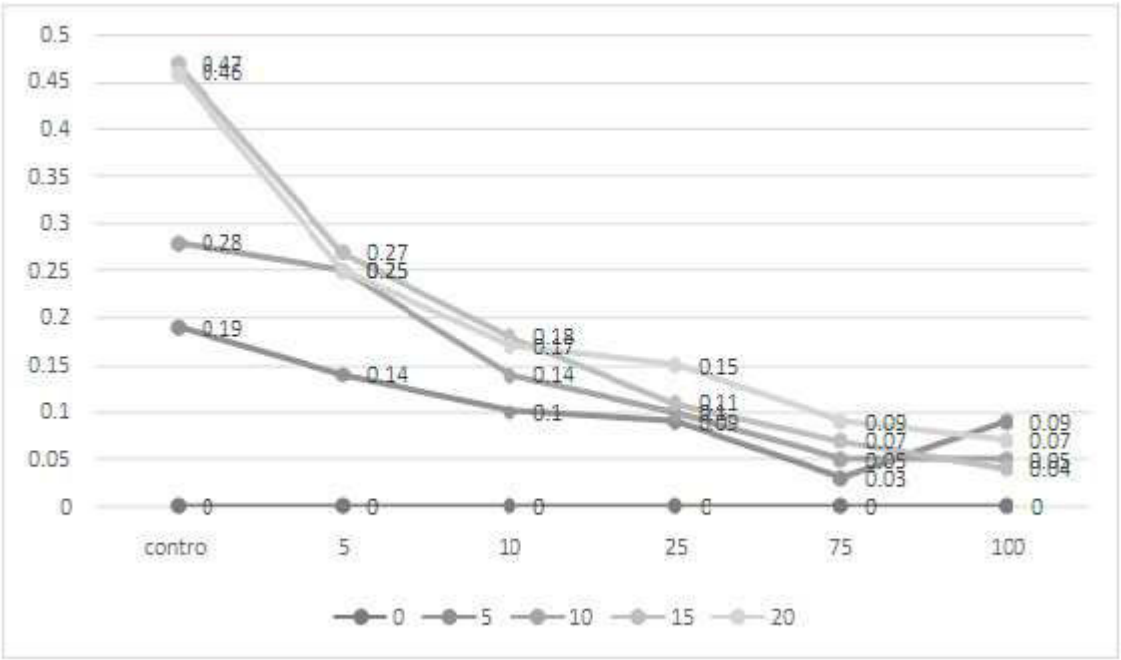


Figure 5
Optical density of Salmonella typhi grown in LB broth containing different concentration of Nomuraea rileyi metabolites

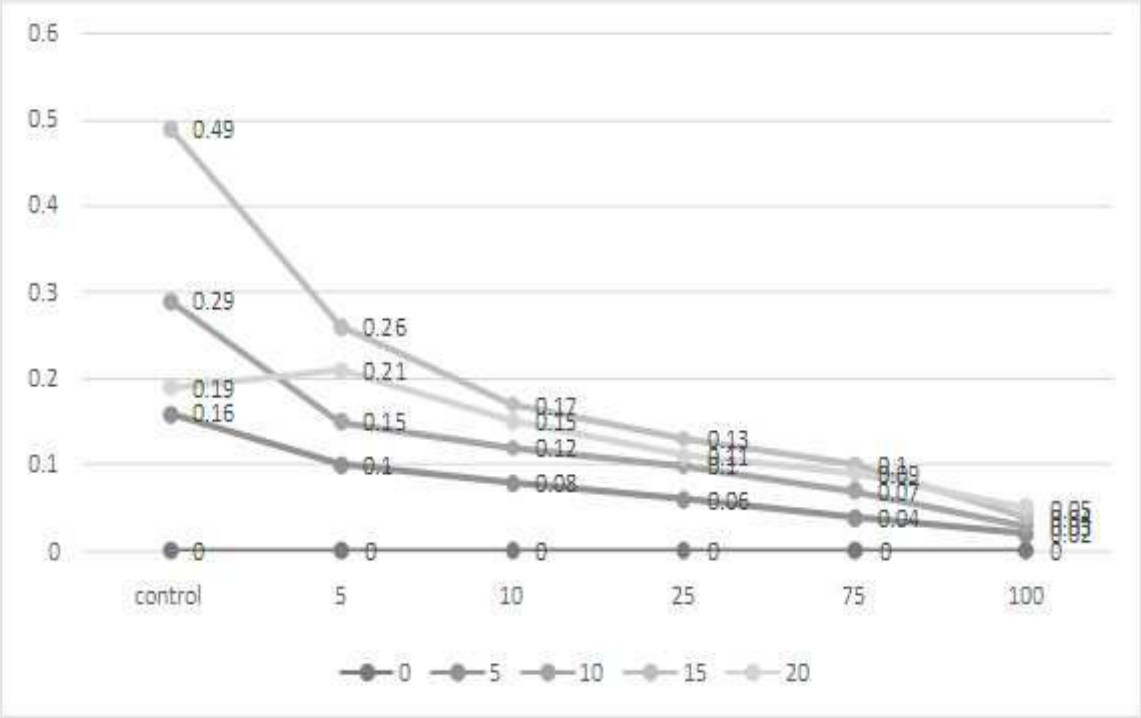


Figure 6
Optical density of E.coli grown in LB broth containing different concentration of Beauveria bassiana metabolites

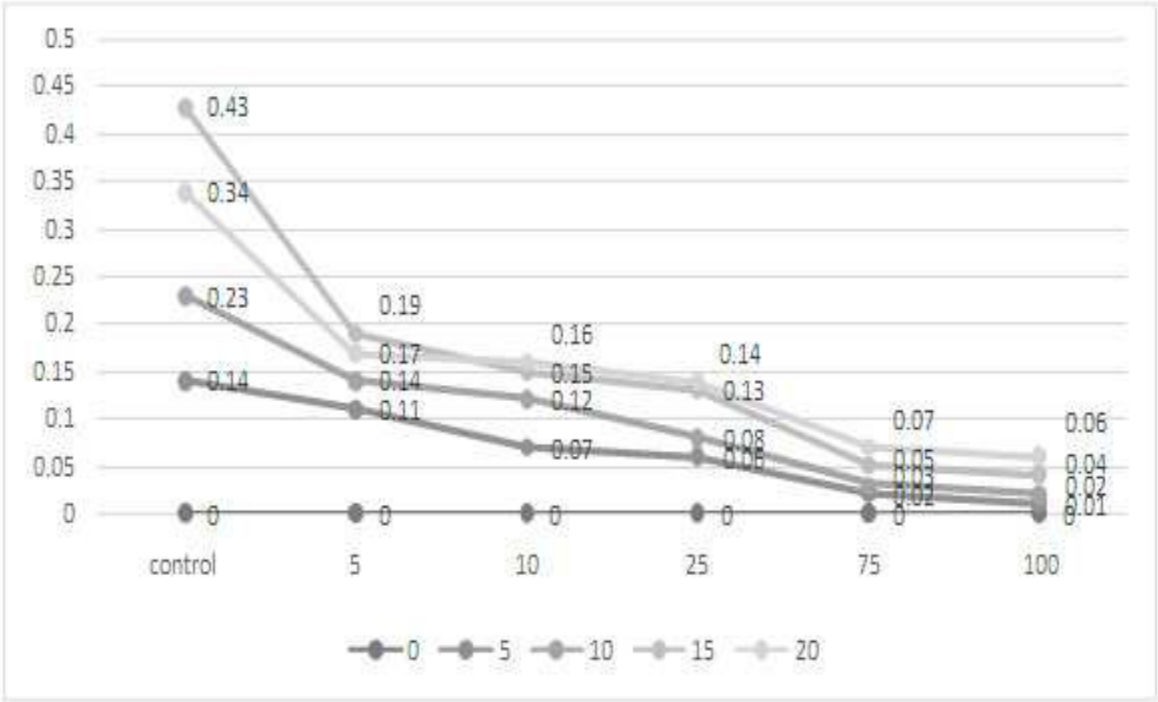


Figure 7
Optical density of Enterococci grown in LB broth containing different concentration of Beauveria bassiana metabolites

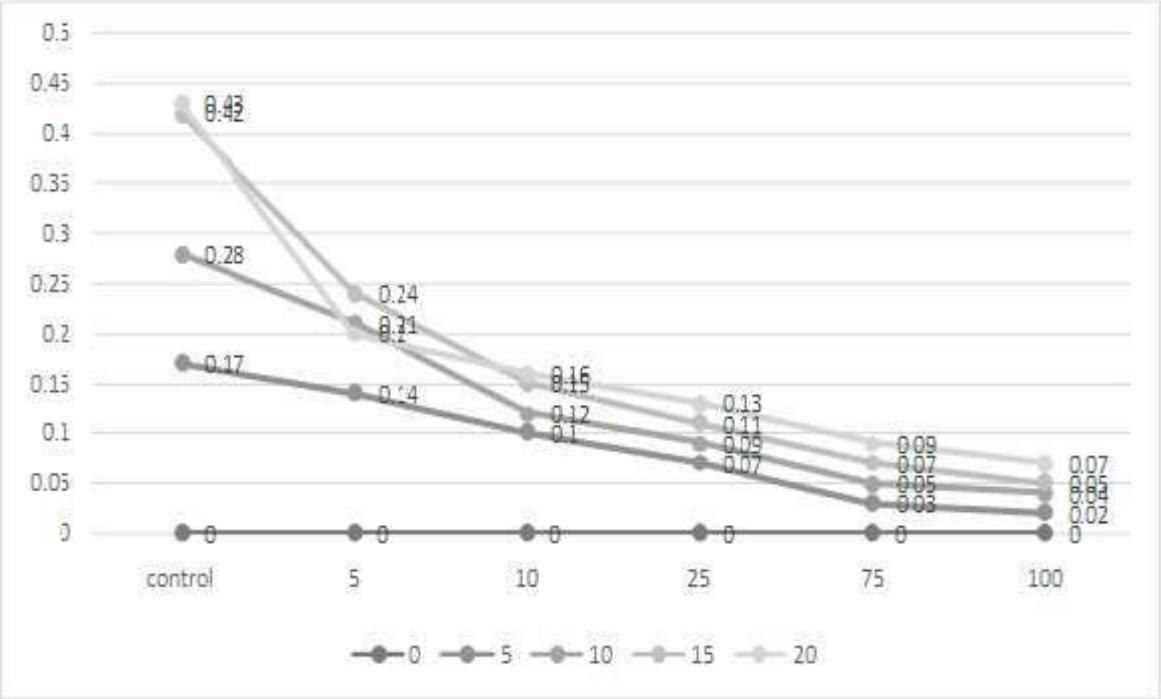


Figure 8
Optical density of Salmonella typhi grown in LB broth containing different concentration of Beauveria bassiana metabolites

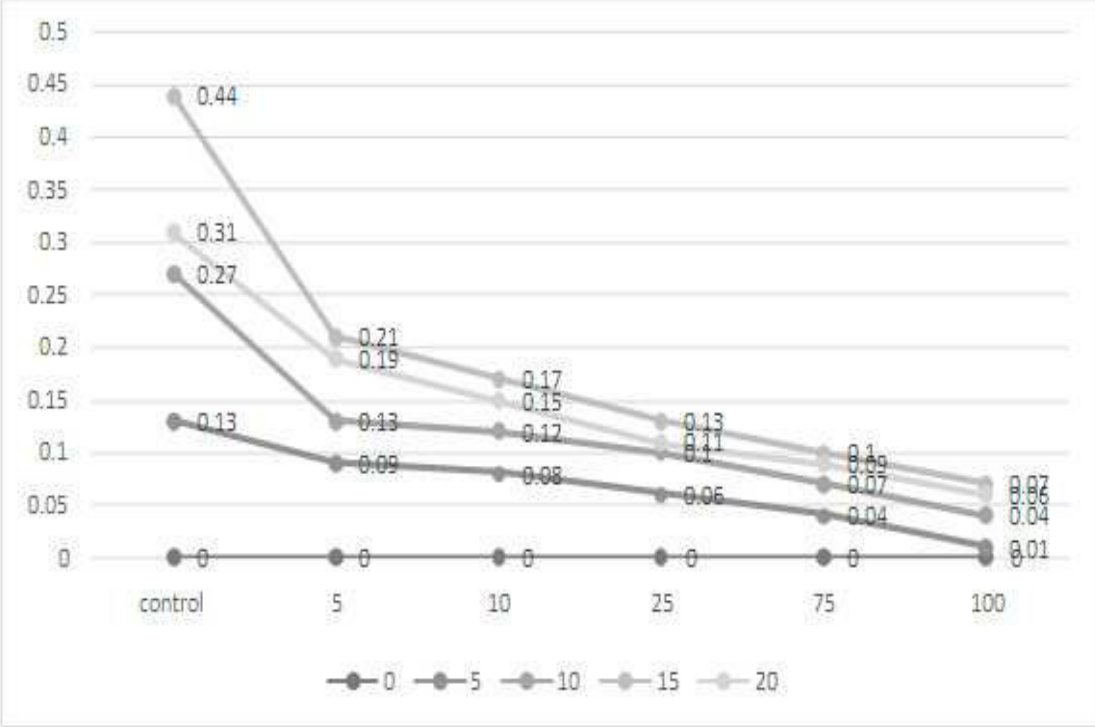


Figure 9
Cell viability (%) of Sf -21 cells treated with Nomuraea rileyi metabolite

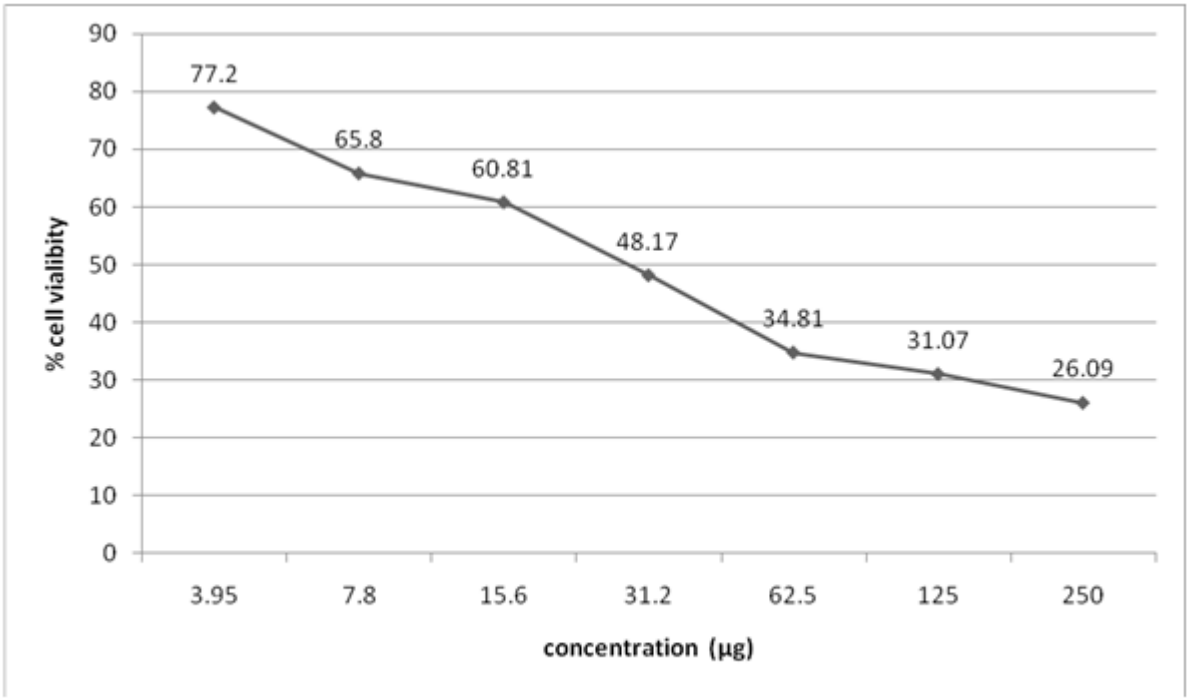


Figure 10
Microscopic examination of Sf-21 cell line a) control b) Nomuraea rileyi metabolite

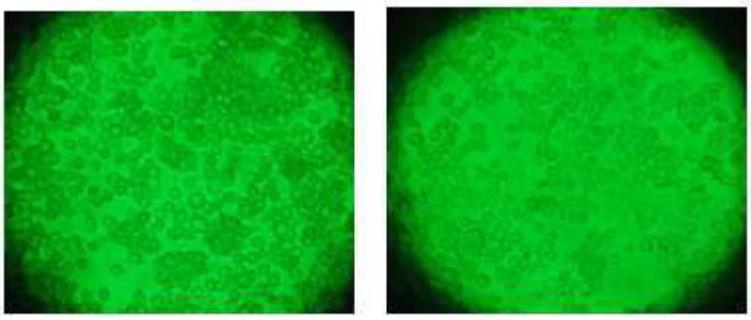


Figure 11
Cell viability (%) of Sf -21 cells treated with Beauveria bassiana metabolite

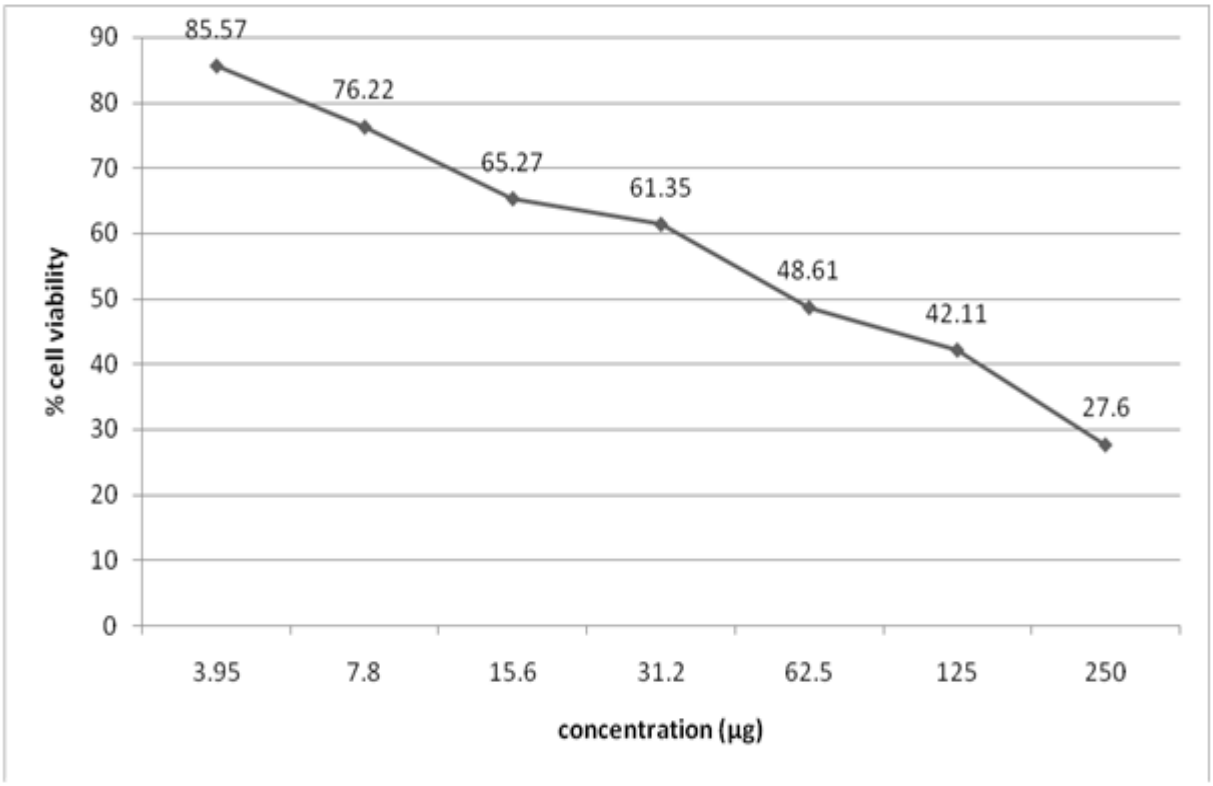
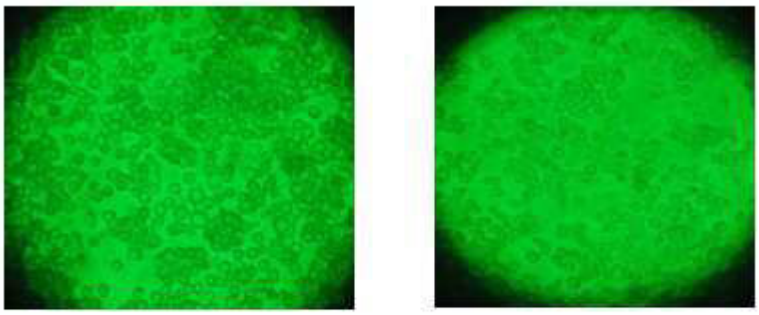


Figure 12
Microscopic examination of Sf-21 cell line a) control b) Beauveria bassiana metabolite



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