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Screening for Antiviral, Cytotoxic and Anti-Inflammatory Activity of Crude Extract from Entomopathogenic Fungal Strain of *Beauveria bassiana*

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ABSTRACT

Entomopathogenic fungus *Beauveria bassiana* is widely used as the microbial pesticide (biological control agent) in agriculture and many studies are reported that Beauveria also possesses active secondary metabolites which are medicinally significant. The present study is to assay the antiviral, anticancer and anti-inflammatory activity of metabolites from *B. bassiana* local isolate that is extracted in ethyl acetate solvent. Antiviral activity was measured by using Hepatitis B antigen binding inhibition assay. Anticancer activity was measured against A-549 cell lines by using SRB method. The anti-inflammatory activity of *Beauveria bassiana* was studied by using HRB membrane stabilization test. In the present study, it is observed that ethyl acetate extract from *B. bassiana* local isolate has antiproliferative, antiviral and anti-inflammatory activity. The research needs to be further continued in the determination of the structure of metabolite(s) may be used for developing new compounds exhibiting medicinal activity.

INTRODUCTION

Fungi are one of the most diversified groups of the microorganisms and having the very prominent role across the fields of biology. Fungi produce various metabolites having significant pharmacological activities like antivirals, anticancer and anti-inflammatory and other. Fungal metabolites those are considered for their antiviral activity due to inhibiting deadly virus-like simplex, HIV, influenza and other human pathogenic viruses those are isolated from *Aspergillus, Penicillium, Fusarium, Cladosporium* and other fungi [1,2]. Antiviral activity processing compounds starchyflin and acetylstachyflin are derived from fungus *Stachybotrys* are effective against Influenza A virus (H1N1) and other deadly viruses. Viral diseases are afflicted for the patients due to drug resistance and cellular toxicity [3, 4]. Synthetic antiviral drugs are developed by mimicking the natural antiviral compounds like laninamivir, zanamivir, etc. Resistance to antiviral drugs is a major constraint and there is a need to research for new compounds with antiviral or viral inhibition [5].Fungal metabolites have diversified structures exerts various anti-cancer activities like apoptotic, antiangiogenic, antiproliferative by activating different metabolic pathways. Fungal metabolites are having promising activity in controlling and preventing cancers isolated from fungi which are pathogenic, nonpathogenic and toxigenic with significant anti-tumor activities. Compounds like cytochalasins isolated and discovered from phytopathogenic fungi are processing distinct biological activities. There are more than 60 types of cytochalasins discovered till now where some of these metabolites are affecting cell division in cancer tissues by capping actin filaments and also inhibiting cancer migration. Other compounds from fungi like cotylenin from *Fusicoccum* sps, alternethanoxins from *Alternaria* sps, phyllostictines from *Phyllosticta* sps, pyrones from *Phomopsis* sps, Sphaeropsidins from *Diploidia*, bisorbicillinoids from *Trichoderma* sps, sesquiterpene from *Neosartorya* sps, tryprostatins isolated from *Aspergillus* sps, Pintulin discovered from *Penicillium* sps, fusarisetin A from *Fusarium* sps and many other metabolites are proven for having anticancer activity in vitro[6]. Fungi metabolites are having an evidential role on human immunity modulations that includes inflammations at surfaces of the mucosa by repairing epithelial barrier leakages and also acting against pro-inflammatory stimulus which is having the association with endotoxemia which was a physiological process that associated with chronic inflammation of gastrointestinal epithelial cells hyper permeability. Butyrates from fungi can induce fatty acid production along with antimicrobial peptides which are having anti-inflammatory activity and also that can act as a ligand for receptors of the immune system that influence pro-inflammatory metabolic

pathways and stimulate immunity cells like T cells and dendritic cells activity and also induce cytokines and interleukins production. Metabolites like glutamine, zinc carnosine, quercetin, occludin, cingulin can reduce inflammations by strengthening the integrity of the mucosa membrane and also repair the epithelial barrier and its hyper permeability [7, 8, 9]. Fungal metabolites are able to reduce the production of TNF (tumor necrosis factor alpha) which is a pro-inflammatory due to leakage of barriers by effecting integrity between epithelial cells. Sclerotinin A, hydroxyemodin, penicitrinone, citrine are the metabolites derived from *Penicillium* having inhibitory activity against production of nitric oxide and production of prostaglandin in microglia which causes inflammations. Metabolites produced from *Penicillium* are proved for the anti-inflammatory activity in many ways like suppressing the expression of the gene like cyclooxygenase-2, phosphorylation of kappa β-α inhibition, interruption of nuclear translocation of Kappa β nuclear factor [10, 11]. Microbial metabolites suppress inflammations by acting indirectly like inducing production of small peptides like defensins β-2, AP-1, bacteriocins that act as barriers in the first line of defense at mucosa and also in controlling acute inflammation responses and restoring homeostasis for the inflamed tissue[12].

Entomopathogenic fungal based biological control agents like *Pacilomyces*, *Metarhizium*, and *Beauveria* is known to produce toxic metabolites. Among these metabolites, few have been found to show fungicidal, antibiotic or insecticidal activities against diseases and insect pests and some of the entomopathogenic fungal cultures are known to produce metabolites like brassinolide, cyclosporine A, beauverolides, beauvericin and oosporein[13]. Antifungal and antibacterial properties were exhibited by some of the secondary metabolites derived from *B. bassiana* against the pathogens [14].

Based on the research findings available in literature the current research is aimed to evaluate antiviral, cytotoxic and anti-inflammatory effects of *B. bassiana* culture mycelial extract using organic solvents.

MATERIALS AND METHODS

Isolation and culturing of *Beauveria bassiana* (Bb2)

B. bassiana (Bb2) was isolated from local field infected *Helicoverpa armigera* and cultured on Sabouraud's dextrose broth (SDB) media supplemented with yeast extract (1%) and

incubated at 25ºC temperature for 10 days in shaking incubator. After incubation, mycelium was separated using Whatman No.1 fitter paper under aseptic conditions, rinsed with sterile double distilled water and mycelium was dried at 45ºC for 48 hrs in hot air oven.

Solvent extraction of *B. bassiana* mycelia

A hundred grams of *B. bassiana* dried mycelia was homogenized and extracted with 1000ml of ethyl acetate solvent using soxhlet extractor. Then the extract obtained (830ml) was concentrated at 40ºC and dried using rotavaporator (Buchi R-300) apparatus and concentrated extract (7.42 gm) was stored in the deep freezer at -20ºC[15].

Preparation of Hela and A-549 cell suspension

A subculture of Hela cells in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized separately, after discarding the culture medium. To the disaggregated cells in the flask, 25 mL of DMEM with 10% fetal calf serum (FCS) was added. The cells were then suspended in the medium by a gentle passage with the pipette and the cells homogenized.

Seeding of Hela cells

One mL (3 x 10^5 cells /ml) of the homogenized cell suspension was added to each well of a 24 well culture plate along with different concentrations of Bb extract (0 to 10 µg/mL) dissolved in 0.01% DMSO was added to cells and incubated at 37ºC in a humidified CO_2 incubator with 5% CO_2. After 48 hrs incubation, the cells were observed under an inverted microscope. With 80% confluence of cells without any cytotoxic assay was further used for the anti-viral activity. Lamivudine at 1µg/ mL was used as the standard antiviral drug for reference and cells added with 10 µl of DMSO solution has used as the control.

Anti-HBV activity of sample Test -1: HBsAg binding inhibition assay

One of the major viral diseases of human liver was Hepatitis B (HBs) and it was tested using a model Virus for developing antiviral drugs. Standard Hepatitis B serological antigen was obtained from Axis laboratories Chennai and test were performed by adding of Bb2 ethyl acetate extract of different concentrations to 10 µg of HBsAg serum and incubated for 5 days at 37ºC after that ELISA was performed and plates are read for absorbance at 450 nm. Standard drug was used as reference and control added with the only 10µl concentration of

HBsAg that was present in the plasma was estimated and quantified by using ELISA and percent inhibition was calculated by using formula [16].

$$Percent\ inhibition = \frac{OD\ of\ control - OD\ of\ test\ at\ 450\ nm}{OD\ of\ control\ at\ 450\ nm}\ X\ 100$$

Cytotoxicity assay by using A-549 cell lines

The cytotoxicity of the extracts was determined by testing on human cell line lung carcinoma (A-549). Cell viability was measured by using SRB assay by using the colorimeter. Each well was added with 190µl of cells with 5 $\times 10^3$ in the microtitre plate (96-welled) and cultured for 18 h in drug-free media. Mycelium extracts were added to wells in aliquots of 10 µl for final concentration of 0- 1000µg/ml and after 48h of exposure, the cytotoxicity was measured by the SRB methodology (Peter *et al.*) [17] by using 50% TCA (cold) cells are fixed and incubated at 4°C for 1hr. After incubation plates are washed and dried. 0.4% SRB solution was prepared in 1% acetic acid and 100µl of prepared SRB solution was added to microtiter well and incubated at room temperature for 10 minutes. Unbound SRB was removed by washing with 1% acetic acid. After incubation, plates were air dried and added with tris buffer. Optical densities were read using automated spectrophotometric plate reader at a single wavelength of 560nm. The activity of the mycelium extracts was given in % GI (growth inhibition) for concentration tested (50µg/ml).Values could be as follows. The experiment was repeated in triplicates in time [18]. Percent growth inhibition more than 100 % was considered as cytotoxic by the compound against tested cells.

HRB membrane stabilization test

HRB membrane stabilization test was performed by the following method.10 mL of human whole blood (freshly collected) was centrifuged for 10 min at 3000 rpm and washed with an equal volume of saline water. RBC pellet was mixed in saline to obtain 10% of final volume. For the test to 1000µl of ethyl acetate, Bb2 mycelia extract and 1000µl of 10% RBC was mixed and a positive control was prepared by adding 100µl of the standard aspirin solution along with blank or negative control by adding 1000µl of saline in place of test solution. Tubes were incubated at 56ºC by using a water bath for 30 min after incubation tubes were cooled to room temperature by placing under running tap water. Tubes were then centrifuged at 2500 rpm for 5 min and OD of the supernatant was recorded at 560nm [19].

RESULTS AND DISCUSSION

HBsAg binding inhibition assay as antiviral assay

B. bassiana local isolate Bb 2 ethyl acetate extract has significant antiviral activity against tested virus Hepatitis B virus. Antiviral activity was increased by increasing concentration of the extract. Standard antiviral drug lamivudine at 1µg/ml has the potentiality of 85% inhibition and negative control did not show any activity. Highest viral inhibition was observed at a maximum concentration of 5µg/ml concentration. There was linearity in a dosage of compound and inhibition activity with maximum inhibition of 61% at 5µg/ml concentration of ethyl acetate extract of Bb2. Further concentrations above 5 µg/ml did not enhance inhibition activity of HBs. Antiviral activity of entomopathogenic fungus *B. bassiana* was reported by Shin et al.,[20] from the culture filtrates and it was later identified as beauvericin a toxic compound against insect pests and it's a cyclic depsi peptide inhibited HIV by inhibiting its integrase enzyme [20] Other studies by Dong observed that fungi *B. bassiana* are able to inhibit dengue virus by activating anti-dengue pathways like Toll and JAK-STAT in mosquitoes [21]

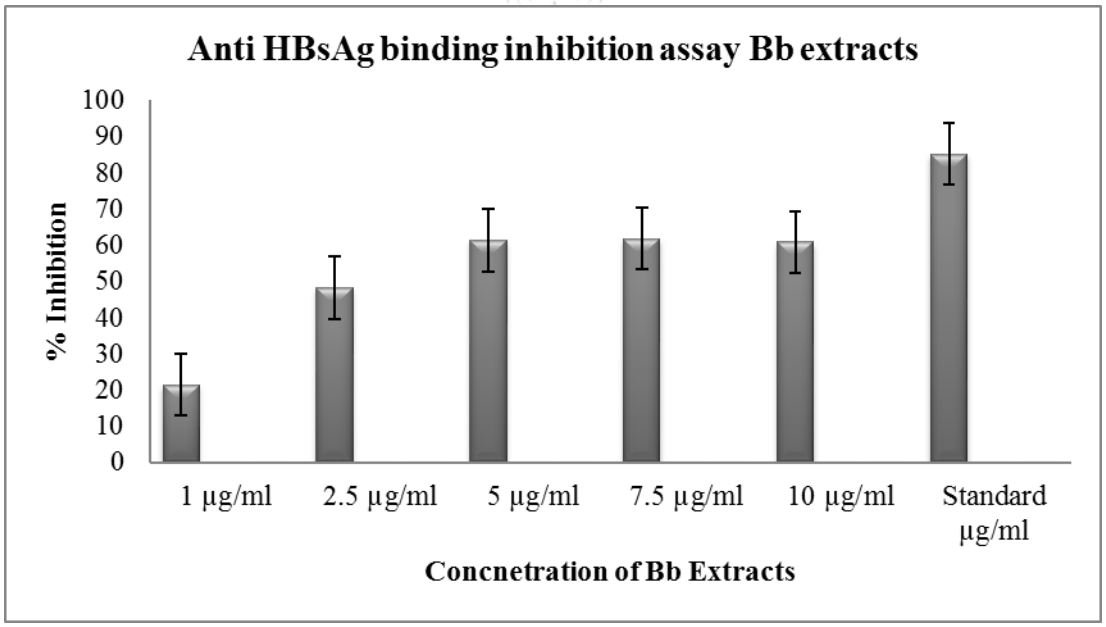

Figure 1: Antiviral activity of ethyl acetate extract of *Beauveria bassiana* local isolate against hepatitis B virus

Standard error (SE) is mean of triplicate

Cytotoxic activity

Cytotoxic activity was one of the key activities for the entomopathogenic fungi that affect the cells of the insect to make them parlayed. For this process, entomopathogenic fungi release many compounds that are toxic to the insect cells. As insect cells belong to eukaryotic there might be similar effect observed in human cells. Cytotoxic activity one of the key searches for the developing compounds against cancers. Ethyl acetate extract of *B. bassiana* showing the strong cytotoxic activity of 117% growth inhibition (GI) on lung carcinoma cell line (A-549), similar results were reported with *Beauveri feline* strains on HL-60 (leukemia), B16 (melanoma) and HCT8 (colon) cancer cell lines[22].

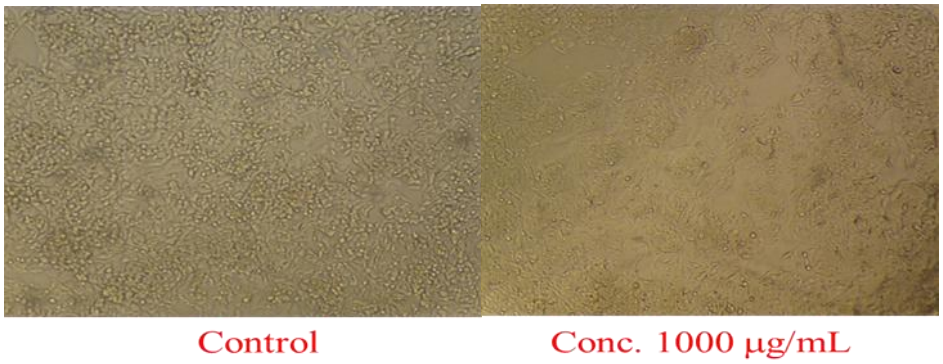

Figure 2: Cytotoxic activity on A- 549 Cell lines by *Beauveria bassiana* ethyl acetate-ethanol extract

HRB membrane stabilization test for anti-inflammatory activity

Anti-inflammatory activity of the Bb2 ethyl acetate extract by using membrane stability test was performed with the help of standard positive drug aspirin. Membrane lysis was a similar process to lysosome that releases various components which cause inflammations [23]. During inflammations that were caused by inflammatory substance lyse the lysosome releasing various enzymes and other compounds which cause inflammations. Similarly, when RBC was exposed to factors like heat, hypotonic medium, and chemical factors causes lysis of RBC membrane [24]. Hypotonicity inhibition and heat-induced membrane destabilization are the two factors that cause membrane lysis and compounds that prevent membrane damage by membrane stabilization assay in which preventing the membrane from lysis by the compounds can be used as anti-inflammatory compounds [25]. Membrane destabilization has adverse effects like leakage of serum proteins into tissues. *B. bassiana* metabolites are having

anti-inflammatory activity by stabilizing RBC membrane in the presence of lytic factors. Cyclodepsipeptide class anti-inflammatory compound hydroxamic acids from Beauveria felina that capable of inhibiting histone deacetylase are studied by Chung (2013) [26].

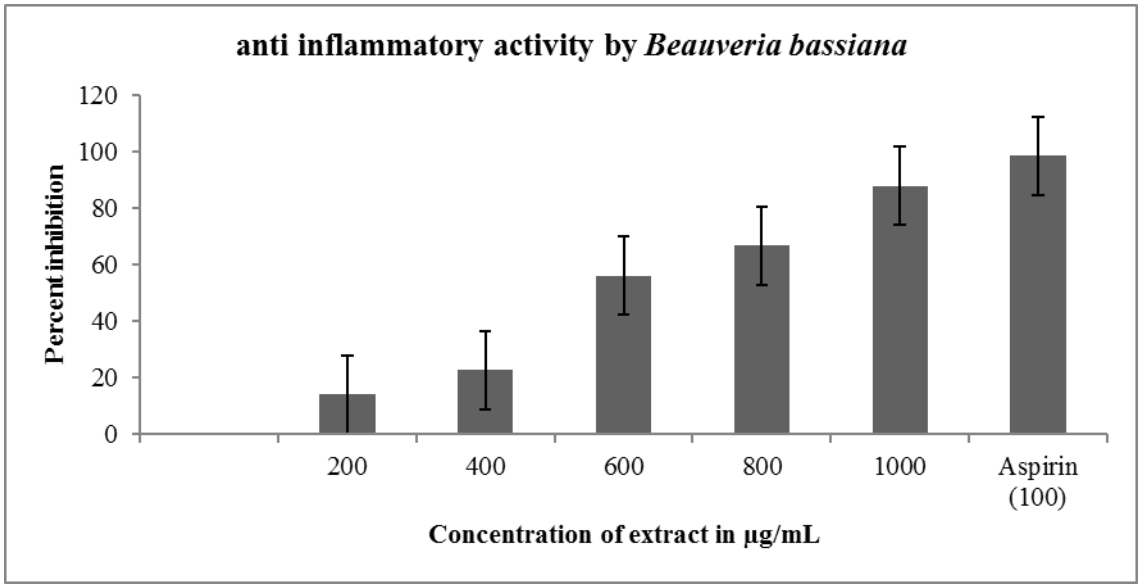

Figure 3: Anti-inflammatory activity of ethyl acetate extract of *Beauveria bassiana*

Standard error (SE) is mean of triplicate

CONCLUSION

Bb2 ethyl acetate extract contains metabolites with antiviral, cytotoxic and anti-inflammatory activity and further screening these compounds can develop new anti-viral activities, cytotoxic and anti-inflammatory drugs.

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