BIOEFFICACY OF CRUDE EXTRACT FROM ASPERGILLUS SP. ISOLATED FROM SOIL

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ABSTRACT

Secondary metabolites are particularly produced from plants, fungi and actinomycetes, where as yeasts and animals produce them to a lesser extent. Fungi are well known as prolific sources of distinctive biologically active secondary metabolites. In order to find out the efficacy of secondary metabolites, the crude extract of metabolite is extracted from the fungi. The soil sample is collected and screened for the fungal colonies by using Rose Bengal chloramphenicol media which is specific for the growth of fungi and inhibits the growth of bacteria. Among the colonies obtained, pure culture was made to grow on slants with Potato Dextrose Agar Medium. The structure of fungi was identified by SEM analysis. The fungi was mass cultivated in Potato Dextrose Broth and incubated for 15 days for the release of secondary metabolite. The secondary metabolite was extracted by ethyl acetate and concentrated using Rota vapor. The crude extract obtained was examined for various biological assays. Antimicrobial activity was tested against various bacteria and fungi. Extract was tested for antioxidant scavenging activity. Cytotoxicity was tested upon Brine Shrimps. Larvicidal activity was done to find out the efficacy against mosquito larvae. Then the metabolite was fractionated by using thin layer chromatography.

Key words: Bio-efficacy, Rota vapor, Extraction, Cytotoxicity, Thin Layer Chromatography.

I. INTRODUCTION

The term secondary metabolism, first used by plant physiologists more than 50 years ago, comprises all parts of metabolism specific for certain organisms. In general, the term secondary metabolites are assigned to low molecular weight compounds produced by living organisms that apparently lack life-sustaining functions. Instead, they are assumed to contribute to the producing organism's survival in the ecosystem. Metabolites are naturally produced by organisms as a means of defense and hence survival.

A. Secondary metabolites

Secondary metabolite is a chemical compound produced by a limited number of fungal species in a genus, an order, or phylum. They are biologically produced chemical compounds though not directly involved in the normal growth, development, or reproduction of an organism, but the organisms do not cause death if they are absent. Secondary metabolites are used as medicines, flavorings, and recreational drugs, generally produced following active growth, and many have an unusual chemical structure.

Bio-efficacy is the ability of a drug to control or cure an illness. It should be distinguished from activity, which is limited to a drug's immediate effect on the microbe triggering the disease. Hence it has a role more than an activity as the term bio-efficacy refers for a longer time situation. Through natural selection, organisms that fall prey to antibiotics begin to develop a resistance, and the effects of the antibiotics diminish over time until the organisms are immune to its effect.

B. Characteristics of secondary metabolites

- Structurally diverse, produced in mixtures with other members of the same chemical family, and usually are formed at low specific growth rates.
- Produced as members of a particular chemical family because of the low specificity of enzymes involved in secondary metabolism. They include mycotoxins, antibiotics, pigments and pheromones.
- Compartmentalization of enzymes, precursors, intermediates and products involved in biosynthesis, storage and breakdown.

II. NEED FOR THE PRESENT INVESTIGATION

Although there are ample of antibiotics existing in the nature, new discoveries are being made to find out cure for the new mysterious diseases arising day by day. This is due to increasing resistance of bacteria and fungi, the high mortality of some common bacterial diseases, the problems of viral infections. So the need for new agents becomes the target of research in today's world to treat such situations. Hence new metabolites are being tested against different microbes and insects to find their efficacy against them.

Considering the importance and applications of fungal metabolites, the present work was aimed at:

- Isolation of Fungi from the soil.
- Identification of fungi.
- Production of metabolite by Batch Fermentation.
- Extraction of secondary metabolite from fungi.
- Biological activities (Antimicrobial, Larvicidal, Cytotoxicity, Antioxidant activity) of Crude extract.
- Separation of compounds using TLC.

III. MATERIALS AND METHODS

A. Soil sample Collection

Soil sample was collected from Srisailam situated in Nallamala hills of Kurnool district, Andhra Pradesh.

B. Test organisms

Bacteria used

Bacillus subtilis, Staphylococcus aureus, Staphylococcus epidermis, Escherichia coli, Pseudomonas aeroginosa, Klebsiella pneumonia.

Fungi used

Candida albicans, Candida rugosa, Saccharomyces cerevisiae, Aspergillus niger, Aspergillus flavus.

Test organisms (bacteria, fungi) obtained from the Institute of Microbial Technology, Chandigarh, India.

Larvae used for larvicidal test

Culex 3rd instar larvae.

Larvae used for Cytotoxicity

Brine Shrimp larvae.

C. Isolation of Soil Fungi using Soil Dilution Method

A wide variety of microorganisms can be isolated from the soil environment and cultivated on media in the laboratory. Different media will encourage the growth of different types of microbes through the use of inhibitors and specialized growth substrates. We will use several different media to isolate microbes from soil. For fungi, Rose Bengal media is used as its composition encourages growth of fungi only. Weigh about 2 g of soil sample in a 500ml conical flask containing 200 ml sterile distilled water. Keep the conical flask on orbitary shaker for 30 min at 200 rpm. Prepare about 250 ml Rose Bengal medium and sterilize it at 121°C 15lbs pressure. After cooling down pour it into Petri plates leave it for some time for solidification. The soil solution (100 µl) from the conical flask was spread it over the Petri plates with Rose Bengal medium. Incubate the plates at 25°C – 27°C for 24-72 hrs. After incubation, check for the individual colonies and subculture unique and isolated colonies onto the potato dextrose agar slants.

Preparation of Rose Bengal Media

Weigh 32gm of Rose Bengal medium into a conical flask of 1000 ml of distilled Water.

D. Preparation of Sample for SEM analysis

The scanning electron microscope (SEM) is a type of electron microscope that images the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern. The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties such as electrical conductivity. The types of signals produced by an SEM include secondary electrons, back-scattered electrons, characteristic X-rays, specimen current and transmitted electrons. SEM can produce very high-resolution images of a sample surface, revealing details about less than 1 to 5 nm in size.

Method

- Fix the samples in 40% Glutaraldehyde in phosphate buffer (pH -6.9, 0.02M).
- Wash thoroughly with distilled water twice.

- Pass through with alcohol series (2 times each) 10 %, 20 %, 30 %, 50 %, 70 %, 90 %, 100 % two times each for 30 minutes after each step.

E. Extraction of Secondary Metabolites

After incubation ethyl acetate was added to the culture flasks and kept for 5 hr. Minimal shaking is required for the dissolving of metabolites into ethyl acetate solvent.

Fig 1: Culturing of Fungi in PDB broth

Separation of Metabolites

The metabolites which are now dissolved in ethyl acetate solvent are separated by using separating funnels. In the separating funnel, add the media with ethyl acetate. To that add some amount of ethyl acetate, shake well and allow it to settle for a few minutes. Two layers were observed. The bottom layer is discarded and the upper layers of ethyl acetate with metabolites are collected which is the organic layer. The washes were repeated for 3 times to extract the complete metabolites. The separated extract was concentrated for the collection of crude extract.

Fig 2: Separation of metabolite extract from the broth

F. Antibacterial Activity

The antibacterial activity of the crude extract was studied against the 6 bacterial strains by agar cup diffusion method. The plates were kept at 37°C for 48 h. Inhibition zones were measured and the diameter was calculated in millimeters.

G. Antifungal activity

The in-vitro antifungal activity of the crude extract was studied against the 5 fungal strains by agar cup diffusion method. The protocol used for the antibacterial activity was same except the media used Potato Dextrose agar instead of nutrient agar. The treated and the controls were kept at 27°C for 48 h. Inhibition zones were measured and the diameter was calculated in millimeter.

H. Larvicidal Activity

The crude extract was dissolved Acetone and concentrations of 1000 ppm, 100 ppm, 10 ppm were prepared. One ml of each of 3 concentrations of the extract prepared in acetone was added to plastic disposable cups containing 50 ml of water. Control was made with the same volume of solvent without the addition of compound. They were kept at room temperature to evaporate the solvent. Then 10 larvae were added to each cup. The cups were placed under the illumination at room temperature and after 24 hours the number of survivors was counted and LD 50 value were calculated.

I. Cytotoxicity Assay

Brine shrimp eggs were hatched in artificial salt water at constant illumination and air supply.The shrimps were hatched and active shrimps were

collected and used for the experimental bioassay. The crude extract was dissolved in DMSO and concentrations of 1000 ppm, 100 ppm, 10 ppm were prepared.10 ml of water was taken in plastic disposable cups and 0.5 ml of the compound was added. 10 active shrimps were added to the each cup and were incubated for 24 hours. The survived shrimps were counted after 24 h and percentage of the mortality was assessed.

J. Antioxidant assay: (DPPH Scavenging Activity)

1 mg extract powder was dissolved in 1 ml of 50% ethanol solution to obtain 1000 µg/ml sample solution. Amounts tested were 20 µg, 40 µg, 60 µg, 80 µg and 100 µg. In each reaction, the solutions were mixed with 1 ml of 0.1 mM 1,1-Diphenyl-2 -picrylhydrazyl (DPPH), 0.45 ml of 50 Mm Tris-HCl buffer (pH 7.4), and 0.05 ml samples for 30 min. The reduction of the DPPH free radical was measured by reading the absorbance at 517nm. DPPH, a purple-colored, stable free radical is reduced to the yellow-colored diphenyl-picrylhydrazine when antioxidants are added. L-ascorbic acid was used as positive control. The inhibition ratio (percent) was calculated from the following equation:

% inhibition = [(absorbance of control – absorbance of test sample)/absorbance of control] × 100%.

K. Purification by TLC

A method for the separation and purification of fungal secondary metabolites was developed. Pre-coated silica gel chromate-plates were used for purification of the compounds. The metabolite was spot at several places on a line drawn 1cm from the bottom. The Solvent system consisted of ethyl acetate / hexane (40:60). The fractions were collected in separate vials and labeled. Thus obtained fractions were checked for their purity by spotting them again on a small TLC.

IV. RESULTS AND DISCUSSIONS

A. Isolation of Fungi

Soil sample collected was serially diluted and was inoculated onto the Chloramphenicol Rose Bengal agar media and incubated at 27°C. After 3 days, the growth of various fungal species was observed. Dark green colony was isolated and sub cultured.

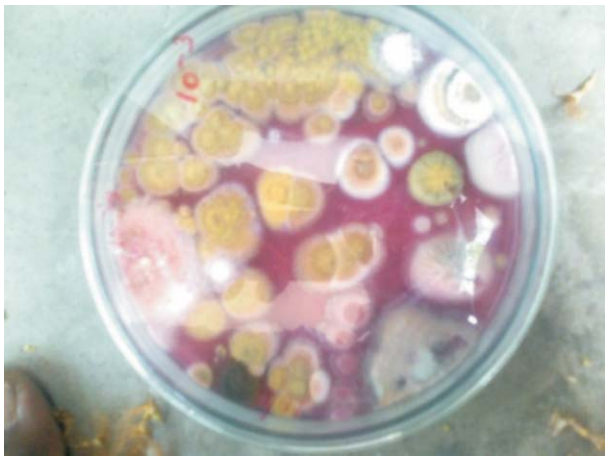

Fig 3: Isolation of Fungi from the soil

B. Identification of Fungi by SEM Analysis

Aspergillus sp.,

The culture from dark green colony of fungi was observed by Scanning Electron Microscopy (SEM) to identify the genus of the fungi. Long undifferentiated hyphae were observed in the SEM image. Spherical molds on the top layer resembled the conidiospores.

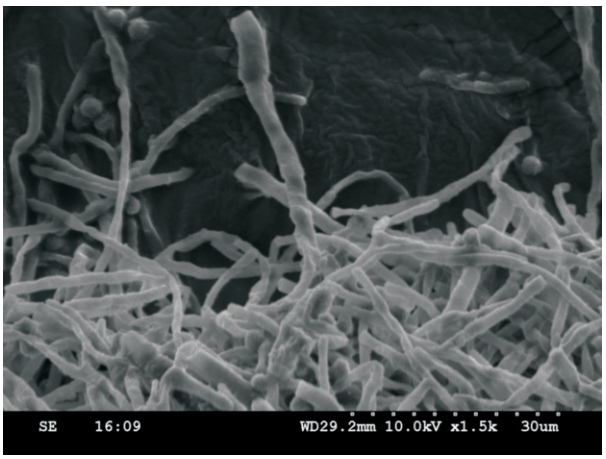

Fig 4: SEM image of *Aspergillus sp.,*

These spores are asexual, non-motile spores of fungi. These are formed in asexual reproductive stage in Ascomycetes classification of fungi. *Aspergillus* species are capable of growing in nutrient-depleted environments and in this stage, they produce conidiospores. The fungal culture was grown till the nutrients were depleted and then analyzed. Thus the fungi were identified as Aspergillus species.

C. Amount of crude metabolite extracted

Table 1: Quantity of crude metabolite extract

Volume of the media inoculated (lts)	Amount of compound obtained (mg)
3 lts	450 mg

The weight was measured after complete drying the ethyl acetate in a glass vial.

Table 2: Anti-Bacterial Activity – Zones of inhibition

Conc (ul)	*B. subtilis*	*S. aureus*	*S. epi-dermidis*	*E. coli*	*P.Aeuro-ginosa*	*K aero-genes*
10	14	14	13	13	13	10
20	16	15	13	13	16	10
30	16	17	15	14	16	10
40	17	17	16	15	19	10
50	18	18	17	16	16	11
60	20	20	18	19	19	11

D. Antibacterial activity

The crude extract isolated, was active against both gram positive and gram negative bacteria. When compared, the compound is showed similar activity on *Bacillus subtilis* and *Staphylococcus aureus* and more activity on these bacteria than others.

E. Larvicidal activity

The crude extract of *Aspergillus species* had no activity against the 3rd instar culex larvae of mosquito.

F. Cytotoxic Assay

The crude extract was tested for cytotoxic bioassay against the brine shrimp larvae. The brine shrimp lethality assay is considered a useful tool for 'preliminary assessment of toxicity and to know the antitumor activity of the compound. We compared the dead larvae in each treatment to the dead larvae in the control. A lethal effect of 100, 80 and 20% against brine shrimp (*Artemia salina* Leach) was exhibited by crude extract at 1000 -10 ppm concentrations.

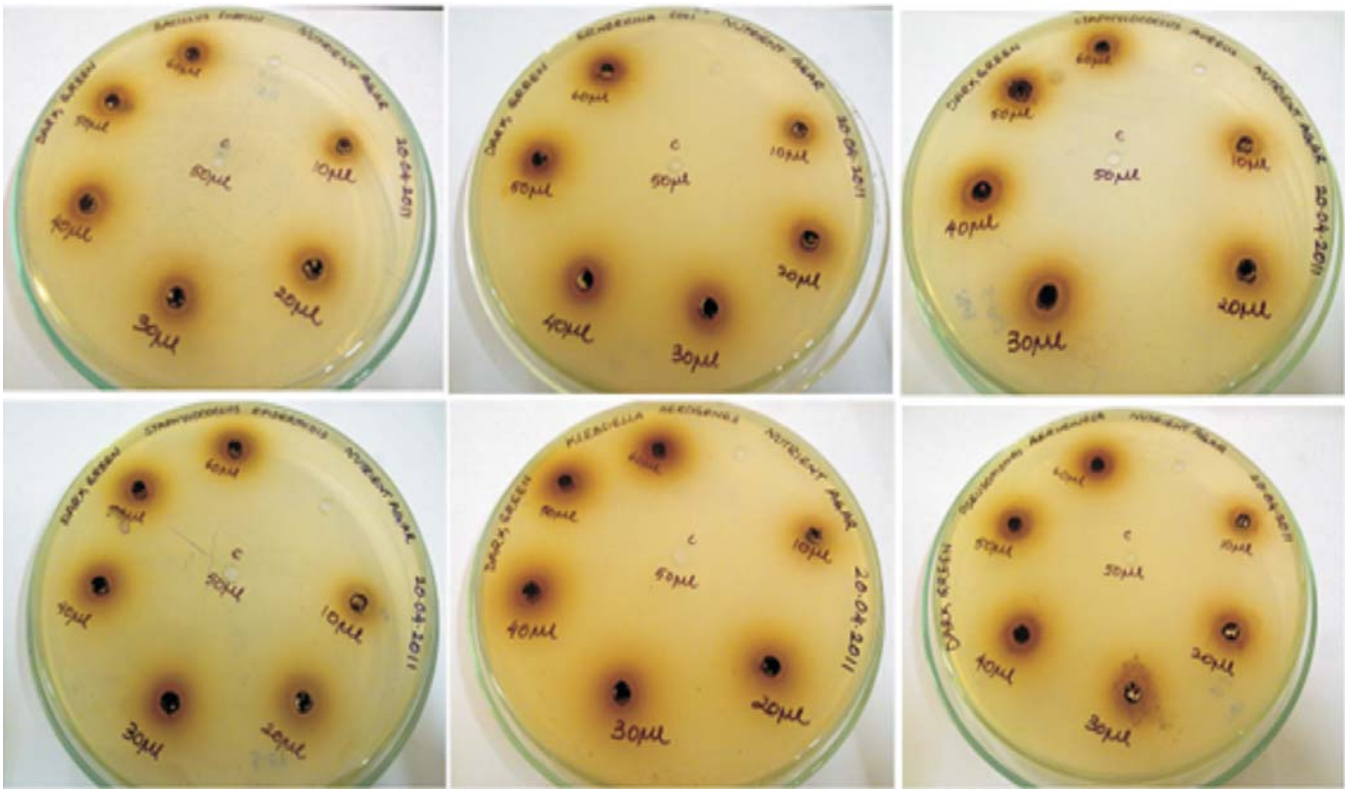

Fig 5: Antibacterial assay – Plates with zones of inhibition

Table 3: Cytotoxicity Activity of Aspergillus Sp.,

Conc (ppm)	No of larvae tested	No of larvae died	% of mortality
1000	10	10	100%
100	10	8	80%
10	10	2	20%

G. Antifungal activity

The antifungal activity of crude extract on fungi like *Candida rugosa, Candida albicans, Aspergillus niger, Aspergillus flavus* and *Saccharomyces* cerevisiae was found to be ineffective. No activity was found *against 5 types of fungi.*

H. Antioxidant Assay

The crude compound was tested for the antioxidant activity by DPPH assay. The compound was characterized on the basis of their spectroscopic data. The compounds were tested for their radical-scavenging and antioxidant activities by measuring their capacity to scavenge DPPH. Fungal extract *has* shown the significant antioxidant activity.

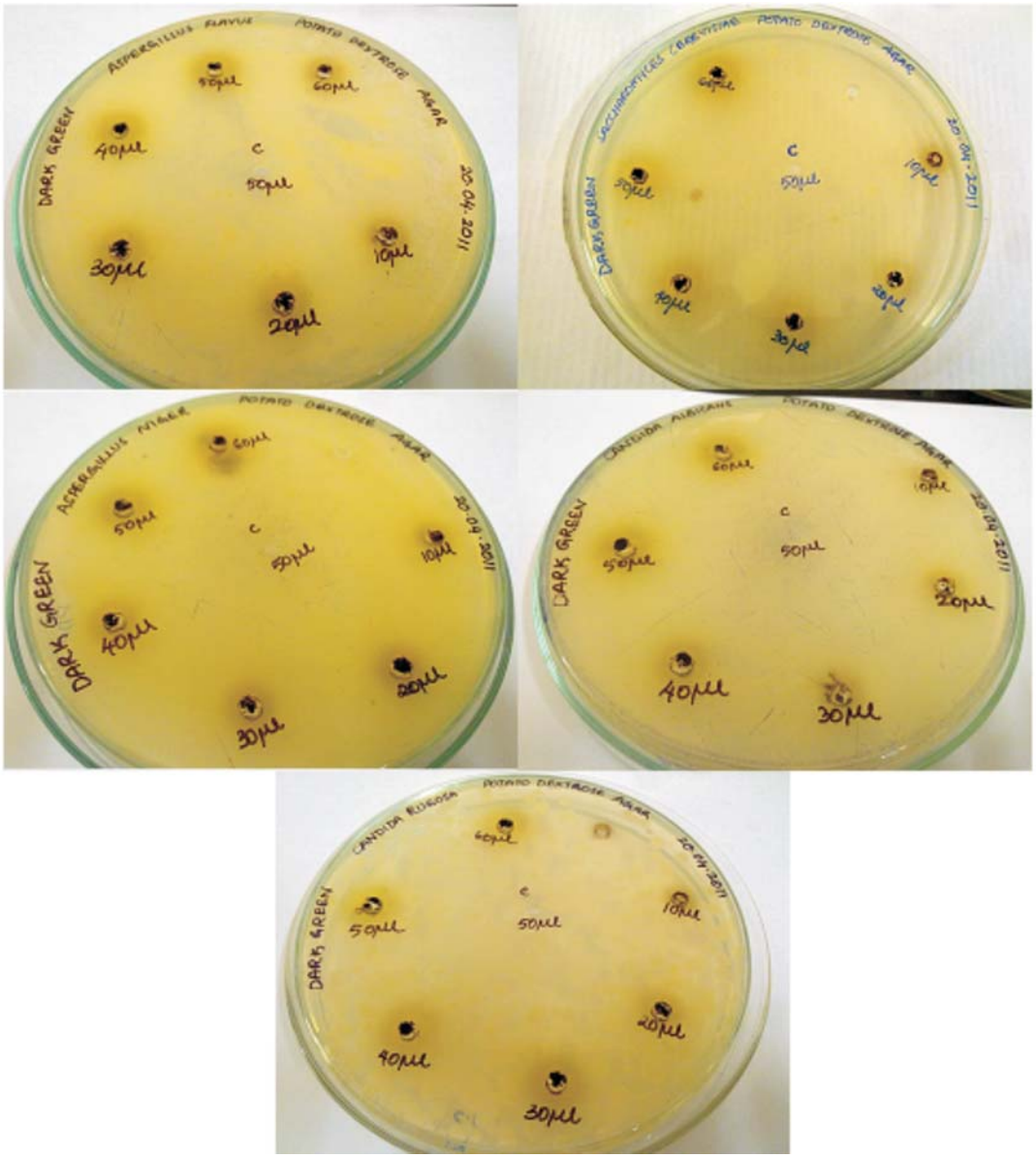

Fig 6: Antifungal assay – Plates with no zones of inhibition

Table 4: Scavenging activity of metabolite extract

15 min	20 ug	40 ug	60 ug	80 ug
Dark Green	85.16	86.09	86.65	86.93
Rutin	85.57	86.09	-------	--------

I. Thin Layer Chromatography of the crude extract

Crude extract of the *Aspergillus Sp.,* was spotted on the silica gel coated TLC plate and run in 40% solvent. The solvents used were Hexane and ethyl acetate. 4 fractions were observed in crude extract with the 40% solvent after stained with Iodine vapors.

Fig 7: TLC plate with 4 fractions in crude extract

V. CONCLUSION

Biological control methods aimed to improve the resistance of the host or favoring microorganisms to antagonist the pathogen, such as bacteria and fungi. Control of pathogens by biological means was environmentally advantageous in comparison to chemical methods which had many risks to human health and environment. Natural products have been a stepping-stone in fundamental and applied researches in bioscience and life science. Fungi were isolated from the soil by soil dilution method. The diluted soil sample was spread onto the selective medium. After the incubation of 3-5 days, the unique isolated colonies were picked and inoculated into a freshly prepared potato dextrose agar plates. The identification of fungi was done by Scanning electron microscopy based on the Morphology of the spores. The fungi were identified as *Aspergillus* species. *Aspergillus* species are among the most ubiquitously found fungi throughout the world of high importance in medicine, agriculture and biotechnology. The fungus was used for batch liquid fermentation. The crude extract was obtained by liquid-liquid extraction was concentrated using Rota vapor. The ability to control the microbial toxicity and cytotoxicity by the crude extract was tested by biological assays. The crude extract thus obtained was studied for anti-microbial activity using well diffusion method, larvicidal activity, antioxidant activity and Brine Shrimp cytotoxic assay. The crude extracts of *Aspergillus* species have shown moderate antibacterial activity and significant cytotoxicity and antioxidant activities but no effect was observed against the fungi and mosquito larvae. Then the crude metabolite was fractionated by using thin layer chromatography.

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