**In vitro Antifungal and Phytotoxic Study of Metabolites Extracted from Aspergillus flavus**

**Bashir Ahmad, Muhammad Rizwan, Sadiq Azam, Abdur Rauf, Shumaila Bashir and Jamshaid Ahmad**

**Center of Biotechnology and Microbiology, University of Peshawar, KPK-Pakistan**

**Department of Geology, University of Swabi, KPK-Pakistan**

**Department of Pharmacy, University of Peshawar, KPK-Pakistan**

**Abstract:** *Aspergillus flavus* is one of the most important plant as well as human pathogen. *A. flavus*, has gained worldwide attention for its industrial use and toxigenic potential. Besides their pathogenicity, *A. flavus* produces a wide range of secondary metabolites, used in food and pharmaceuticals industry. To determine the bioactivity of *A. flavus*, it was isolated from soil of Khyber Pukhtunkhwa, Pakistan. Crude metabolites were extracted through broth culture in bulky quantity under shaking condition. EtOAc and *n*-hexane fraction were checked for *in vitro* screening. Crude metabolites show a significant results against tested bacterial strains, insects and weeds. The crude metabolites showed a moderate effect against fungal pathogen, inhibiting only the growth of *Candida albicans* (80±1.414 % inhibition), while moderate active against *Penicillium notatum*, *Aspergillus fumigatus* and *Acronomion alternatum* (44.88±0.77, 66.66±1.4 and 53.33±0.0 % inhibition, respectively), while showed no activity against *Verticillium chlamydosporium* (0±0 mm). The *EtOAc* fraction was more active as compared to *n*-hexane fraction and at a higher concentration (1000 µg/mL) showed a significant activity (90%) against *L. minor*. At 100 µg/mL, a good activity was recorded (70%), while at a low concentration (10 µg/mL), a moderate activity (40%) was observed. The *n*-hexane fraction exhibited a moderate activity (40%) at a higher concentration (1000 µg/mL). Further it was evaluated that *EtOAc* was more active than *n*-hexane fraction. It was concluded that the *A. flavus* have a potential to produce bioactive secondary metabolites. Compounds of *A. flavus* should be exploited for the development of phytotoxic, insecticidal and antifungal compounds.

**Key words:** Secondary metabolites • Lamina minor • Insecticidal • EtOAc, *n*-hexane

**INTRODUCTION**

*Aspergillus flavus* is one of the most important fungal species in tropical environments. It can be found in soil and other medium. *Aspergillus flavus*, has attracted worldwide attention for its industrial use and toxigenic potential. *Aspergillus flavus* associated with many diseases of human, most severe of which is invasive *Aspergillus* is also causing diseases in insects [1] as well as in crops (such as rice, peanuts and maize etc.). Agricultural products including cereals e.g. maize, wheat, sorghum and by products thereof and variety of oilseeds are major constituents of poultry feed [2]. Some fungal species produced a special type of secondary metabolites known as mycotoxins harmful to animals or humans. Until now 16 structurally close aflatoxins have been discovered. Among these, aflatoxins B1, B2, G1 and G2 are the most important aflatoxin [3]. *A. flavus* only produces aflatoxins B1 and B2, [4]. Aflatoxin B1 is identified to be most toxic and potentially hepatocarcinogenic [5]. Agricultural products contaminated with toxigenic fungi like *A. flavus* producing mycotoxin are injurious to animals and human health. Identification and characterization of toxigenic fungi is necessary as Production of mycotoxins is species specific. [6] a large amount of economical crop and livestock losses occur every year, due to contamination of toxigenic fungi and mycotoxin. However, it should be remember that, not all strains of *A. flavus* produce aflatoxins; since several strains are non-toxigenic aflatoxin [7]. Two types of allergens (Asp fl 13 and Asp fl 18) have been detected in *A.flavus* [8]. Besides their pathogenicity, *A. flavus* produced a wide range of secondary metabolites, used in agricultural, pharmaceuticals and food industry. The present study was carried out to exploit the metabolites secreted by *A. flavus*. 
MATERIALS AND METHODS

Collection of Soil Samples: Soil samples were collected from different localities of Khyber Pakhtunkhwa, Pakistan in a sterilized polythene bags and transferred to Microbiology Lab II for further processing (Fig. 1). 

A. flavus was purified by serial dilution and sub culturing technique.

Extraction of Crude Metabolites: A. flavus was grown by using already optimized condition to produce secondary metabolites in potato dextrose broth (PDB) media under shaking incubation. After completion of incubation period, the crude metabolites were extracted by mixing with EtOAcby 1:1 ratio. Filtration was carried by Mucilage cloth. The same process were repeated three times. Crude secondary metabolites were concentrated by rotary evaporator at 45°C under vacuum. Further, the crude metabolites were fractionized in two fractions i.e. EtOAc and n-hexane fractions.

Antifungal Assay: The EtOAc and n-hexane fractions of crude secondary metabolites were screened against the above mentioned pathogenic fungi. Antifungal susceptibility testing was evaluated using the agar tube diffusion method to detect the antifungal potential of the metabolites [9]. In order to obtain fresh culture, the test fungi were inoculated in potato dextrose agar and incubated for 5 days at 28°C. For the preparation of slants, 5 mL of PDA was added to each test tube and autoclaved. Then, 66.6 µL of the test sample was added to each test tube and a small piece (5×5 mm) of 7 days old culture of test fungi was placed in the test tube. The test tubes were incubated at 28°C for seven days. Stock solutions (24 mg/ml) were prepared in DMSO (<1%). Miconazole and DMSO (≤1%) were used as positive and negative controls, respectively. The experiments were performed in triplicate. After seven days, the percent growth inhibition was calculated using the following formula:

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\text{Growth inhibition} = \frac{100 \times \text{No of fronds in sample}}{\text{No of fronds in control}}
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RESULTS AND DISCUSSION

Fungal secondary metabolites are known as a valuable source of new and biologically active compounds. Aspergillus spp produce a wide range of secondary metabolites, used in diverse fields. Genomic analysis predicted that Aspergillus spp have the capability to produce many more biological active secondary metabolites. Genomic analysis evaluated that there are 55 genes clusters associated with secondary metabolites were present in A. flavus. Fungi produces secondary metabolites which possess numerous pharmacological uses, in special as antibacterial and antifungal agents [12,13,14]. Secondary metabolites were more interesting because these are more effective and with less adverse effects. Production of antimicrobial metabolites by fungi is natural, since their survival in the natural habitats depends on their ability to stop the growth of other co-habitant microorganisms. The present study was carried out to determine the bioactivity of A. flavus isolated from soil of Khyber Pakhtunkhwa. Using optimized growth condition, crude metabolites were extracted and applied for different bioassays. The antifungal potential of the EtOAc and n-hexane fractions was determined against different pathogenic fungi as shown in Fig. 1. The EtOAc fraction showed significant activity against Candida spp. (80 ±1.414% inhibition), while moderate effect was exerted against P. notatum (44.8 ±0.77%), A. fumigatus (66.66 ±1.4%) and A. alternatum (53.33 ±0.0%). The EtOAc fraction was inactive against V. chlamydosporium.
Fungi produce several secondary metabolites that have numerous pharmacological uses, particularly as antibacterial and antifungal agents [14]. The search for new antimicrobial drugs is critically important since the incidence of fungal diseases has grown extremely in the last few years. The production of antifungal metabolites by fungi is a natural process, because their survival in the natural ecosystem depends on their capability to inhibit the growth of other co-habitant microorganisms. Several fungi from Aspergillus genus are known for their capability to produce various active secondary metabolites of pharmaceutical importance, of which echinocandin B has antifungal activity [15]. Aspergillus spp. also produces a remarkable metabolite called kojic acid, a compound currently used in the pharmaceutical industry for various purposes due to its wide range of biological activities, including antibacterial, antifungal, anti-inflammatory, antitumor and insecticidal effects [16].

*A. flavus* was also found to produce some bioactive secondary metabolites having antifungal properties [17]. Fungal metabolites are recognized as a valuable source of new biologically active compounds. In our study, the EtOAc and *n*-hexane fractions of secondary metabolites produced by *A. flavus* were applied for determination of their phytotoxic effect. The results are shown in Fig. 2. The EtOAc fraction was more active as compared to *n*-hexane fraction and at a higher concentration (1000 µg/mL) showed a significant activity (90%) against *L. minor*. At 100 µg/mL, a good activity was recorded (70%), while at a low concentration (10 µg/mL), a moderate activity (40%) was observed. The *n*-hexane fraction exhibited a moderate activity (40%) at a higher concentration (1000 µg/mL) *A. flavus* is a pathogenic fungus causing different types of diseases in humans and
plants. It was discovered that Aspergillus spp. produced cichorine, a secondary metabolite which has phytotoxic effects [18]. Phthalides, which are structurally the most diverse class, are secondary metabolites consisting of approximately 180 natural compounds. They are produced by a number of organisms, including Aspergillus spp. Phthalides show a broad spectrum of bioactivities, including phytotoxic activity [19,20,21].

REFERENCES