



RESEARCH PAPER

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Phytotoxic, cytotoxic and antimicrobial effect of the organic extract of *Aspergillus niger*

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Abstract

Biological screening of *Aspergillus niger* culture extract was carried out on antimicrobial, cytotoxic and phytotoxic activities with the aim to identify bioactive natural products in the extract as new drug candidates. The investigation was carried out using disc diffusion (antibacterial), agar diffusion (antifungal), brine shrimps lethality (cytotoxicity) and *Lemna minor* (herbicidal) bioassays. The crude extract subjected to the bioassays exhibited no antibacterial property at concentration up to 200 µg. mL⁻¹. The extract showed antifungal potential against tested fungal pathogens. Growth of *Fusarium oxysporum* and *Alternaria alternata* was inhibited up to 36% and 64% when compared to negative reference. The phytopathogen *A. alternata* was found highly sensitive to the antifungal effect of the extract among the tested fungal species. Potent activity in brine shrimp bioassays was shown by the extract and mortality up to 96.7% was observed at concentration of 200 µg. mL⁻¹ after 48 hrs. LC₅₀ of the extract against brine shrimp was calculated as 9.25 µg. mL⁻¹. Plant inhibitory effect was observed at all concentrations. Mortality up to 83% of *Lemna minor* frond with reference to negative control was observed at 200 µg. mL⁻¹. FI₅₀ against *Lemna minor* was determined as 15.4 µg. mL⁻¹. The observed high toxicity against *Lemna minor* and brine shrimps larvae make the fungal extract worthy of further exploration for isolation and structural characterization of bioactive compounds.

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Introduction

Fungi survive in a very competitive environment with other organisms such as bacteria, other fungi, algae, protozoans and small metazoans (Demain *et al.*, 2000). For successful competition they possess a complex mechanism of biosynthesizing secondary metabolites that kill or inhibit growth of other organisms (Praveena *et al.*, 2011). An array of these fungal metabolites is now commercially available pharmaceutical products utilized for treatment of various ailments. In ancient times too, fungi were used a great deal for their supposed medicinal properties (Wainwright *et al.*, 2003). The antibiotic penicillin 1 from *Penicillium chrysogenum*, cholesterol lowering agent lovastatin 2 from *Aspergillus terreus* and antitumor lentinan 3 from Shiitake mushroom are the landmark of fungal-derived pharmaceutical products in today's world market (Chihara *et al.*, 1987; Hajjaj *et al.*, 2001; Elander, 2003). Other bioactive compounds produced by fungi include cyclosporine A 4 (anti lyphocytic) and fusidic acid 5 (anti-infective) used to help control infections from methicillin resistant *Staphylococcus aureus* (Borel *et al.*, 1994; Collignon *et al.*, 1999). The values of these pharmaceuticals have developed multi-billion dollar industry.

Fungi also produce toxic compound like ergot alkaloids (Keller *et al.*, 2005). The toxins of fungal origin are called mycotoxins. The toxicological effects are caused by various alkaloids the ergot produce, but in low and controlled concentrations these alkaloids are valuable drugs causing dilation of veins and a decrease in blood pressure and contraction of smooth muscles as well (De Groot *et al.*, 1998). They are drugs of folk-lore too and had been used during childbirth to hasten contraction of the uterus (Aleck *et al.*, 1927).

Aspergillus niger is an ascomycete fungus, widely used industrially for production of metabolites and as a source of enzymes α -amylase, cellulose and pectinases (Chiang *et al.*, 2010). The fungus is also known for production of various mycotoxins including ochratoxin A 5 and fumonisin B1 6 (Nielsen

et al., 2009). In this project, it was investigated the ethyl acetate extract of *A. niger* from mycelia produced during static fermentation for screening of antimicrobial, cytotoxic and phytotoxic activities.

Materials and methods

Microbial specimen collection

The pure fungal culture of *Aspergillus niger* was obtained from Department of Plant Pathology, University of Agriculture Peshawar. *Aspergillus niger* culture was refreshed on potato dextrose agar (PDA) medium by boiling potatoes (200 g) in 200 mL distilled water following filtration *via* muslin cloth. Glucose and agar (20 g each) was added and the volume was made up to 1L. The fungal culture was grown to 25 °C on the PDA medium in pre-sterilized plates till a substantial amount of biomass was obtained.

Fermentation of *A. niger* and crude extraction

A portion of mature agar slant was inoculated in 200 mL potato dextrose broth (PDB) in 500 mL Erlenmeyer flask and incubated at room temperature (28 ± 4 °C) as stationary mat culture for a period of 10 days. The whole cell culture (5 L) was extracted with EtOAc (1:1 v/v) overnight with stirring. The organics were separated and dried over anhydrous magnesium sulphate. After concentration in *vacuo* 300 mg of crude extract was obtained from 1 L *A. niger* culture.

Bioassays of whole cell extract of *A. niger* Antifungal activity

The antifungal activity of the crude extract has been evaluated against *Fusarium oxysporum*, *Alternaria alternata*, *Aspergillus flavus* and *Penicillium* sp. The crude extracts of the *A. niger* were prepared into two different concentrations 100 and 200 $\mu\text{g. mL}^{-1}$ and tested against the fungal strains, following the procedure of Rusman (2006). The extract was spread uniformly on agar plate and the tested fungal strains mycelia (5 mm) were placed in the centre of the agar plate. A positive control (clotrimazol, 50 $\mu\text{g. mL}^{-1}$) and negative control was also run in parallel. The tested cultures were kept at room temperature for 10 days

and growth of the fungal strains was observed daily. For each test, three replicates were performed and the diameter of zone of growth in mm for negative, positive and test culture was measured. Percent growth inhibition was determined using the restriction of growth of the tested fungal species by comparing the zone of growth with negative reference (Grover *et al.*, 1962; Rusman, 2006).

$$I = (C-T)/C \times 100$$

I = Percentage of inhibition, C = Diameter of fungal biomass in control, T = Diameter of the fungal biomass in treatment.

Antibacterial activity

Approximately 0.5 mg of crude extract was dissolved in 500 μ L MeOH and its antibacterial activity against *E. coli*, *S. Arvenia* sp., *S. aureus* and *B. subtilis* was determined by disc diffusion bioassays using 5 mm disc, which had 10 μ g of crude extract. Discs containing MeOH were used as negative control in all experiments and positive control with antibiotic. The test strains of bacteria were inoculated by streaking method to form colonies. After incubation at 35 °C for 30 hrs, the zones of growth inhibition were measured (Yin *et al.*, 2010).

$$IE \% = \frac{DC - DS}{DC} \times 100$$

DC – 5.

IE % = Inhibition effect (%), DC = Diameter of negative control (mm), DS = Diameter of test sample or positive control (mm), 5 is diameter of crude disk.

Brine shrimp lethality test

Brine shrimp lethality test is a routine test for evaluation of the extract, fractions and purified compounds. Brine solution (38% sea salt solution) was prepared and poured in hatching tray. Toxicity of the fungal ethyl acetate extract was tested at 10, 50, 100 and 200 μ g. mL⁻¹ concentrations in 15 mL brine solution with 1% DMSO (v/v). Ten, one-day nauplii were used in each test and the dead larvae counted after 24 hrs. Three replications were used for each concentration and data were recorded as the mean $\pm$

SD of the three findings. A standard potassium dichromate solution as positive control and the blank were also carried out and their toxicities were compared (Atta-ur-Rahman, 1991; McLaughlin *et al.*, 1998). LC₅₀ values were calculated using the prism pad version 6.00 software.

Phytotoxicity test (herbicidal activity)

Phytotoxic test was performed according to the modified method of Atta Ur Rehman (1991). Crude extract (10 mg) of *A. niger* was taken in a vial and dissolved in 1 mL MeOH. Different concentrations of the extract i.e 10, 50, 100, and 200 μ g. mL⁻¹ were prepared from the stock solution and allowed to evaporate overnight. Sterilized E-medium was poured (100 mL) in a glass with crude and then 10 *Lemna minor* each containing a rosette of three fonds was placed on E-medium. Other glasses were supplemented with MeOH as negative control and the reference inhibitor atrazine as a positive control. Treatment was replicated three times and all the glasses were kept under the uninterrupted light at room temperature. Growth of *Lemna minor* in extract containing glasses was determined by counting the number of fonds against concentrations and phytotoxicity effect of the extract was calculated with reference to negative control. FI₅₀ value was determined using graph pad software.

Statistical analysis

The statistical analysis of the recorded data was conducted using CR design. Means were compared with Std. using MSTAT version 2.00 software. The Ld₅₀ value was calculated through probit analysis in excel.

Results

Antimicrobial activity

In the present study, the crude ethyl acetate (EtOAc) extract of *A. niger* mycellia was tested against 4 fungal (*Fusarium* sp., *A. alternate*, *A. flavus* and *Penicillium* sp.) and 4 bacterial strains (*E. cloi*, *Ervenia* sp., *S. aureus* and *B. subtilis*). In disc diffusion antibacterial activity no zone of inhibition was observed against the all tested bacterial strains at

concentration of 10 µg on 5 mm disc. Control treatments (positive and negative both) were also run in parallel. The results clearly showed that the extract possess no antibacterial activity against the tested

bacterial pathogens at the applied concentration. As anticipated in blank no zone of inhibition was detected while in the positive control (streptomycin) the zone of inhibition upto 26 mm was measured.

Table 1. Antifungal activity of the EtOAc whole cell extract of *A. niger* against selected fungal isolates as well as reference negative control and the antifungal Diathane M45.

Diameter of zone of growth in mm ± SD, values of % inhibition in enclosed parenthesis with reference to negative control				
Fungal isolates	Concentration (µg. mL ⁻¹)			
	200	100	-ve control	Diathan M45
<i>Fusarium. Oxysporum</i>	12.6 ± 1.1 (46.6)	15.0 ± 1.0 (36.4)	23.6 ± 1.5	-
<i>Alternaria alternate</i>	9.3 ± 0.6 (60.6)	12.3 ± 0.5 (47.9)	-	-
<i>Aspergillus flavus</i>	15.0 ± 1.0 (36.4)	16.3 ± 0.6 (30.9)	-	-
<i>Penicillium sp</i>	14.0 ± 1.0 (38.0)	15.0 ± 1.0 (36.4)	-	-

Two different concentrations of the organic extract of *A. niger* were tested for antifungal activity against the selected fungal pathogens. The extract exhibited inhibitory effect on the growth of tested pathogens. Of all the tested fungi, *A. alternata* was found highly

sensitive with 64% growth inhibition in concentration of 200 µg. mL⁻¹ with reference to negative control. *F. oxysporum* was found least sensitive with 36% growth inhibition as shown in table 1.

Table 2. Brine shrimp lethality test shown as mean of three replicates ± SD values at different concentrations for 24 and 48 hrs.

Mean Mortality ± SD				
Concentration	24 h		48 h	
(µg mL ⁻¹)	Dead Larvae*	% Lethality	Dead larvae*	% Lethality
0	0.00±0.0	0.00	1.67 ± 0.6	16.7
10	3.67 ± 0.6	36.7	7.00 ± 1.0	70.0
50	4.33 ± 0.6	43.3	8.33 ± 0.6	83.3
100	6.00 ± 1.0	60.0	9.33 ± 0.6	93.3
200	7.67 ± 0.6	76.7	9.67 ± 0.6	96.7

* Mean of three replicates of dead brine shrimps out of total ten larvae.

Brine shrimp cytotoxicity bioassay

To test the cytotoxicity, the EtOAc extract of *A. niger* was subjected to bioscreening study by the brine shrimp lethality bioassay. Different concentrations of the crude extract were prepared i.e 10, 50, 100 and 200 µg. mL⁻¹. The tests were performed in triplicate and compared with control for systematic result. The morality in percent was calculated after 24 and 48 hrs. The organic extract studied in this work showed significant lethality against brine shrimps as the results are shown in table 2. The maximum morality

was observed after 24 hrs at 200 ppm (70%) while minimum at 10 ppm (36.7%). The highest mortality rate was observed after 48 hrs at 200 ppm (96.7 %). LC₅₀ of the extract against brine shrimps was calculated as 9.25 µg. mL⁻¹ (1 µg. mL⁻¹ = 1 ppm) after 24hrs.

Bioassay for plant growth inhibitor (*Lemna minor* Phytotoxicity test)

A. niger crude extract was tested against the *Lemna minor* to test its effect on plant growth. Various

concentrations of extract i.e. 10, 50, 100, and 200 $\mu\text{g.mL}^{-1}$ were prepared in E. medium. *Lemna minor* plants (10 in number) each containing three fronds were placed on E-medium in plastic translucent glasses. Negative reference (E-medium only) and positive control (atrazine herbicide) were prepared and tested against proliferation of fronds. In control no frond was affected while in standard (Atrazine) all fronds of *Lemna minor* were severely damaged as

evident from the dark color of the fronds. In tested solution 200 $\mu\text{g.mL}^{-1}$ organic extract showed 82% mortality when compared to atrazine. The mortality of fronds declined with decreasing concentration of extract as 10 $\mu\text{g.mL}^{-1}$ exhibited only 36.7% mortality of fronds. The data was processed using graph pad prism software to determine the FI_{50} value of the crude extract against *Lemna minor* were calculated as 15.8 $\mu\text{g.mL}^{-1}$ shown in table 3.

Table 3. Phytotoxicity test of *A. Niger* crude extract against *Lemna minor*.

Concentration ($\mu\text{g mL}^{-1}$)	Dead fronds*	% Mortality
0	0.0 $\pm$ 0.0	0
10	11.3 $\pm$ 1.5	37.7
50	14.0 $\pm$ 1.0	46.7
100	23.3 $\pm$ 0.6	77.7
20	24.6 $\pm$ 1.6	82

* Number of dead from the total thirty fronds tested against different concentrations, Atrazine was used as a positive control with 100% mortality.

Discussion

A. niger is a known producer of bioactive natural products including mycotoxins. During our antibacterial bioassay no inhibitory effect was observed with the applied concentration against the

tested strains of bacteria. However, Praveena and Padmini (2011) reported weak antibacterial potential of mycotoxins viz., aflatoxin, patulin and deoxynivalenone (DON) against *E. coli*, *Micrococcus luteus*, *S. aureus* and *Proteus mirabilis*.

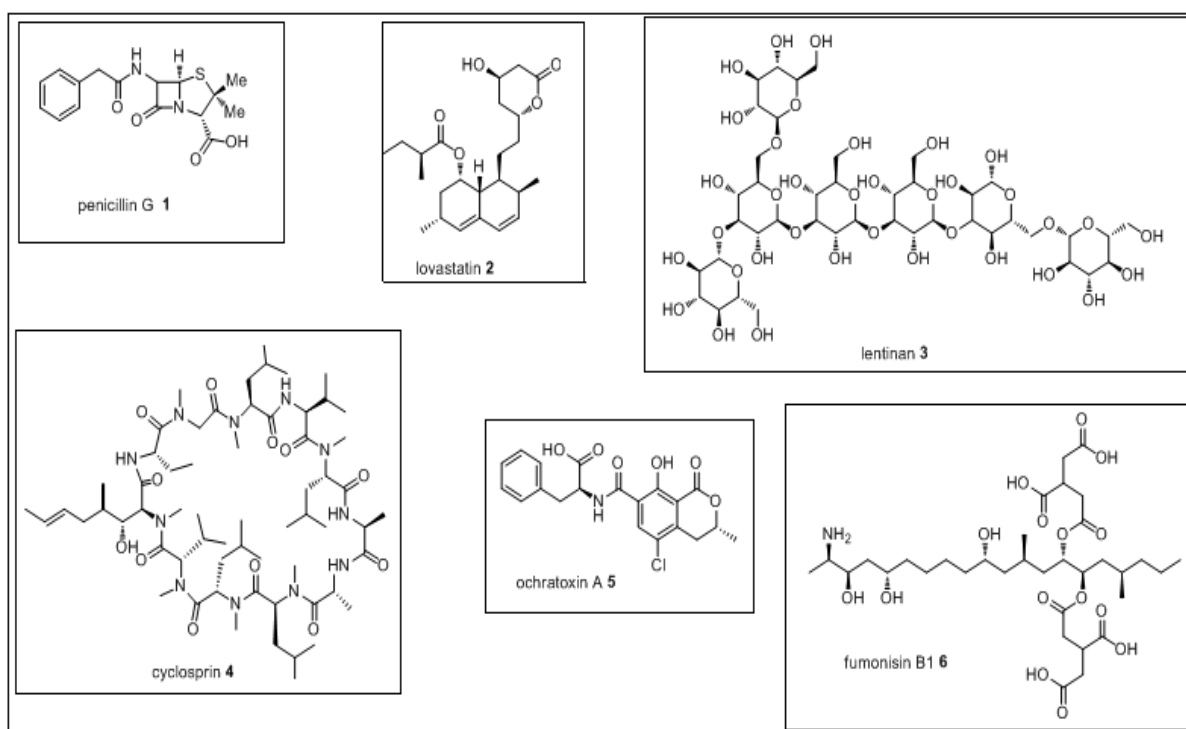


Fig. 1. Structures of important natural products. Their bioactivities are mentioned in the text.

The antifungal bioassay against test isolates showed that the crude extract of the cells contain antifungal compounds and may possess potential for pharmaceutical and/or agricultural applications. Thenmozhi and Kannabiran (2010) also observed antifungal activity of *A. niger* extract against *A. fumigatus* and *A. flavus*.

Brine shrimp (*Artemia salina* L.) larvae were found to be convenient test organism for detecting fungal metabolites in crude fungal extracts. The bioassay has been employed for determination of fungal toxins by many researchers (Harwig *et al.*, 1971). Jerry *et al.* (1998) reported a positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity as well. The brine shrimp lethality bioassay has good correlation with the human solid tumor cell lines (McLaughlin *et al.*, 1991). LC₅₀ values < 1000 ppm are considered significant for crude extracts. Due to toxicity of the extract for brine shrimps it is opined that *A. niger* may produce metabolite (s) possessing anticancer activity.

Lemna minor commonly known as duck weed is an aquatic monocot consisting of three fronds (one mother and two daughter fronds). The plant has been used in bioassays to screen extracts and chemical substances for their effects on plant growth (Einhellig *et al.*, 1985). The test is useful to determine the factors of plant growth inhibitor by measuring FI₅₀ values (concentrations necessary to inhibit 50% of frond proliferation) or to determine plant growth stimulant activity by FP₅₀ values (concentrations causing 50% increase in proliferation of fronds (McLaughlin *et al.*, 1998). The crude extract obtained from the *A. niger* mycelia may contain herbicidal components and further study to isolate bioactive compounds is required.

Based on the results of this study the whole cells extract from *A. niger* possess cytotoxic, plant growth inhibitory and antifungal activities. No antibacterial activity of the extract was found using disc diffusion method in tested concentrations. These results

concluded that further investigations on structural characterization of active components and advanced bioassays are required as the extract may contain useful bioactive factors for future applications in pharmaceutical and agriculture.

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