In vitro Antibacterial, Cytotoxic and Free Radical Scavenging activities of an *Aspergillus* species

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Abstract

Aspergillus is a genus of saprophytic, asexual reproducing molds commonly found in soil and compost piles. These molds can produce antibiotics, mycotoxins, immunosuppressants, cholesterol-lowering agents, and so forth. The ethyl acetate extract (EAE) of the broth culture of an *Aspergillus* species showed moderate antibacterial activity. The chloroform and ethyl acetate extracts of the culture showed significant cytotoxicity having LC_{50} 78.67 µg/ml and 0.064µg/ml, respectively. Total phenolic content of extractives was measured involving Folin-Ciocalteu reagent and it was found to be higher in EAE than that of chloroform extract (CE). The antioxidant potential of ethyl acetate and chloroform extracts was evaluated by using free radical (DPPH) scavenging assay. The ethyl acetate extract showed significant free radical (DPPH) scavenging activity having IC_{50} 70.45µg/ml.

Keywords: *Aspergillus*, antibacterial, brine shrimp lethality bioassay, free radical scavenging, total phenolic content

Introduction

Few fungi are as important as members of the genus *Aspergillus*. This taxonomic group encompasses saprophytic organisms whose characteristics are of high pathological, industrial, pharmaceutical, agricultural, scientific, and cultural importance. Members of the genus *Aspergillus* can produce antibiotics, mycotoxins, immunosuppressants, and cholesterol-lowering agents. These secondary metabolites are classified chemically by their biosynthetic origin as polyketides, nonribosomal peptides, sesquiterpenes, and so forth (Keller *et al*., 2005).

The first statin, lovastatin was developed for human use as a drug which was isolated from *A. terreus*. The Merck Research Laboratories patented this statin in 1980 (Tobert, 2003). As other secondary metabolites, statins are synthesized by complex sequential steps, involving polyketide synthases, the genes coding for the cognate enzymes map in a 64kb gene cluster (Kennedy *et al*., 1999). The status of *Aspergillus* genomics was reviewed by Jones (2007).

The aflatoxin biosynthetic pathway has become a model for studying the biochemistry and molecular biology of fungal secondary metabolism (Keller *et al*., 2005; Payne *et al*., 1998; Yu *et al*., 2004). The genes for aflatoxin biosynthesis along with the pathway specific regulator aflR, reside in a 70kb DNA cluster near the telomere of chromosome 3 (Chang *et al*., 1993; Payne *et al*., 2006; Woloshuk *et al*., 1994; Yu *et al*., 2004).

Paclitaxel is a potent and widely used antitumor agent. Considerable worldwide research efforts have been carried out on different production alternatives of paclitxel. Some *Aspergillus* species were reported as paclitaxel producers (Caruso *et al*., 2000; Zhao *et al*., 2009).

Since *Aspergillus* species have potential biological applications, here authors describe the isolation and biological investigations of ethyl acetate extract (EAE) and chloroform extract (CE) from the fermentation broth of an *Aspergillus* species. The present work was an endeavor to screen the extractives for probable antibacterial, cytotoxic and antioxidant activities.

Materials and Methods

Microorganisms and culture conditions

The organism was isolated from a soil sample collected from Naryangang, Bangladesh. For small

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scale liquid fermentation the Czapek-dox acidic broth media was used which consisted of the following (in grams per liter): KCl, 0.5; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; $NaNO_3$, 2.0; $FeSO_4.7H_2O$, 0.01 and Sucrose, 30. The pH was adjusted to 5.30 prior to autoclaving. Different carbon sources other than sucrose were also tried.

Production of Extract

For the small-scale liquid fermentations the preserved slant cultures of the isolates were used as inoculums. A loopful of the inoculums of the strain was added to an Erlenmeyer flask (500ml) containing nutrient broth medium to obtain a seed culture. The flask was kept in an incubator (at 37.5°C) for 5 days. This seed culture was used to inoculate a number of 1000ml Erlenmeyer flasks containing 300ml Czapek-dox acidic broth medium. The flasks were then kept in an incubator (at 37.5°C) for 8 days (without changing other conditions).

Extraction from the broth media

The broth media was filtered to separate the biomass from the broth. The filtrate was then partitioned with chloroform and then ethyl acetate. These fractions were evaporated under reduced pressure to obtain orange-yellow solid masses. Thus 130mg ethyl acetate extract (named as EAE) and 85mg chloroform extract (named as CE) were obtained from 5liter fermentation broth.

***In vitro* antibacterial activity**

The extracts were tested for antibacterial activity by the standard disc diffusion method (Bauer *et al.*, 1966 and Serrano *et al.*, 2004). The screening was done against 13 strains of bacteria. The results obtained were compared with a standard antibiotic, kanamycin (30 µg/disc).

Cytotoxicity by brine shrimp lethality bioassay

In brine shrimp lethality bioassay (Meyer *et al*., 1982; Persoone, 1988; McLaughlin, 1982) DMSO was used as a solvent and negative control. Vincristine sulphate served as the positive control. For cytotoxicity screening, DMSO solutions of the plant extractives were applied against *Artemia salina* in a 1-day *in vivo* assay. For the experiment, 4mg of each of the extract was dissolved in DMSO and solutions of varying concentrations (400, 200, 100, 50, 25, 12.50, 6.25, 3.125, 1.563, 0.781 µg/ ml) were obtained by serial dilution technique.

Total phenolics analysis

Total phenolic content of extractives was measured by employing the method described by Skerget *et al.*, 2005 involving Folin-Ciocalteu reagent as an oxidizing agent and gallic acid as a standard. To 0.5ml of extract solution (0.25mg/ml) in water, 2.5ml of Folin-Ciocalteu reagent (diluted 10times with water) and 2.0ml of sodium carbonate (7.5% w/v) solution were added. After 20 minutes incubation at room temperature the absorbance was measured at 760nm using UV-visible spectrophotometer. Total phenolics were quantified by calibration curve obtained from measuring the known concentrations of gallic acid (0-100µg/ml). The phenolic contents of the sample were expressed as mg of GAE (gallic acid equivalent)/gm of the extract.

Free radical scavenging activity

The free radical scavenging activity (antioxidant capacity) of the extractives on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was estimated by the method established by Brand-Williams *et al*., 1995. Here 2.0ml of a methanol solution of the sample (extractive/ standard) at different concentration (500µg/ml to 0.977µg/ml) were mixed with 3.0ml of a DPPH methanol solution (20µg/ml). After 30min of reaction at room temperature in dark place the absorbance was measured at 517nm against methanol as blank by UV spectrophotometer. Inhibition of free radical DPPH in percent (I%) was calculated as follows:

$$(I\%) = (1 - A_{sample}/ A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test material). Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted with inhibition percentage against extractive / standard concentration.

Statistical analysis

Three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD (n=3). Probability (P) value of 0.05 or less ($P < 0.05$) was considered significant.

Results and Discussion

***In vitro* antibacterial activity**

The EA extract showed moderate antibacterial activity (Table 1) but chloroform extract was

antibacterially inactive. The zones of inhibition of EA extract were compared with the zones of inhibition of kanamycin 30µg/disc.

Table 1: Zones of inhibition of ethyl acetate extract against a series of test bacteria

Test organisms	Zone of inhibition (in mm)	
	EA extract (400 µg/disc)	Kanamycin (30 µg/disc)
Gram positive bacteria:		
Bacillus cereus	11±0.13	30±0.18
Bacillus megaterium	11±0.21	32±0.36
Bacillus subtilis	11±0.37	31±0.58
Sarcina lutea	14±0.32	31±0.64
Staphylococcus aureus	12±0.16	31±0.29
Gram negative bacteria:		
Escherichia coli	11±0.43	34±0.73
Pseudomonas aeruginosa	12±0.18	32±0.62
Salmonella paratyphi	12±0.19	31±1.05
Salmonella typhi	11±0.34	30±0.82
Shigella boydii	11±0.22	32±0.27
Shigella dysenteriae	17±0.39	32±0.64
Vibrio mimicus	12±0.06	32±0.81
Vibrio parahaemolyticus	12±0.17	31±0.37

The average values of three calculations are presented as mean ± S.D. (Standard Deviation)

Cytotoxicity by brine shrimp lethality bioassay

In case of brine shrimp lethality bioassay, the lethality of the EA extract and chloroform extract to brine shrimp was determined on *A. salina*. Table 2 shows the results of the brine shrimp lethality testing after 24 hours of exposure to the samples and the positive control, vincristine sulphate (VS). The LC_{50} obtained from the best-fit line slope were found to be 0.064, 78.67 and 0.28µg/ml for EA extract, chloroform extract and vincristine sulfate, respectively. In comparison with the positive control (vincristine sulphate), the cytotoxicity exhibited by the extracts were significant.

Table 2: Cytotoxicity samples by brine shrimp lethality bioassay

Samples	LC_{50} (µg/ml)
VS	0.28±0.01
EAE	0.064±0.002
CE	78.67±2.93

The average values of three calculations are presented as mean ± S.D. (Standard Deviation)

Total phenolics analysis

Total phenolics was found to be higher in EA extract (71.98mg of GAE/gm of extract) than that of chloroform extract (24.81mg of GAE/gm of extract) (Table 3).

Table 3: Total phenolic content and free radical scavenging activity of the samples

Sample	**Total Phenolic Content** (mg of GAE/gm of extract)	**Free radical scavenging activity** (IC_{50} in µg/ml)
BHT	-	20.39±0.72
ASA	-	2.88±0.09
EAE	71.98±1.63	70.45±1.48
CE	24.81±0.63	634.16±5.92

The average values of three calculations are presented as mean ± S.D. (Standard Deviation)

Free radical scavenging activity

Free radical scavenging activity (Figure 1) was found to be much higher in EA extract (IC_{50} value is 70.45µg/ml) than that of chloroform extract (IC_{50} value is 634.16µg/ml) (Table 3).

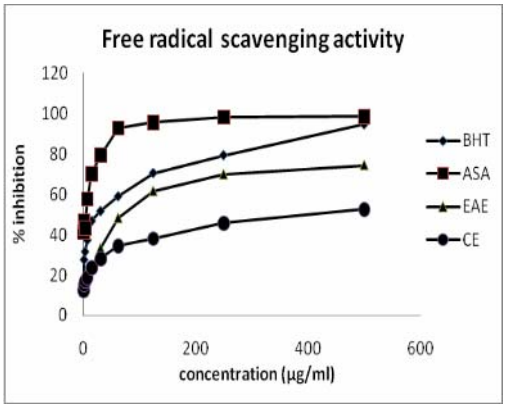

Figure 1: DPPH free radical scavenging activity of different samples

Therefore it can be concluded that, in the preliminary studies, the EA extract of the *Aspergillus* species demonstrated moderate antibacterial activity and free radical scavenging activity as well as strong cytotoxic activity. On the other hand, the Chloroform extract of the *Aspergillus* species demonstrated moderate free radical scavenging activity and cytotoxic activity.

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