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Antimicrobial susceptibility assessment of compound from *Aspergillus fumigatus*

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Ethyl acetate extract of the culture filtrate *of Aspergillus fumigatus* on chromatographic analysis has led to the isolation of the compound, AF-1 which exhibited a significant *in vitro* antimicrobial activity against the tested pathogenic microorganism. The structure of the isolated compound, AF-1 was identified as 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one from its IR, UV, EI-MS, ^{13}C-NMR, ^{1}H-NMR, HMBC and HMQC data. This is the first report of the compound, AF-1 from the *A. fumigatus.*

Key words: *Aspergillus,* 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, antimicrobial screening, bioactive compound.

INTRODUCTION

Ever since mankind started suffering from ailments, the quest for finding remedies to treat the diseases started. The science of antibiotics has remained and will remain for many years, one of the most interesting natural sciences, in both theoretical and practical aspects. Determining new molecules which are potent bioactive component from newer sources to fight against pathogens is of great interest now-a-days. Fungi have proved to be capable of biosynthesizing secondary metabolites bearing conspicuous structural diversity, which could be further enlarged by structural modifications (Czarnik, 1996).

Fungal secondary metabolites are gaining importance in Pharmaceutical and Biotechnological applications. At present, at least six different components were commonly prescribed as medications isolated from *Aspergillus* origin (Varga and Toth, 2003). *Aspergillus* strains have been reported to be producers of metabolites with broad range of biological activities (Cheng-Bin et al., 1997). During recent decades, bacteria resistance to most clinically available antimicrobial agents has emerged at an alarming rate (Wu et al., 1999; Arias and Murray, 2009). Consequently, discovery of new antimicrobials has become increasingly crucial.

As a part of continuing research on the microbial metabolites from the soil samples we have isolated a number of antagonistic microorganisms, among the isolates selected one was identified by morphological and other criteria. Finally, we screened for the production of antimicrobial and pharmacological

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active compound.

MATERIALS AND METHODS

Isolation and identification of antagonistic microoganisms from soil samples

In a systematic screening program for the isolation of bioactive compound from microorganism, soil samples were collected into sterile boiling tubes with a sterile spatula near to the Rajshahi University Campus. Care was taken to see that the points of collection had widely varying characteristics as possible with regard to the organic matter, moisture content, particle size and color of soil (Reiner, 1982; Mythili and Das, 2011).

About 1 g of sample was transferred to a sterile flask containing 50 ml sterile water. The flasks were shaken on rotary shaker for 30 min and were kept aside for 30 min to settle down the particulate matter. The clear supernatant was diluted with sterile water. These dilutions (1/10 - 1/1000) were used as inocula. One ml of each of these dilutions were pipetted out into the medium, plated into petridishes 10 cm diameters and incubated at 28°C for three to seven days. For the isolation of antagonistic microorganisms from the above mentioned samples, the following media were used: starch casein agar medium, Czapek Dox agar, Czapek yeast agar and malt extract agar medium.

For the identification of the selected organism we emphasize the morphological methods including; macroscopic and microscopic characteristics according to Klich (2003) and McClenny's (2005) Identification Aspergillus Species. Finally we compared the morphological characteristics of tested *Aspergillus* species with those of standard *Aspergillus fumigatus*.

Production, isolation and purification of the compounds

The organism was allowed to grow in a number of culture flasks of 500 ml capacity containing Czapek-Dox broth acidic medium at 35°C. The broth was separated from the mycelial mat on 8^{th} day to get the maximum yield of antimicrobial activity (data not shown). The culture filtrate was then subjected to repeated ethyl acetate extraction and the extract was evaporated under reduced pressure. The crude extract was resolved by thin layer chromatography (TLC), preparative TLC (PTLC) (Stahl, 1969) and obtained on large scale on column chromatography (CC) (Beckett and Stenlake, 1986). For checking purity of the compound, TLC was carried out using pre-coated silica gel 60 F_{254} plates (Mercks) and detection was made by visualization under UV light (254 nm) and spraying with 0.1% vanillin sulfate spray reagent followed by heating.

Spectral measurement

Ultra-violet (UV) spectra were recorded on a Beckman double beam spectrometer. IR spectra were obtained by a Perkin Elemer 1600 FTIR spectrometer. ^{1}H-NMR (500MHz) and ^{13}C-NMR (125 MHz) spectra were acquired on a JEOL JNM alpha spectrometer using TMS as internal standard. Electro spray ionization mass (EI-MS) was recorded on a JEOL DX-300 spectrometer. ^{1}H-^{13}C HMBC and HMQC were recorded at 500 MHZ (proton) and 125 MHZ (carbon), respectively.

Antimicrobial screening

The disc diffusion method (Radovanović et al., 2009) was used to test antimicrobial activity against 15 bacteria and three fungi. Solutions of known concentration (µg/ml) of the test samples were made by dissolving measured amount of the samples in calculated volume of ethyl acetate. Dried and sterilized filter paper discs (7 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test material were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic (Kanamycin 30 µg/disc) and blank discs (impregnated with solvents) were used as positive and negative control, respectively. These plates were then kept at low temperature (4°C) for 24 h to allow maximum diffusion. The plates were then incubated at 37°C for 24 h to allow maximum growth of the organisms. The antimicrobial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out in triplicate and the mean of the readings were taken. Griseofulvin (20 µg/disc) was used as standard for antifungal activity. The MIC values of the compound were determined against *Shigella dysenteriae, Salmonella typhi, Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus* by serial dilution technique (Hammond and Lambert, 1998).

RESULTS AND DISCUSSION

Identification of the organism

The isolated organism was identified as *A. fumigatus* based upon the following morphological characteristics:

Macroscopic feature

Growth rate is rapid; the colony size can reach 3 ± 1 cm within a week when grown on Czapek-Dox agar at 25°C; growth occurring at temperatures as high as 40°C and survival were maintained at temperatures up to 60°C; the texture of colonies varies from wooly to cottony to granular; surface colony color is smoky gray to green and the reverse is yellow due to the diffusible pigment; and color of very mature colonies turn to slate gray while atypical colonies may remain white with slight conidiation.

Microscopic appearance

Conidial heads are in the form of compact columns in an undisturbed culture. The chains of conidia are borne directly on broadly clavate vesicles. The conidiophores are smooth-walled up to 300 µM long, and terminate in a dome-shaped vesicle with a diameter of 20-30 µM long; Hyphae are septate and hyaline; the species is uniseriate producing a closely compacted phialides with size ranging from 5-10 × 2-3 µM, and only occurring on the upper portion of the vesicle; and conidia are round to sub-globse, smooth to finely roughened, and with diameter size of 2.0 - 3.5 µM.

Characterization of the compound

The compound was obtained as colorless crystalline needle having melting point of 153°C.

EI-MS analysis

In the mass spectrum the compound showed molecular

Table 1. ^{1}H-^{13}C correlation using HMQC (500 MHz, CD30D).

^{1}H-NMR	^{13}C-NMR
6.49 (1H, s, H-3)	110.8 (C-3)
7.95 (1H, s, H-6)	141.0 (C-6)
4.41 (2H, s, H-7)	61.2 (C-7)

Table 2. ^{1}H-^{13}C correlation using HMBC (500 MHz, CD30D).

^{1}H-NMR data	^{13}C-NMR data
6.49 (1H, s, H-3)	61.2,147.4,170.4,176.8
7.95 (1H, s, H-6)	147.4,170.4,176.8
4.41 (2H, s, H-7)	110.8,170.4

Table 3. Antibacterial activity of the compound and kanamycin standard.

Test organism	Strain No.	Compound (30 µg/disc)	Compound (60 µg/disc)	Kanamycin (30 µg/disc)	Crude extract (60 µg/disc)
Gram negative					
Shigella dysenteriae	AL-35587	16	33	33	20
Shigella shiga	ATCC-26107	17	27	31	20
Shigella boydii	AL-17313	14	28	30	16
Shigella sonnei	AJ-8992	14	27	29	17
Shigella flexneri	AL-30372	15	27	33	19
Escherichia coli	FPFC-1407	17	30	31	23
Pseudomonas aeruginosa	QL-147	14	26	26	20
Klebsiella spp.	CRL	17	28	25	22
Salmonella typhi	CRL	16	32	27	18
Gram positive					
Bacillus subtilis	QL-40	15	28	29	29
Bacillus cereu	QL-29	16	27	30	30
Bacillus megatrium	QL-38	14	25	27	26
Sarcina lutea	QL-166	14	22	25	25
Staphylococcus aureus	ATCC-259233	10	17	26	22
Streptococcus-β- haemolyticus	CRL	10	16	27	20

ion peak at m/z 142 *(W)* and (M*+1) peak at *m/z* = 142, which correspond to the molecular formulae $C_6H_5 4_4$.

UV Spectra

In UV spectrum the compound showed absorption maximum, at 242 nM, which indicates the presence of aromatic carbonyl compound.

IR Spectrum

In IR spectrum, the compound showed absorption band at 1725 and 3620 cm^{-1}, these might be due to carbonyl and hydroxyl group, respectively. ^{1}H-NMR (500 MHz, CD_3OD) δppm: 6.49(IH, s, H-3), 7.95 (1H, s, H-6), 4.41(2H, s, H-7); ^{13}C-NMR (125 MHz, CD_3OD) δppm: 170.4 (C-2.), 110.8 (C-3), 176.6 (C-4), 147.4 (C-5), 141.0 (C-6) and 61.2 (C-7) (Table 1).

The compound AF-1 was elucidated as 5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one, on the basis of UV, IR, EIMS, ^{1}H-NMR, ^{13}C-NMR, HMBC and HMQC spectra (Table 2); and comparing the pyran derivatives (Budavari, 2000; CIS Information Services, 2000 and Lide and Milne, 1996). The structure of the compound is shown in Figure 1.

Antimicrobial activity

The ethyl acetate extract of the culture filtrate of *A. fumigatus* and compound showed significant antibacterial activity against pathogenic organisms at a concentration of 30 and 60 µg/disc (Table 3). However, the activity of

Table 4. Antifungal activity of the compound.

Test fungus	Diameter of zone of inhibition (mm)			
	Compound (100 µg/disc)	Compound (200 µg/disc)	Griseofulvin (20 µg/disc)	Crude extract (200 µg/disc)
Tinea pedis	11	15	19	14
Tinea corporis	10	15	20	13
Candida species	13	18	22	12

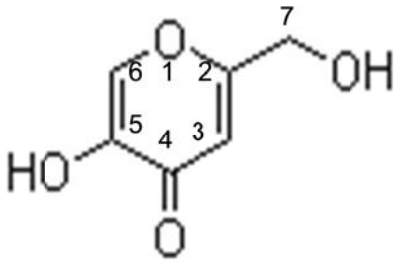

Figure 1. 5-Hydroxy-2-(hydroxymethyl)-4H-pyran-4-one.

the compound was potent against *S. dysenteriae, S. typhi* and *Escherichia coli* as compared to that of standard. The compound also possesses antifungal activity (Table 4). The antifungal activity was ten time less as compared to the standard (griseuofulvin).

Minimum inhibitory concentration

The minimum inhibitory concentration of the compound against *S. dysenteriae, E. coli*, *S. typhi*, *B. subtilis* and *Sarcina lutea* were 64, 64, 128 and 256 µg/ml, respectively.

Conclusions

The compound AF-1 (5-hydroxy-2-(hydroxymethyl)-4H-pyran-4-one) was the first report from *Aspergillus fumigatus*. The potency of antimicrobial activity of the compound was higher against gram-negative bacteria compared to gram-positive bacteria. Interestingly the antimicrobial activity of crude extract were different than the isolated pure compound, which reflect that the crude extract contained antimicrobial compound(s) other than the isolated compound and predominantly effective against gram-positive bacteria. The isolated compound AF-1 may be a very effective therapeutic agent, but further study is necessary for drug development.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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