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Isolation and *in vitro* Antimicrobial Activities of Ethyl acetate Extract from *Streptomyces bangladeshiensis*

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Abstract: The aim of the present study was to investigate the antimicrobial activities of ethyl acetate extracts against Gram-positive and Gram-negative bacteria and fungi. An attempt has been made to investigate the indigenous drugs of choice in infectious diseases for mitigation of suffering of the vast masses of humanity. The organism, *Streptomyces bangladeshiensis* was isolated from a soil sample collected from Natore, Bangladesh at the depth of 0.75 m using crowded plate technique. The maximum secretion of metabolites from the strain was found at the 7th day of incubation in Czapek Dox broth (alkaline pH 8.5) medium at 32.5°C by maintaining the physicochemical factors in optimum level for the culture. *In vitro* antimicrobial susceptibility was determined as per National Committee for Clinical Laboratory Standards guidelines and serial dilution technique for the determination of Minimum Inhibitory Concentration (MIC) of extracts. Ethyl acetate extract from a new actinomycetes, *Streptomyces bangladeshiensis*, showed good antibacterial and antifungal activities against a total of 14 bacteria (5 gram positive plus 9 gram negative) and 8 fungi. The Minimum Inhibitory Concentrations (MIC) were determined and found to be 16 μg mL^{-1} against *Bacillus subtilis*, *Staphylococcus aureus*, *Shigella dysenteriae* and *Aspergillus flavus* while 32 μg mL^{-1} against *Salmonella typhi*, *Candida albicans* and *Aspergillus niger*. Present data shows that all the pathogenic microorganisms (Gram positive and negative bacteria and fungi) showed a substantial sensitivity towards the crude extract. But further work is necessary in order to establish the individual antibiotics present of this isolated compound.

Key words: *Streptomyces bangladeshiensis*, ethyl acetate extract, antimicrobial activity

INTRODUCTION

Infectious diseases are leading health problems with high morbidity and mortality in the developing countries (Black *et al*., 1982; Walsh and Warren, 1974). Although huge numbers of antibiotics have been discovered, the pathogenic organisms are developing resistance to these antibiotics day by day. In third world countries like Bangladesh, irrational use of antibiotics is a major cause of such resistance. So it is no doubt important to discover newer, safer and more effective antibiotics.

Among the antibiotics developed from the microorganisms, the actinomycetes, particularly the genus *Streptomyces*, is reported to produce a number of well known antibiotics (DNP CD-ROM, 2001 and ISIC Database, 2004) including streptomycin, neomycin, tetracycline and chloramphenicol

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(Waksman and Woodruff, 1940; Dienstag and Nue, 1972; Argoudelis *et al*., 1987; Harvey, 1993). As a part of our ongoing research for antimicrobial principles (Jabbar *et al*., 1998, 1999; Biswas *et al*., 2000) from antagonistic organism, by screening a number of soil sample in Bangladesh, we isolated a new actinomycetes, *Streptomyces bangladeshiensis*, from a soil sample collected in the region of Natore (Alim Al-Bari, 2003). We herein report the antimicrobial activities of the ethyl acetate extract from its culture broth against a number of bacteria (both Gram positive and Gram negative) and fungi.

MATERIALS AND METHODS

Organism

The organism was isolated from a soil sample collected from Natore, Bangladesh at the depth of 0.75 m during September-October 2001 using crowded plate technique (Hammond and Lambert, 1978). The organism was identified as new actinomycetes and named *Streptomyces bangladeshiensis* on the basis of morphological, physiological, biochemical and sequencing of 16S rDNA studies (Alim Al-Bari *et al*., 2005). The strain has been deposited under the accession number NRRL B-24326^T (ARS Culture Collection, Peoria, Illinois) and LMG 22738^T (BCCM/LMG Bacteria Collection, Belgium).

Extraction

The maximum secretion of metabolites from the strain was found at the 7th day of incubation in modified Czapek Dox broth (Difco) (alkaline pH 8.5) medium at 32.5°C by maintaining all the physiochemical factors at optimum level for the culture (Alim Al- Bari, 2003). The extraction of the metabolites was carried out by ethyl acetate on the basis of best solubility and maximum antimicrobial activities. The solvent, ethyl acetate, was evaporated using a rotary evaporator at 40°C under reduced pressure. Two hundred and fifty milliliter of the culture filtrate was taken in a 500 mL separating funnel which was shaken with 3×70 mL of ethyl acetate for 3×15 min. After settlement for 5 min, the ethyl acetate layer was separated by removing the aqueous filtrate layer and collected in a beaker (1000 mL) by covering with aluminum foil. In this way, total culture filtrate was extracted with ethyl acetate and collected in the same beaker. On average, 1 L of culture filtrate gave 515 mg of crude extract.

Antimicrobial Assay

Test pathogenic microorganisms employed for *in vitro* antimicrobial assay were obtained from the Institute of Nutrition and Food Science (INFS), University of Dhaka, International Center for Diarrhea Disease and Research, Bangladesh (ICDDRB) Dhaka. *In vitro* antibacterial screening is performed by disc diffusion method (Bauer *et al*., 1966; Rios *et al*., 1988; NCC Laboratory Standards, 1999) for primary selection of the compounds as therapeutic agent. The method is essentially a qualitative or semi quantitative test indicating sensitivity or resistance of microorganisms to the test materials as well as bacteriostatic or bactericidal activity of a compound (Reiner, 1982). The sample solution of the extract to be tested was prepared by dissolving 1 mg of the extract in 1 mL of in ethyl acetate to attain the desired concentrations (200 μg disc^{-1} for fungi, 100 and 30 μg disc^{-1} for bacteria). Sample solutions of desired concentrations were applied on the sterilized filter paper discs (6 mm in diameter) with the help of a micropipette in an aseptic condition and left these discs for a few minutes in the aseptic hood for complete removal of the solvent. To compare the antibacterial and antifungal activities, kanamycin; 30 μg disc^{-1} (Oxoid Ltd. England) and nystatin; 20 μg disc^{-1} (Square Pharmaceutical Ltd., Bangladesh) were used as standard antibiotics respectively. As a negative control, a blank disc impregnated with solvent followed by drying off was used.

A total of five gram positive and nine Gram-negative bacteria (Table 1) and eight pathogenic fungi (Table 2) were used in this antimicrobial screening. Briefly in disc diffusion method, the molten nutrient agar medium (Difco) for bacteria and molten potato dextrose agar medium for fungi

Table 1: Antibacterial activity of the ethyl acetate extract

		Diameter of zone of inhibition (mm)		
Test organisms	Strain No.	100 μg disc^{-1}	30 μg disc^{-1}	Kanamycin 30 μg disc^{-1}
Gram positive bacteria				
Staphylococcus aureus	-	24	14	34
Streptococcus-β-haemolyticus	CRL	23	11	25
Bacillus megaterium	QL-38	20	10	21
Bacillus subtilis	QL-40	19	9	16
Sarcina lutea	QL-166	20	8	16
Gram negative bacteria				
Salmonella typhi	-	25	10	18
Shigella dysenteriae	AL-35587	27	12	21
Shigella shiga	-	21	10	19
Shigella flexneri	AL-30372	24	10	22
Shigella sonnei	AJ-8992	19	11	25
Shigella boydii	AL-17313	21	10	34
Escherichia coli	FPFC-281	23	12	24
Pseudomonas aeruginosa	CRL	28	13	30
Klebsiella species	-	26	12	24

Table 2: Antifungal activity of the ethyl acetate extract

		Diameter of zone of inhibition (mm)	
Test fungus	Strain No.	Ethyl acetate extract (200 μg disc^{-1})	Nystatin (20 μg disc^{-1})
Candida albicans	ATCC-10231	19	22
Aspergillus fumigatus	-	12	19
Aspergillus flavus	CRL	22	23
Aspergillus niger	CRL	21	24
Epidermophyton floccosum	CRL	18	21
Trichoderma species	-	19	30
Fusarium species	-	18	26
Bipolavis species	-	16	24

respectively was poured in 15 mL quantity in each in the clean test tubes and plugged with cotton. The test tubes and a number of Petridishes were sterilized in an autoclave at 121°C and 15 Ibs^{-1} sq. inch pressure for 30 min and were transferred into laminar airflow unit and then allowed to cool to about 45 to 50°C. The test organisms (both bacteria and fungi) were transferred from the fresh subculture to the test tube containing 15 mL autoclaved medium with the help of an inoculating loop in an aseptic condition. Then the test tube was shaken by rotation to get a uniform suspension of these organisms. These suspensions were immediately transferred to the sterile Petridishes in an aseptic area. The Petridishes were rotated several times, first clockwise and then anticlockwise to assure homogenous distribution of the test organisms. The media were poured into Petridishes in such a way as to give a uniform depth of approximately 4 mm. For the placement of the discs, by means of a pair of sterile forceps, the sample impregnated discs and standard disc were placed gently on the solidified agar plates seeded with the test organisms to ensure contact with the medium. The plates were then kept in a refrigerator at 4°C for 24 h in order to provide sufficient time to diffuse the extract into the medium.

The petridishes were then incubated at 37°C for overnight to allow the bacterial growth and 72 h for fungal growth. The antibacterial and antifungal activities of the extract were then determined by measuring the respective zones of inhibition in mm.

For demonstrating the antibacterial activity and subculture of the test organisms nutrient agar medium (Difco) was used. Nutrient broth (Difco) was used as liquid culture of all the tested bacteria and is used in the minimum inhibitory concentration determination experiments. Potato Dextrose Agar (PDA) media was prepared in the lab to maintain the fungal growth. Antifungal activity of the complexes was done on PDA media spreading with fungal spores and kept at 28°C for about 72 h. For

PDA preparation 20 g potato was extracted with distilled water 100 mL at 100°C for 1 h and it was then filtered off by cotton filter. The potato juice (100 mL) was then mixed with 2 g Dextrose and 1.5 g agar and finally the pH of the prepared media (PDA) was adjusted at 7.00.

A current definition of the Minimum Inhibitory Concentration, MIC, is the lowest concentration which resulted in maintenance or reduction of inoculum viability (Carson *et al*., 1995). The determination of the MIC involves a semi quantitative test procedure, which gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth. The method displays tubes of growth broth containing a test level of preservative, into which an inoculum of microbes was added. The end result of the test was the minimum concentration of antimicrobial (test materials) which gave a clear solution, i.e., no visual growth (Collins, 1964; Davidson and Parish, 1989). Serial dilution technique (Reiner, 1982) was applied for the determination of minimum inhibitory concentration of the extracts. The minimum inhibitory concentrations (MICs) of the crude extract against *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhi*, *Shigella dysenteriae*, *Candida albicans*, *Aspergillus flavus* and *Aspergillus niger* were determined by serial dilution technique (Reiner, 1982; Noble and Sykes, 1977) because of maximum antimicrobial activities against these organisms. In serial dilution technique, Bacterial suspensions and fungal suspensions were prepared in sterile nutrient broth medium (Difco) and potato dextrose broth medium respectively in such a manner so that the suspension contains 10^7 cells mL^{-1}. These suspensions were used as inoculums. Thirteen autoclaved test tubes were taken, ten of which marked as 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 and the rest three were assigned as C_m (medium), C_S (medium + compound) and C_i (Medium + inoculum). One milliliter of sterile nutrient broth medium or potato dextrose broth medium was added to each of the twelve test tubes. One milliliter of the sample solution was added to the first test tube and mixed well. One milliliter content from the first test tube was transferred by the sterile pipette to the second test tube and mixed uniformly. Then 1 mL of this mixture was transferred to the third test tube. This process of serial dilution was continued up to the tenth test tube. One drop (10 μL) of properly diluted inoculum was added to each of the ten test tubes and mixed well. For the control test tube, C_S 1 mL of the sample solution was added, mixed well and 1 mL of this mixed content was discarded. This was to check the clarity of the medium in presence of diluted solution of the compound. Ten microliter of the inoculum was added to the control test tube, C_i to observe the growth of the organism in the medium used. The control test tube, C_m containing medium only was used to confirm the sterility of the medium. All the test tubes were incubated at 37.5°C for 18-24 h.

RESULTS AND DISCUSSION

The ethyl acetate extract showed significant antimicrobial activities (both antibacterial and antifungal activities) against the selected test pathogens in comparison with that of standard kanamycin and nystatin (Table 1 and 2). However, the activity profile of the extract was investigated that all the Gram positive and Gram negative bacteria showed a potent sensitivity towards the crude extract. Moreover, better antibacterial activities were observed against *Staphylococcus aureu*, *Streptococcus-β-haemolyticus*, *Salmonella typhi*, *Shigella dysenteriae*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Klebsiella* species in comparison with other pathogens.

During the antifungal testing, the crude ethyl acetate extract showed better antifungal activities against *Aspergillus niger*, *Aspergillus flavus*, *Candida albicans* and *Trichoderma* species than the other fungi ranging from 19-22 mm in diameter in zone of inhibition in comparison with that of standard nystatin ranging from 19-30 mm in diameter.

The Minimum Inhibitory Concentrations (MICs), observed by serial dilution technique (Reiner, 1982) were found to be 4 μg mL^{-1} against *Shigella dysenteriae* and *Aspergillus niger*; 8 μg mL^{-1} against *Bacillus subtilis*, *Bacillus megaterium*, *Salmonella typhi*, *Klebsiella* species and *Bipolavis* species;

Table 3: The results of MIC values of ethyl acetate extract (μg mL^{-1})

Test organisms	Extract	Kanamycin	Nystatin
Bacillus subtilis	8	2	-
Staphylococcus aureus	16	8	-
Streptococcus-β-haemolyticus	32	8	
Bacillus megaterium	8	4	
Sarcina lutea	16	8	
Shigella dysenteriae	4	2	-
Salmonella typhi	8	4	-
Shigella shiga	32	8	-
Shigella flexneri	-	16	-
Shigella sonnei	16	4	-
Shigella boydii	16	4	-
Escherichia coli	16	8	-
Pseudomonas aeruginosa	-	16	-
Klebsiella species	8	4	-
Aspergillus flavus	16	-	8
Aspergillus niger	4	-	4
Candida albicans	16	-	4
Aspergillus fumigatus	16	-	8
Epidermophyton floccosum	-	-	8
Trichoderma species	16	-	8
Fusarium species	16	-	8
Bipolavis species	8	-	4

16 μg mL^{-1} against *Staphylococcus aureus*, *Sarcina lutea*, *Shigella sonnei*, *Shigella boydii*, *Escherichia coli*, *Aspergillus flavus*, *Candida albicans*, *Aspergillus fumigatus*, *Trichoderma* species and *Fusarium* species while 32 μg mL^{-1} against *Streptococcus-β-haemolyticus*, *Shigella shiga*. The MIC values of the extract against the tested organisms indicated their noticeable antibacterial and antifungal potencies compared with standard antibiotic, kanamycin and nyststin respectively (Table 3). However, further work is necessary in order to isolate active antimicrobial compounds from this organism.

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