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Antimicrobial and Cytotoxic Activities of Secondary Metabolites Obtained from a Novel Species of *Streptomyces*

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Abstract

With an aim to isolate actinomycetes having antimicrobial and cytotoxic activities, five colonies of actinomycetes were collected from soils of a vegetable garden of Rajshahi District, Bangladesh and designated as ANTS-1 to ANTS-5. Initial screening for antibacterial activity showed that the isolate ANTS-1 produced potent antimicrobial metabolites in the culture media. The crude ethyl acetate extract obtained from the yeast-extract glucose agar (YEGA) medium showed strong activity against pathogenic bacteria and moderate activity against fungi. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the extract against the test bacteria were in the range of 1-2 μg/ml and 4-8 μg/ml, respectively. The LC_{50} value of the crude extract against brine-shrimp nauplii was only 0.4μg/ml indicating its potent cytotoxic activity. One component of the extract was identified as actinomycin D by interpretation of the spectral data and by comparison with published values.

Keywords: Actinomycetes, secondary metabolites, MIC, MBC, brine shrimp lethality

Introduction

The actinomycetes are an important part of the microbial community in the soil environment, responsible for degradation and recycling of natural biopolymers, such as cellulose, lignin and chitin (Semeˆdo *et al*., 2001). It is also a source of a wide range of other types of bioactive compounds for biotechnological applications (Okami and Hotta, 1988; Bull *et al*., 1992). The species belonging to the genus *Streptomyces* constitute 50% of the total population of soil actinomycetes and are well known for producing a variety of bioactive secondary metabolites including antibiotics, immunomodulators, anticancer & antiviral drugs, herbicides, and insecticides (Rahman *et al*., 2010). Although thousands of antibiotics have been isolated from *Streptomyces*, these represent only a small fraction of the repertoire of bioactive compounds produced (Berdy, 1995; Watve *et al*., 2001). So, still there is a chance of discovery of new *Streptomyces* species producing novel compounds from this genus. However, the frequency of rediscovery of known compounds by *Streptomyces* strains was fairly high (Huck *et al.*, 1991).

From the soil samples of Bangladesh, previously we discovered several new species of actinomycetes (e.g., *Streptomyces bangladeshensis*) as well as some novel bioactive compounds having significant biological activities (Al-Bari *et al.*, 2005; Rahman *et al.*, 2010). Recently, we reported the isolation and characterization of a *Streptomyces* sp. ANBS-15 along with its antibacterial activities. This is also derived from soils and the initial screening showed interesting antimicrobial and antifungal activities (Biswas *et al.*, 2011).

In the present study, we report the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and brine shrimp lethality bioassay of a crude extract obtained from the solid fermentation media of a novel species of actinomycetes, *Streptomyces banglaensis* ANTS-1.

Materials and Methods

Sampling and isolation of actinomycetes: The soil sample was collected from a vegetable garden of Rajshahi city, Bangladesh from the depth of 3 inches. In this isolation method soil suspension made from dried soil was used for isolation of actinomycetes (especially *Streptomyces*) with antibiotic activity. The isolation media contains starch and casein as sole carbon and energy sources. Only organisms capable of degrading these complex polymers (mostly molds and *Streptomyces*) are

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able to grow (Figure 1). Total five actinobacteria isolates, ANTS-1, ANTS-2, ANTS-3, ANTS-4 and ANTS-5, were isolated from the dilution plates on yeast-extract glucose agar (YEGA) supplemented with cycloheximide (Figure 2). The isolates were then tested for antimicrobial activity (Figure 3) and the isolate ANTS-1 was selected.

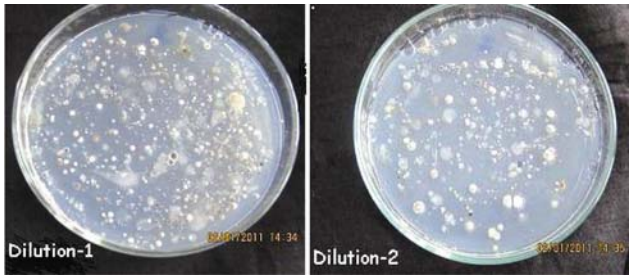

Figure 1. Colonies of actinomycetes appeared on the dilution plates using the soil samples collected from vegetable garden

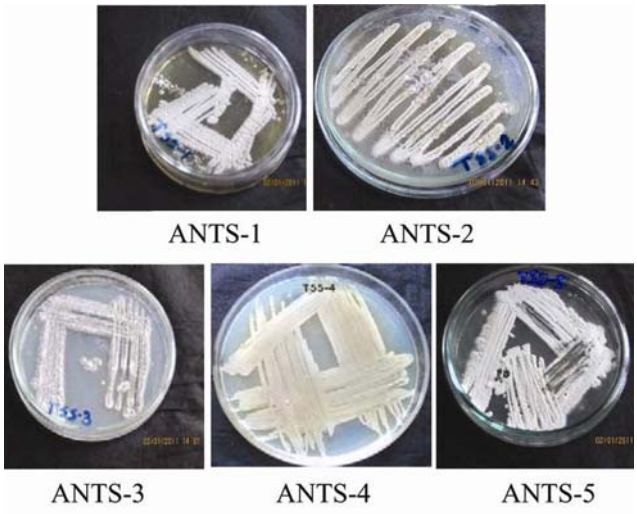

Figure 2. Actinomycete isolates collected from a vegetable garden of Rajshahi city.

Optimization and cultural conditions: The isolate ANTS-1 was grown on different culture media yeast-extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), tyrosine agar (ISP-7), trypton-yeast extract agar (ISP-1), nutrient agar, czapek-Dox (acidic), czapek-Dox (basic) and yeast-extract glucose agar (YEGA) (Shirling and Gottlieb, 1966) and the growth was observed. The strain ANTS-1 was assigned to the genus *Streptomyces* based on it phenotypic and cultural characteristics. The 16s rDNA sequence of the isolate ANTS-1 has been submitted to the GenBank with the accession number JF812169. The isolate was sub cultured and maintained in slant culture at 4°C. For small scale fermentation yeast-extract glucose agar (YEGA) medium consisting of yeast-extract-1.0g, glucose- 10.0g, KNO_3- 0.5g, K_2HPO_4- 0.1 g & agar - 15 g per liter) was used.

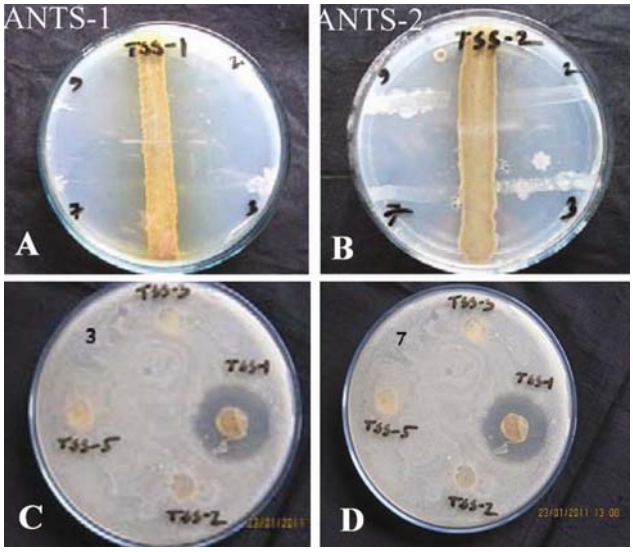

Figure 3. Screening for the antibacterial activities of the isolates through cross streaking method (A, B) and plug technique (C, D) on *Streptococcus agalactiae* (2), *Bacillus* cereus (3), *Pseudomonas aeruginosa* (7) & *Escherichia coli* (9).

Fermentation, extraction and purification of secondary metabolites: For the secondary metabolites small scale solid state fermentation was performed. The isolate ANTS-1 from the slant cultures was used as inoculum and streaked on a number of petri dishes containing YEGA medium. The petri dishes were then incubated at 28°C for 14 days. After 14 days the medium became yellowish orange due to the production of secondary metabolites. The medium from all the petri dishes were cut into pieces and collected in beakers containing ethyl acetate. The beakers were then covered and kept for extraction for three days. The extracts were then filtered and evaporated under reduced pressure with a rotary vacuum evaporator at 45°C until a reddish orange solid mass was obtained. In this way 400mg of crude extract was obtained from 5 liter of fermentation medium.

For separation of constituents from the crude extract, silica gel plates (20 x 20 cm, 1 mm thick) were prepared. They were activated at 150°C for half an hour. Ten microliters of the ethyl acetate soluble fractions were applied on the plates and the chromatogram was developed using 100% ethyl acetate as solvent system. The separated bands were cut off and eluted with solvent

and air-dried. The purity was further confirmed by analytical TLC.

In vitro antimicrobial screening: The antibacterial and antifungal activities of the crude extract was evaluated by the disc diffusion method (Bauer *et al*., 1996; Serrano *et al.*, 2004) against 3 Gram positive and 2 Gram negative pathogenic bacteria and 3 fungi (Table 1) using kanamycin and nystatin as standards. The antibacterial and antifungal activity tests of the crude extract were carried out at 30 μg/disc. The discs were placed on test organism seeded plates. The activity was determined after 24 hours of incubation at 37ºC. The diameter of the zone of inhibition was measured with a transparent scale in mm.

The minimum inhibitory concentration (MIC)) was determined (Table 1) using serial tube dilution technique (Reiner 1982; Tyler *et al.,* 1988). The minimum bactericidal concentration (MBC) was determined by sub-culturing the contents of the tubes of MIC showing no growth after adding 5ml of nutrient broth medium and incubating at 30°C for 24 hrs.

Test microorganisms: In this study three gram-positive and two gram negative bacteria were used. The gram-positive bacteria were *Bacillus cereus, Streptococcus agalactiae, and Bacillus subtilis* and the gram-negative bacteria were *Escherichia coli* and *Pseudomonas aeruginosa*. The test fungi were *Candida albicans, Aspergillus niger* and *Aspergillus flavous*. The microorganisms were obtained from the Institute of Nutrition and Food Science (INFS), University of Dhaka and International Center for Diarrheal Disease Research, Bangladesh (ICDDR, B).

Brine shrimp lethality bioassay: Brine shrimp lethality bioassay (Meyer *et al.,* 1982; Mclaughlin *et al.,* 1988) technique was applied for determination of general toxic properties of the extracts. DMSO solutions of the extract were applied against *Artemia salina* in a 1-day *ex-vivo* assay (Table 2). The eggs of the brine shrimp, *Artemia salina*, were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial sea water (3.8% NaCl solution) for 48 hr to mature shrimp called nauplii. 10 mg of the crude extract was accurately weighed and dissolved in 1 ml DMSO. Thus a concentration of 10 mg/ml was obtained which was used as a stock solution. Then a series of concentrations 160, 80, 40, 20, 10, 8, 6, 5, 4, 2, 1, 0.1, 0.2, 0.3, 0.4 and 0.5 μg/ml were made from the stock solution. Vials containing 10 μl and 0.5μl of DMSO diluted to 5ml were used as a control. Then 10 matured shrimp nauplii were applied to each of all experimental and control vials. The number of the nauplii that died after 24 hr was counted.

The findings were presented graphically by plotting concentration versus percentage of mortality of nauplii from which LC_{50} was determined by extrapolation.

Result and Discussion

Among the five isolates, ANTS-1 was selected due to its potent antimicrobial activity (Figure 3). The small scale fermentation was carried out on YEGA medium because the isolate ANTS-1 greater production of secondary metabolite on solid medium than on broth medium.

The crude extract exhibited strong antimicrobial activity against the test organisms (Table 1). The result indicates that the compound is more active against gram positive bacteria than gram-negative bacteria. The MIC value and the MBC of the extract against *Bacillus cereus, Streptococcus agalactiae, Pseudomonas aeruginosa* and *Escherichia coli* were 1, 1, 1 & 2 μg/ml and 4, 4, 4 & 8 μg/ml respectively. Therefore, the extract is bactriostatic not bactericidal. The crude extract was also active against *Candida albicans* and *Aspergillus flavous* and the minimum antifungal concentration values were 64 and 8 μg/ml, respectively.

Brine shrimp lethality bioassay was performed to determine the cytotoxicity of the compound (Table 2). 100% mortality of the nauplii was found at concentrations of 1, 2, 4, 5,6, 8, 10, 20, 40, 80 and 160 μg/ml, and the test was again performed at lower concentrations 0.1, 0.2, 0.3, 0.4 and 0.5 μg/ml and the LC_{50}, value was determined to be 0.4μg/ml (Figure 4).

Total three compounds, AS-1, AS-2 & AS-3, were separated and purified from the crude extract by preparative thin-layer chromatography (PTLC). Among the three PTLC fractions, the compound AS-3 was further analyzed due to its higher antimicrobial activity and greater purity. AS-3 was soluble in methanol, ethyl acetate and DMSO. Its melting point was in the range of 240-246°C. The structure of the compound was elucidated by - ^{1}H-NMR and ^{13}C-NMR. These spectral data were compared with the published data (Chin and Tseng, 1992) and AS-3 was identified as Actinomycin D.

Table 1. Antibacterial activity of the crude extract against the test organisms (MIC, Minimum Inhibitory Concentration, MBC, Minimum Bactericidal Concentration)

Test microorganisms	Diameter of zone of inhibition (mm)		MIC (µg/ml)	MBC (µg/ml)
	Crude Extract (30µg/disc)	Standard antibiotic (30µg/disc)		
Gram positive bacteria		**Kanamycin**		
Bacillus cereus	23	23	1	4
B. subtilis	20	22	ND	ND
Streptococcus agalactiae	23	22	1	4
Gram negative bacteria		**Kanamycin**		
Escherichia coli	23	18	2	8
Pseudomonas aeruginosa	23	12	1	4
Fungi		**Nystatin**		
Aspergillus niger	0	22	ND	ND
A. flavous	20	23	8	16
Candida albicans	8	22	64	64

ND = Not determined

Table 2. The results of brine shrimp lethality bioassay of the crude extract obtained from the solid state fermentation medium of *Streptomyces* sp. ANTS-1

Group	Conc. of sample (µg/ml)	No. of shrimp added	No. of death in each vial			Average No. of death	% of Mortality	LC_{50} µg/ml
			1	2	3			
Control	0.5µl DMSO	10	0	0	0	0	0%	0.4
Crude extract	0.1	10	0	0	0	0	0%	
	0.2	10	3	2	4	3	30%	
	0.3	10	4	4	4	4	40%	
	0.4	10	5	6	4	5	50%	
	0.5	10	6	6	6	6	60%	

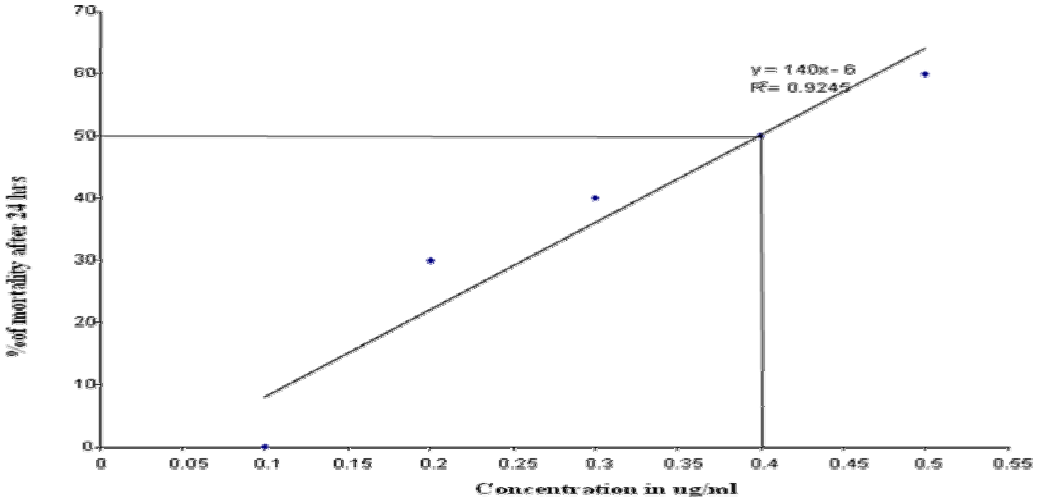

Figure 4. Relation between concentration of the crude extract and percentage of mortality of brine shrimp nauplii.

Conclusion

The isolate described in this study was identified as a novel species of the genus *Streptomyces* (unpublished report). Small-scale solid fermentation using YEGA media was used to produce bioactive metabolites from this isolate. The crude extract was used to determine the MIC and MBC against the test bacteria and fungi. As the MIC of the crude extract was lower that MBC the extract was bacteriostatic not bactericidal. Furthermore, the potential cytotoxicity of the compounds also suggests that the

metabolites present in the extract have the potential to exhibit antitumor or anticancer activities. Due to very-low yield of the crude extract, we could not use the separated compounds for biological investigations. Further works are required to increase the yield of secondary metabolites under optimized conditions. The structures of all of the compounds along with their detailed biological activities also need to be determined.

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