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Antibacterial and Cytotoxic Activities of Crude Ethyl Acetate Extract of *Streptomyces* sp. FEAI-1 Isolated From Soil Samples of Rajshahi, Bangladesh

Most. Farida Khatun, Md. Uzzal Haque and Md. Anwar Ul Islam

Department of Pharmacy, University of Rajshahi, Rajshahi-6205, Bangladesh

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Abstract

The present study was aimed to isolate and investigate actinomycetes having antimicrobial and cytotoxic activities from soil samples of Mirzapur, Rajshahi, Bangladesh. Total 27 bacteria were isolated and initial screening found that *Streptomyces* species have low to moderate antagonistic property against various pathogenic bacteria and among them EFAI-1was quite interesting. The antimicrobial activity of crude ethyl acetate extract obtained from EFAI-1was determined using broth-dilution method against *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Shigella sonnei* and *Salmonella* typhi. The crude extract was almost equally active against both Gram-positive and Gram-negative bacteria. The highest zone of inhibition was found 21 mm at a concentration of 100 µg/disc against *Salmonella* typhi. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the crude extract against the test bacteria were in the range of 15.6-125 µg/ml and 62.5-250µg/ml, respectively. We found that the LC_{50} value of the crude extract was only 0.13 µg/ml against brine-shrimp nauplii indicating its potent cytotoxic nature. Our results indicate that this bacterium would be an excellent source of potent antibiotic and anticancer drugs.

Key words: Actinomycetes, *Streptomyces* sp. FEAI-1, Antibacterial, cytotoxic, Bangladesh.

Introduction

The problems of drug resistance, patient's sensitivity and inability to control certain infectious diseases have given an importance for continuous search of new antibiotic all over the world. To combat the multidrug resistant organisms, production of new potent antibiotics from new source is urgently needed (El-khawaga and Megahed, 2012). Screening of microorganisms for the production of potent antibiotics has been intensively used for many years by researchers (Oskay*et al.,* 2004). Actinomycetes are the most widely distributed groups of microorganisms in the nature. They are attractive, bodacious and charming filamentous gram-positive bacteria with DNA rich in G+C from 57-75%. They make up in many cases, especially under dry alkaline conditions, a large part of the microbial population of the soil (Athalye *et al.,* 1981; Goodfellow and Williams, 1983; Lacey, 1997).They produce numerous substances essential for health such as antibiotics, enzymes, immunomodulators, hormones, anticancer and antiviral drugs, herbicides, and insecticides (Bachmann and McCarthy, 1991; Tamamura *et al.,* 1985). The productivity of *Streptomyces* strains as antibiotic producers remains unique amongst Actinomycetes strains. 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 the isolation and characterization of new *Streptomyces* species producing potent bioactive compounds from this genus.

A few studies have been conducted for isolation of potent *Streptomyces* sp. from soils of Bangladesh. *Streptomyces bangladeshensis* was the first report of

Correspondence to: Md. Anwar Ul Islam; E- mail: profanwarulislam@yahoo.com; Tel: +88-0721-750071

the discovery of a new species of *Streptomyces*, from soil samples of Bangladesh producing bis-(2-ethylhexyl)-phthalate (Al-Bari *et al.,* 2005). Therefore the present study was undertaken to isolate and screen bioactive *Streptomyces* sp. from soils of Rajshahi city, Bangladesh. This report describes the antimicrobial and cytotoxic activities of *Streptomyces* sp. FEAI-I isolated from soils of Rajshahi, Bangladesh.

Materials and Methods

Sample collection and isolation of actinomycetes: Total 11 soil samples were collected from different places of Mirzapur, Rajshahi city, Bangladesh from the depth of 8 inches to 1.5 feet. The soil samples were dried in a hot air oven at 60-65^{o}C for about three hours to reduce the number of bacteria in the soil other than actinomycetes (especially *Streptomyces*) and soil suspensions were made using sterilized distilled water. The isolation media contains starch and casein as sole carbon and energy sources was used for isolation of bacteria. The organisms whose are capable of degrading these complex substances able to grow the plate (Figure 1). By using spread plate technique (Bernard, 2007), isolation of the *Streptomyces* sp. from these soil samples were done. A total of twenty eight actinomycetes [FEAI-1 to FEAI-27] were isolated and purified as pure culture from these soil samples. All of these purified isolates were preserved on yeast-extract-glucose-agar slants at 4^{o}C. Then by using streak plate technique (Alcamo *et al.,* 2004) on yeast extract-glucose-agar medium, all of these pure isolates were preliminary screened for antibacterial activity and FEAI-1 was selected (Figure 2).

Optimization and cultural conditions: The isolate FEAI-1 was grown on different ISP culture media. Among the minorganic salt-starch agar media (ISP Medium 4) was best for the culture of FEAI-1. The strain FEAI-1 was assigned to the genus *Streptomyces* sp. based on it microscopic and cultural characteristic (Figure 3).

Figure 1. Colonies of actinomycetes appeared on the dilution plates using the soil samples.

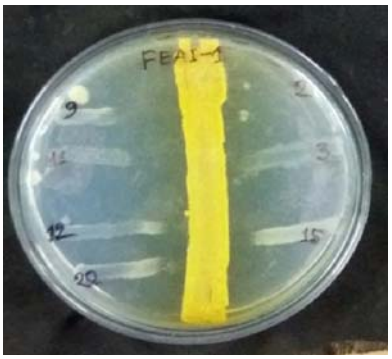

Figure 2. Screening for the antibacterial activities of the isolate FEAI-1 through cross streaking method, *Bacillus cereus* (2), *Staphylococcus aureus* (3), *Escherichia coli* (9), *Shigella dysenteriae* (11), *Shigella sonnei* (12), *Agrobacterium* sp. (15), *Shigella boydii* (20).

Figure 3.Microscopic view of actinomycetes FEAI-1.

*Fermentation and extraction of secondary metabolites:*For small scale fermentation inorganic salt-starch broth medium (consisting of soluble starch 10. 0 g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 1.0g, NaCl 1.0 g, $(NH_4)_2SO_4$ 2.0 g, $CaCO_3$ 2.0 g, Trace salts solution 1.0 ml &Distilled water 1000 ml) was used. A loopful of the organisms of the strain FEAI-1 was added to 200 ml flask containing inorganic salt-starch broth medium. The flasks were shaken in a rotary shaker at 220 rpm at 30^{o}C for 3 days. This seed culture was used to inoculate a number of 500 ml conical flasks containing 200 ml broth medium. These flasks were then incubated at 30 °C for 7 days. After the incubation the cell biomass were separated by filtration through sterile Whatman filter paper no. 1 and the filtrate was partitioned with ethyl acetate on the basis of best solubility and maximum antimicrobial activities. The ethyl acetate fraction was evaporated under reduce pressure in a rotary vacuum evaporator at 45^{o}C until a solid mass was obtained and the weight of the solid extract was determined. This crude bacterial extract was then evaluated for antibacterial and cytotoxic activities.

Determination of antibacterial activity: Antimicrobial activity of the crude extract of FEAI-1 was evaluated *in vitro* against three gram-positive bacteria (*Bacillus cereus, Listeria monocytogenes* and *Staphylococcus aureus*) and three gram-negative bacteria (*Escherichia coli, Shigella sonnei* and *Salmonella* typhi) by disc diffusion assay (Bauer *et al.,* 1966). These organisms were available in the Pharmaceutical Microbiology Research Laboratory of the Pharmacy Department, Rajshahi University, Bangladesh. Nutrient agar was sterilized in a flask and cooled to 45-50°C and then taken in sterilized petridishes with a diameter of 120 mm. The filter paper discs (6 mm in diameter) were impregnated with the crude extract at 100µg/disc and then placed onto the previously inoculated agar plates with the test microorganisms. Kanamycin was used as standard at the dose of 30 µg/disc. The petridishes were kept at 4°C for 1 hour. The plates were incubated at 37°C for 16 h to allow the growth of the microorganisms. The antimicrobial activity was determined by measuring the size of the inhibition zone in mm.

Determination of minimum inhibitory concentration (MIC) & minimum bactericidal concentration (MBC): MIC of the extract was determined by serial tube dilution technique or turbidimetric assay against *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Shigella sonnei* and *Salmonella* typhi (Reiner, 1982). The crude extract was serially diluted in sterile nutrient broth media. Various concentrations of the extract were prepared from the stock solution and 60 µl of properly diluted inoculums was added to the broth containing the extract. The tubes were incubated aerobically at 37ºC for 18-24 h. Two separate control tubes for each organism were maintained. The lowest concentration of the extract that produced no visible bacterial growth (no turbidity) when compared with the control tubes was regarded as the MIC (Hassan *et al.,* 2009).The minimum bactericidal concentration (MBC) was determined by subculturing the contents of the tubes

of MIC showing no growth after adding 5ml of nutrient broth medium and incubating at 37ºC for 24 hrs.

Cytotoxic Bioassay: The cytotoxicity assay of the crude bacterial extract was determined by brine shrimp lethality bioassay (Mayer *et al.,* 1982). The result of the brine shrimp lethality bioassay was given in Table 2. In this method, the eggs of the brine shrimp, *Artemia salina*, were collected from an aquarium shop (Dhaka, Bangladesh) and hatched for 48 h to mature shrimp called nauplii. Constant oxygen supply was provided and temperature (37±1)°C was maintained for 48 h in seawater to hatch and mature the shrimp. The test sample was prepared by dissolving them into DMSO (not more than 50 µl in 5 ml solution) plus sea water (3.8% NaCl in water) to attain concentrations 05, 0.1, 0.2 and 0.3 µg/ml. A vial containing 20 µl DMSO diluted to 5 ml with water was used as a control. Vincristine sulfate was used as standard in this test. 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

The antibacterial activity of the extract was investigated against six pathogenic bacteria by agar well diffusion method. The result of the antibacterial activity of the crude bacterial extract (100 µg/disc) was given in the Table 1.The zone of inhibition of this extract was lower than standard kanamycin (30 µg/disc) against both gram-positive bacteria and gram-negative bacteria. We found that the extract showed antibacterial activity against *Bacillus cereus* (19 mm), *Listeria monocytogenes* (15 mm), *Staphylococcus aureus* (13 mm), *Escherichia coli* (14 mm), *Shigella sonnei* (18 mm) and *Salmonella* typhi (21 mm).Among the test bacteria, *Salmonella* typhi and *Bacillus cereus* were inhibited to high extent followed by *Shigella sonnei*, *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus*. The results of our study we found that our extract was moderately active against pathogenic bacteria which is comparable with our previous studies. In a previous study found that the extract had 32 mm (50 µg/disc) inhibition zone against *Klebsiella pneumoniae* (Oskay *et al.,* 2004). Another study conducted by Singh *et al.,* (2009) reports that the extract of *Streptomyces tanashiensis* strain A2D showed antibacterial activity against *B. subtilis* was 15 mm, *Staphylococcus aureus* 25 mm, *E. coli* 21 mm and *Klebsiella pneumoniae* 23 mm.

MIC of crude bacterial metabolite was determined by broth dilution method against six pathogenic bacteria *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus, Escherichia coli, Shigella sonnei* and *Salmonella* typhi. The results of MIC determination against bacteria are shown in the Table 1. The MIC value of crude extract varies in between 15.6-125 µg/ml. The lowest MIC value 15.6 µg/ml was found against *Listeria monocytogenes*, whereas 62.5 µg/ml MIC value for *Shigella sonnei* and *Salmonella typhi*. The highest MIC value 125 µg/ml was found against *Bacillus cereus, Staphylococcus aureus and Escherichia coli.*The minimum bactericidal concentration (MBC) ranges in between 62.5-250 µg/ml. This value of MIC was lower than the values of MFC. Therefore, the crude extract is bacteriostatic not bactericidal.

The results of the brine shrimp lethality bioassay are shown in the Table 2. In the experiment, mortality rate of the brine shrimp nauplii increased with the increase in the concentration of the test sample i.e., at higher concentration, the mortality was higher. The lethal concentration 50 (LC_{50}) of our crude extract was found to be 0.13 µg/ml (Figure 1). LC_{50} values greater than 1000 µg/ml were regarded as nontoxic. Therefore the crude bacterial extract is highly toxic.

A lot of previous studies have been conducted on cytotoxic nature of *Streptomyces* sp. extracts and purified metabolites on brine shrimp. A study conducted by Sharmin *et al.,* (2013) a novel *Streptomyces* sp. isolated from soils of Rajshahi city, Bangladesh showed marked cytotoxic effect against

brine shrimp. The ethyl acetate extract and a purified compound of *Streptomyces rajshahiensis* exhibited potent lethal effect against brine shrimp (Ripa*et al.*, 2010). In our study, we found that the ethyl acetate extract of *Streptomyces* sp. FEAI-1 showed potential cytotoxic effect. So our extract is toxic and may have potential anticancer activity.

Table 1. Antibacterial activity of the crude extract of FEAI-1 against the pathogenic bacteria.

Pathogenic bacteria	Diameter of zone of inhibition (mm)		MIC (µg/ml)	MBC (µg/ml)
	Crude extract (100 µg/disc)	Kanamycin (30 µg/disc)		
Gram positive bacteria				
Bacillus cereus	19	24	125	250
Listeria monocytogenes	15	30	15.6	62.5
Staphylococcus aureus	13	31	125	250
Gram negative bacteria				
Escherichia coli	14	28	125	250
Shigella sonnei	18	24	62.5	125
Salmonella typhi	21	28	62.5	125

MIC = Minimum Inhibitory Concentration; MBC = Minimum Bactericidal Concentration

Table 2. The results of brine shrimp lethality bioassay of the crude extract obtained from the fermentation medium of *Streptomyces* sp. FEAI-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	40µl in 5 ml	10	0	0	0	0.00	0.00	0.00
Crude extract	0.5	10	3	2	1	2	20	0.13
	0.1	10	4	4	6	4.67	46.7	
	0.2	10	8	8	7	7.67	76.7	
	0.3	10	9	10	9	9.33	93.3	

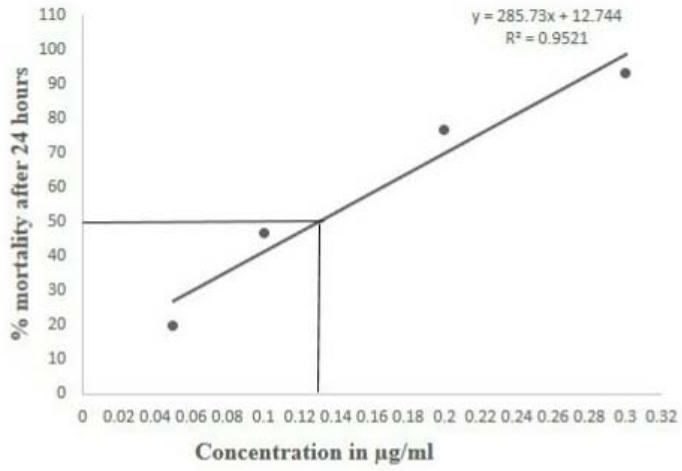

Figure 1. Determination of LC_{50} of extract against *Artemiasalina* larvae.

Conclusion

To discover newpotent antibiotics, screening of bacteria can play a significant role. In our study we isolate a potent *Streptomyces* sp. FEAI-1 from soils of Rajshahi city, Bangladesh. Ethyl acetate extract of this isolate showed promising antibacterial activity. Furthermore, the potential cytotoxicity of the extract also suggests that the extract might have the potential to exhibit antitumor or anticancer activities. This extract might be used in infectious and cancer diseases and further study will be done for the isolation of pure compounds.

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