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In vitro Antibacterial and Cytotoxic Activities of Two Compounds Isolated from *Streptomyces* sp. MBS-15

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Abstract

Two pure bioactive compounds, MB-1a and MB-1b were isolated from a bacterial isolate and the antibacterial activities and brine shrimp lethality bioassay were evaluated in the present investigation. The isolate was collected from soils of an agricultural field located in Rajshahi University campus and characterized as a species of *Streptomyces* genus by polyphasic approach. Two pure compounds were isolated and purified from fermentation broth of this bacterium by solvent extraction followed by chromatographic separation. The MIC and MBC of these compounds were studied against six pathogenic bacteria. The MIC and MBC of both MB1a and MB1b were in the range of 4-8 µg/ml. Same values of MIC and MBC indicates the bactericidal characteristics of both of the compounds. Brine shrimp lethality was evaluated and LC_{50} values of MB1a, MB1b and crude extract was obtained as 9.55 µg/ml, 12.30 µg/ml, and 44.67 µg/ml, respectively.

Key words: MIC, MBC, Antimicrobial, Cytotoxicity and *Streptomyces*.

Introduction

Among the well characterized pharmaceutically relevant microorganisms, actinomycetes remain major sources of novel, therapeutically useful natural products (Jensen and Fenical, 2000). The actinomycetes are gram positive bacteria having high G+C (>55%) content in their DNA. The majority of actinomycetes are free living, saprophytic bacteria, widely distributed in soil, water and colonizing plants. Actinomycetes populations been identified as one of the major group of soil population which may vary with the soil type (Athlete *et al.*, 1981).

Streptomyces are the most well known genus of actinomycete family which always has been notified because of their ability to produce and secrete a large variety of bioactive secondary metabolites. The isolated compounds from soil actinomycetes have a broad spectrum of biological activities such as antibacterial, antifungal, cytotoxic, neurotoxic, antimiotic, antiviral and antineoplastic activities (Black *et al.*, 1982). They produce over two thirds of the clinically useful antibiotics of natural origin. Nevertheless, a periodic replacement of the existing antibiotics is necessary to prevent transmissible resistant microorganisms to the antibiotics already available in the market.

Although thousands of antibiotics have been isolated from *Streptomyces*, these represent only a small fraction of the bioactive compounds produced so far (Berdy, 1995; Watve *et al.*, 2001). Therefore, isolation of new *Streptomyces* from natural resources and characterization of their secondary metabolites is still a valuable endeavor (Rahman *et al.*, 2011).

Using the soil samples of different location 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 (Bari *et al.*, 2005; Hossain *et al.*, 2004; Rahman *et al.*, 2010). Recently, we have 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 intersting antimicrobial and antifungal activities (Biswas *et al.*, 2011). In this present study, we have reported the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and brine shrimp lethality bioassay of two pure compounds obtained form the fermentation broth of this isolate.

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Materials and Methods

Organism: The isolate MBS-15 was isolated from soil sample, collected from a cultivated agricultural field of Rajshahi University campus at a depth of 18 to 36 inch by using selective isolation media. Then it was identified as a representative species of the genus *Streptomyces* by using a polyphasic approach (Biswas *et al.*, 2011).

Fermantation, extraction and purification of secondary metabolites: The maximum secretion of metabolites from the strain was found at the 10th day of incubation in Czapek-dox (acidic, pH 5.3) medium at 37.5°C by maintaining all the physicochemical factors at optimum level for the culture (Sayeed, 2004). The extraction of the metabolites was carried out by ethyl acetate on the basis of best solubility and maximum antimicrobial activities. The ethyl acetate was evaporated using a rotary evaporator at 45-50°C under reduced pressure and lyophilized using a freeze drier (Thermo, USA) (Suthindhiran *et al.*, 2009).

For separation of constituents in 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 ethyl acetate: and *n*-hexane of different ratios as solvent system. The separated bands were cut off and eluted with solvent and air-dried. The purity was further confirmed by analytical TLC.

Minimum inhibitory concentration (MIC): MIC was determined by the broth 2-fold macro dilution method (Andrews, 2001). The pure compounds were serially diluted in Muller Hinton broth for bacteria. Various concentrations of the pure compounds were prepared from the stock solution and 0.1 ml of the culture was added to the broth containing the pure compounds. The tubes were incubated aerobically at 37°C for 24 h for bacteria. Positive controls were prepared separately for bacteria with respective organisms in the same culture media without the pure compounds. After incubation, the tube with least concentration of pure compounds showing no growth was taken as the MIC value for the respective organism and the number of bacteria inhibited was determined by dilution method.

Minimum bactericidal concentration (MBC): The MBC was determined by sub-culturing the contents of the tubes of MIC showing no growth into antibiotic free liquid medium and was examined for bacterial growth. If growth of bacteria was observed in the MIC tubes, it indicated the presence of bacteriostatic agent and in this case the MBC>MIC. No growth of bacteria in the tubes after dilution indicated the presence of bactericidal agent and in this case, MIC=MBC.

Cytotoxicity screening: The cytotoxic activity of the pure compounds and ethyl acetate extract was determined by brine shrimp lethality bioassay (Mayer *et al.*, 1982; Mclaughlin and Anderson, 1988; Mclaughlin, 1992; Persoone, 1988). In this method, the eggs of the brine shrimp, *Artemia salina* Leach, were collected from an aquarium shop (Dhaka, Bangladesh) and hatched for 48 h to mature shrimp. Thirty eight grams of sea salt was weighed, dissolved in 1 L of distilled water, filtered off and was kept in a small tank. They were then added to the divided tank. Constant oxygen supply was provided and temperature (37±1)°C was maintained for 48 h to hatch and mature the shrimp called as nauplii (Larvae). 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 5, 10, 20, 40, and 80 µg/ml. A vial containing 50 µl DMSO diluted to 5 ml with water was used as a control. Then 10 brine shrimp nauplii were applied to each of all experimental vials and control vial. The number of the nauplii that died after 24 h was counted. The findings were presented graphically by plotting log of concentration versus percentage of mortality of nauplii from which LC_{50} was determined by extrapolation using Microsoft Excel 2003. Extracts showing LC_{50} < 1000 were considered to be toxic in the brine shrimp bioassay.

Results and Discussion

In our continuous effort to isolate bioactive compounds from actinomycetes collected from unexplored sources, two distinguished secondary metabolites were isolated from a *Streptomyces* sp. collected from soil sample. In the initial screening, the bacteria showed good antibacterial and antifungal activities. After characterization and optimizing the suitable conditions for maximum metabolite production, small-scale liquid fermentation using Czapek-dox acidic (pH 5.3) and Czapek-dox basic (pH 8) media were carried out to obtain the active principles. The ethyl-acetate extract from these two fermentation media were subjected to chromatographic analysis. Two pure compounds, one

from acidic medium and one form basic medium, were separated and purified by PTLC and designated as MB-1a and MB-1b, respectively. The two compounds were different in their physical characteristics. The R_f values were different under the same solvent system (data not shown).

Preliminary screening of antibacterial activities of the crude extracts as well as the pure compounds showed that both compounds were active against both gram-positive and gram-negative bacteria. Both compounds also showed antifungal activities.

The minimum inhibitory concentrations (MIC) of the compounds were determined by broth dilution method against six test bacteria. The results are shown in Table-1. The MBC of the pure compounds were similar to MIC indicating that the pure compounds MB-1a and MB-1b were bactericidal because the bacteria failed to grow in the tubes of MIC after dilution (Table-1).

Table 1. MIC add MBC values of the isolated compounds

Name of bacteria	Number of bacteria inhibited	Minimum inhibitory concentration (µg/ml)		Minimum bactericidal concentration (µg/ml)	
		MB 1a	MB 1b	MB 1a	MB 1b
Gram-positive					
Bacillus subtilis	0.39×10^6	4	4	4	4
Staphylococcus aureus	0.165×10^6	8	4	8	4
Streptococcus agalactiae	0.325×10^6	4	8	4	8
Gram-negative					
Escherichia coli	0.37×10^6	8	16	8	16
Pseudomonas aeruginosa	0.11×10^6	8	8	8	8
Proteus mirabilis	0.42×10^6	4	16	4	16

Table 2. LC_{50} values of the crude extract and MB-1a and MB-1b from *Streptomyces* sp. MBS-15 on brine shrimp lethality bioassay.

Group	Sample conc. (µg/ml)	Log C	% Mortality after 24 hours	LC_{50} (µg/ml)
Crude ethyl acetate extract	5	0.69	36.6	56.23
	10	1.00	36.6	
	20	1.30	33.3	
	40	1.60	46.6	
	80	2.00	60.0	
MB-1a	5	0.69	36.6	11.22
	10	1.00	50.0	
	20	1.30	60.0	
	40	1.60	66.6	
	80	2.00	70.0	
Mb-1b	5	0.69	36.6	19.95
	10	1.00	40.0	
	20	1.30	50.0	
	40	1.60	60.0	
	80	2.00	66.6	

The cytotoxic activity of the compound MB-1a, MB-1b as well as ethyl acetate extract were investigated in vitro against brine shrimp nauplii. The result is presented in Table-2. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper. The LC_{50} values were obtained by extrapolation. The test samples showing $LC_{50} < 1000$ were considered to be toxic in the brine shrimp bioassay. All of the test samples exhibited significant cytotoxic activities. The pure compound MB-1a and MB-1b was more cytotoxic than crude ethyl acetate extract. The LC_{50} values obtained from the best fit line slope were 11.22µg/ml, 19.95µg/ml and 56.23µg/ml for MB-1a, MB-1b and ethyl acetate extract, respectively. There was no mortality at the control group.

Conclusion

The actinomycete isolate that has been used in this study was a member of the genus *Streptomyces* collected from soil of an agricultural field of Rajshahi University campus. Two compounds MB-1a and MB-1b were isolated form the liquid broth under optimized culture conditions. As the MIC of MB-1a and MB-1b was equal to MBC, they are bactericidal rather than bacteriostatic. Furthermore, the potential cytotoxicity of the compounds

also suggests that they have the potential to exhibit antitumor or anticancer activities. Further studies are required to elucidate the structure of the compounds. The pharmacological activities also need to be confirmed using *in vivo* experiments.

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