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In vitro Toxicological Studies of Metabolites of *Streptomyces* species on Brine Shrimp

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The ethyl acetate extract of yeast extract-glucose broth medium of an Actinomycete strain, *Streptomyces* yielded three active metabolites C-1, C-2 and C-3, among which C-3 was identified as 2-*N*-butanamide-3-methyl-4-methoxy-5-β-L-arabinosyl-propanophenone on the basis of spectral data. The cytotoxic activity of the crude ethyl acetate extract and compound 1, 2 and 3 were determined by brine shrimp lethality bioassay. The compounds showed lower LC_{50} values indicating that these compounds were significantly cytotoxic. The LC_{50} of the crude ethyl acetate extract, C-1, C-2, C-3 and standard ampicillin trihydrate were 6.69, 12.30, 4.67, 6.62 and 10.28 μg ml^{-1} respectively.

Key words: *Streptomyces,* cytotoxicity, brine shrimp and 2-*N*-butanamide-3-methyl-4-methoxy-5-β-L-arabinosyl-propanophenone

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Introduction

Although immeasurable efforts of scientists in antibiotic research as well as commercial success of antibiotics in therapy, have led to the isolation of several thousands of antimicrobial agents from microorganisms, the development of antibiotic resistant strains and new diseases like AIDS have been made attention to scientists and researchers for the development of newer, safer and more effective antimicrobial compounds. *Streptomyces* species is a prominent source of antimicrobial compounds (Reiner, 1982). As part of our on going studies on the bioactive metabolites from microorganisms, soil samples were collected from different parts of Bangladesh and a strain having antimicrobial activity was isolated and identified as *Streptomyces* species which when activated in yeast extract glucose broth medium produced three antimicrobial agents namely C-1, C-2 and C-3 by column and preparative thin layer chromatographic technique (PTLC) (Egon and Stahl, 1969). The compound C-3 was identified by spectral analysis (Sultan, 2002). Owing to paucity of the sample C-1 and C-2, it was not possible to determine the complete structure of these compounds. However, the work is in progress that will be reported elsewhere.

Natural products (extracts and pure compounds) can be tested for their bioactivity by Brine shrimp lethality bioassay method (Mayer, 1982; Persoone, 1980). Here the simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening and fractionation in the discovery of new bioactive natural products. This bioassay is indicative of anticancer, antiviral and cytotoxicity and wide range of pharmacological activities of the compounds. In this paper we are presenting the cytotoxic activities of the ethyl acetate extract and the antibiotic pigment C-1, 2 and 3.

Materials and Methods

Isolation and identification of antagonistic organisms: For screening purpose, dry warm soil samples were collected from 0.25 to 1.5 m depths of different places like roadside, construction site, grave yards, river bank, ploughed field, food wastage, sewage, play ground and under medicinal plant of different parts of Bangladesh. An antagonistic strain was isolated from Shariakandi of Bogra, Bangladesh and its antimicrobial activity was tested by crowded plate technique (Hammond and Lambert, 1978). The organism was identified on the basis of its morphological and biochemical study according to Bergay's Manual of Determinative Bacteriology, 9th edition (John *et al.*, 1994).

Optimum requirements for the production of antimicrobial metabolites: The optimum requirements for maximum yield of antimicrobial compounds from the selected strain of *Streptomyces* species were determined by studying the effect of various physical parameters on the production of antimicrobial compounds against selected test organisms e.g., *Bacillus subtilis* and *Escherichia coli* by disc diffusion method. It was found that the maximum production of antimicrobial compounds from the organism occurred in yeast-extract-glucose broth medium after 10 days of incubation at pH 8 at temperature of 37.5°C and maltose as a carbon source. Ethyl acetate was found to be the most suitable solvent for extraction of antimicrobial compounds from culture filtrate. The ethyl acetate extract was thermostable.

Extraction, isolation and characterization of the compounds: For the collection of metabolites, the *Streptomyces* species was grown optimally in yeast extract glucose broth media at 37.5°C in order to optimum production. The liquid broth was separated through filtration. Then the filtrate was extracted with ethyl acetate and concentrated. By the use of PTLC technique, three compounds C-1, 2 and 3 were isolated using chloroform: methanol (14:1). The compound C-3 was obtained as yellowish red amorphous powder that was tentatively characterized as 3-N-butanamide-4-methyl-5-methoxy-6-L-arabinosyl-propano-phenone on the basis of its spectral data. The structure elucidation of compound C-1 and C-2 are in progress.

Preparation of sea water: Thirty eight grams of sea salt (non-iodized NaCl) was weighed accurately and dissolved in distilled water to make a volume of 1 litre and then filtered off to get a clear solution.

Hatching of brine shrimp: Sea water was kept in small tank and shrimp eggs were added to the one side of the divided tank and this side would attract hatched shrimp through perforation in the dam. Constant oxygen supply was carried out and a constant temperature was maintained. Two days were needed for the shrimps to hatch and mature as nauplii (Larvae). The hatched shrimps were attracted to the lamp on other side of divided tank through the perforations in the dam. These nauplii were taken for bioassay.

Preparation of the sample solution and application of the nauplii to the vial: Three mg of each sample (crude ethyl acetate extract, compound 1, 2 and 3 and standard ampicillin trihydrate) was taken and dissolved in 0.6 ml of Dimethyl sulfoxide (DMSO) to make the concentration of 5 g μl^{-1}.

The experiment was done at five concentrations of each sample. Each concentration contained 3 vials consisting of 10 nauplii in 5 ml of sea water. The concentration of the sample in each vial was made 5,10, 20, 40 and 80 μg ml^{-1}, respectively. The same assay procedure was carried out for standard ampicillin trihydrate. For control group, three vials, each containing 10 brine shrimp nauplii in 5 ml sea water and 20 ml DMSO were taken.

Counting of nauplii: After 24 h, the vials were observed and the number of survived in each vial was counted and the results were noted. From this data, percentage of mortality of the brine shrimp was calculated for each concentration and the median lethal concentration (LC_{50}) values were also determined.

Results and Discussion

The median lethal concentration (LC_{50}) was determined by extrapolation from the graph (Figs. 1 and 2) and the values were found to be 6.69 μg ml^{-1} and 12.30 μg ml^{-1} for ethyl acetate extract and compound 1 respectively. For compound 2, 3 and standard ampicillin trihydrate the LC_{50} values were 4.67, 6.62 and 10.28 μg ml^{-1}, respectively (Table 1).

In this study, the ethyl acetate extract and isolated two compounds C-2 and 3 showed strong positive results, indicating that extract and the compounds are highly cytotoxic as well as biologically active. Each of the test samples showed different mortality rate at different concentrations and was found to be increased with increasing concentration of the sample. Logarithm

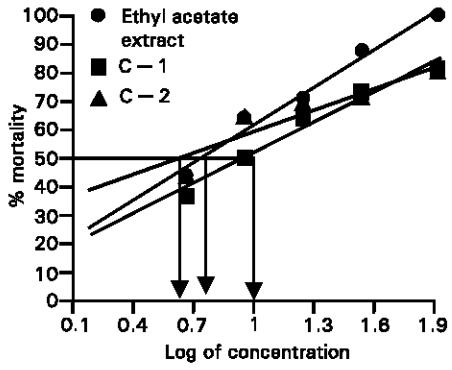

Fig. 1: Brine shrimp lethality bioassay of ethyl acetate extract, C-1 and C-2

Table 1: Results of brine shrimp lethality bioassay

Test samples	Conc. of sample ($\mu g\ ml^{-1}$)	log C	Number of shrimp added	%mortality after 24 h	LC_{50} from the graph ($\mu g\ ml^{-1}$)
Control	0	0.00	10	0.00	0
Ethyl acetate extract	5	0.69	10	43.33	6.69
	10	1.00	10	63.33	
	20	1.30	10	70.00	
	40	1.60	10	86.66	
	80	2.00	10	100.00	
C-1	5	0.69	10	36.66	12.30
	10	1.00	10	50.00	
	20	1.30	10	63.33	
	40	1.60	10	73.33	
	80	2.00	10	80.00	
C-2	5	0.69	10	43.33	4.67
	10	1.00	10	64.45	
	20	1.30	10	68.82	
	40	1.60	10	70.66	
	80	2.00	10	79.49	
C-3	5	0.69	10	47.25	6.62
	10	1.00	10	52.25	
	20	1.30	10	60.00	
	40	1.60	10	65.50	
	80	2.00	10	71.50	
Ampicillin trihydrate	5	0.69	10	44.25	10.28
	10	1.00	10	40.25	
	20	1.30	10	55.00	
	40	1.60	10	63.50	
	80	2.00	10	76.50	

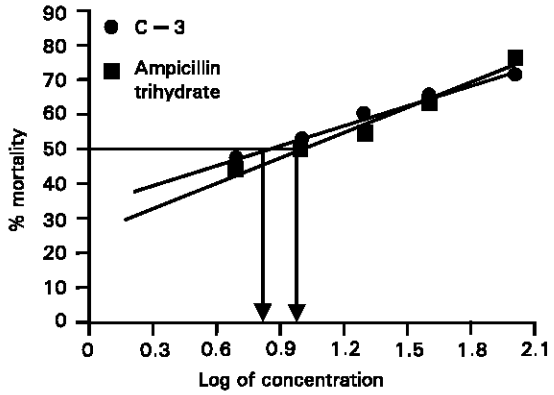

Fig. 2: Brine shrimp lethality bioassay of C-3 and standard ampicillin trihydrate

of concentration versus percentage mortality (Goldstein *et al.*, 1974) was plotted on the graph paper that showed an approximate linear correlation. There was no mortality in the control group. So it is evident that all the test materials were highly lethal to brine shrimp nauplii. However C-2 was more active with minimum LC_{50} value and C-1 was comparatively less active with maximum LC_{50} value.

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