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In vitro Antibacterial and Cytotoxic Activities of a Brown Antibiotic Metabolite from a Strain of *Actinomycetes*

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The research work was conducted to investigate the *in vitro* antibacterial and cytotoxic activities of a antibiotic metabolite. The chloroform ($CHCl_3$) extract of Yeast extract-broth media (acidic) of an *Actinomycetes* strain yielded a brown, amorphous antibiotic pigment Di- (2-ethyl hexyl)-phthalate [AK_2]. The antibiotic and the extract exhibited significant antibacterial activity against both gram positive and gram negative organisms. The zones of inhibition produced by the test materials were found to be between 10 to 22mm. The Minimum inhibitory concentration (MIC) values of the antibiotic were determined against six strains of bacteria and were found to be between 128 to 256µg ml^{-1}. The cytotoxic activity of the antibiotic and $CHCl_3$ extract was done by brine shrimp lethality bioassay and the LC_{50} (median lethal concentration) values were 15.8 and 12.5µg ml^{-1} respectively, calculated by extrapolation from graph.

Key words: *Actinomycetes*, antibacterial activity, cytotoxicity, AK_2

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Introduction

The *Actinomycetes* and in particular the genus *Streptomyces* have been identified as one of the most potent sources for the production of various antibiotics which are used therapeutically (Weese and Smith, 1975) among these streptomycin, kanamycin and neomycin etc. are most important (Goodman and Gilman, 1975). Based on this concept, the field of research for newer antibiotic was broadened. As a part of our continuing studies (Biswas *et al.*, 2000; Azam and Jabbar, 1999), metabolites produced by micro-organisms obtained from soil samples, collected throughout Bangladesh and we isolated a strain of *Actinomycetes* and *Streptomyces* species (Holt *et al.*, 1994) from soil collected in the region of Rajshahi.

From the $CHCl_3$ extract of the yeast extract-glucose-broth culture filtrate of the organism, an antimicrobial agent AK_2 was isolated by preparative thin layer chromatographic technique (PTLC) (Egon and Stahl, 1969) and was identified as Di-(2-ethyl hexyl)-phthalate by spectral analysis.

This paper reported the antimicrobial and cytotoxic activities of the $CHCl_3$ extract and the antibiotic pigment AK_2.

Materials and Methods

Collection of soil sample: The organism was isolated from a soil sample, collected from Upashahar, Rajshahi, Bangladesh at a depth of 1m during July 1999 using crowded plate technique (Hammond and Lambert, 1978). The isolated organism was identified as *Streptomyces* species by its morphological and biochemical characteristics such as carbon utilization test, amino acid utilization test, growth characteristic in milk, potato-agar, cellulose etc. (Holt *et al.*, 1994).

Production of antibiotic: The *Actinomycetes* was cultured in yeast extract broth media at pH 6 (optimum pH) and after 11 days of incubation at 37.5± 0.5°C (optimum temperature) the culture filtrate was extracted with $CHCl_3$. The $CHCl_3$ fraction thus obtained was evaporated to give a brownish solid mass [A mixture of 66mg antibiotic was obtained from 1L of culture filtrate]

Isolation and characterization of the compound: A brown colored compound was isolated by PTLC technique from a crude $CHCl_3$ extract using C_2H_5-O-C_2H_5 and $CHCl_3$ (3:1) solvent system. The isolated compound was named as AK_2. For the characterization of AK_2 physical, chemical and spectroscopic method (^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{1}H COSY and mass spectroscopy) were utilized (Akteruzzaman, 2000).

Antimicrobial assay: The standard test micro organisms were collected from the Institute of Nutrition and Food sciences, university of Dhaka and ICDDRB Dhaka Bangladesh. The antibacterial activity of AK_2 and $CHCl_3$ extract was determined against six gram positive and ten gram negative bacteria by standard disc diffusion method (Barry, 1980; Beur *et al.*, 1966). Kanamycin disk (30µg disc^{-1}) was used as standard. The MIC of the antibiotic AK_2 was determined against six test organisms by simple *in vitro* visual method serial tube dilution technique (Reiner, 1982).

Cytotoxic activity: The cytotoxic activity of the $CHCl_3$ extract and AK_2 was determined by brine shrimp lethality bioassay (Mayer *et al.*, 1982; Mclaughlin and Anderson, 1988).

38g of sea salt was weighted, dissolved in one liter of distilled water, filtered off and was kept in a small tank. *Artemia salina* Leach (brine shrimp eggs) was added to the divided tank. Constant oxygen supply was provided and temperature (37± 1°C) was maintained for 48hr to hatch and mature the shrimp as nauplii (larvae).

1mg of each sample (crude $CHCl_3$ extract and AK_2) were initially dissolved in 200µl of dimethyl sulfoxide (DMSO) to get a concentration of 5µg µl^{-1}. Forty clean vials were taken for the 4 samples in five concentrations (two vials for each concentration) and two vials also taken for control test for each sample. 5ml sea water containing 10 brine shrimp nauplii was given to each of the 5 vials and specific volume of samples were transferred from the stock solutions to the vials to get final concentration of 5, 10, 20, 40 and 80µg ml^{-1}. Control vials contain 5ml of seawater and same volume of DMSO as in the sample vials. After 24hr the number of survivals in each vial was counted. The percentage of mortality of the brine shrimp was calculated for each concentration and the median lethal concentration (LC_{50}) values were determined (Goldstein, 1974).

Results and discussion

A *Sreptomyces* species was isolated from soil sample, collected at Rajshahi, Bangladesh. This local species was subjected to antibiotic production using yeast extract glucose agar media. The $CHCl_3$ soluble portion of the extract yielded a brown antibiotic pigment AK_2.The antibiotic was characterized as Di-(2-ethyl hexyl)-Phthalate (Fig. 1) on the basis of its spectral data (Akteruzzaman, 2000).

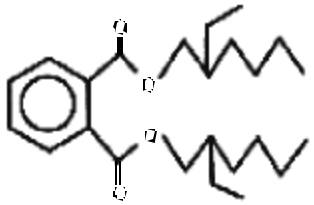

Fig. 1: Structure of Di-(2-ethyl hexyl)-Phthalate

The compound and the extract showed remarkabb antibacterial activity against both gram positive and gram negative bacteria in comparison with standard Kanamycin. The zone of inhibition produced by the test materials was observed between 10 to 13mm and 15 to 22mm for $CHCl_3$ extract (500µg disc^{-1}) and AK_2 (500µg disc^{-1}) respectively (Table 1).

Table 1: Antibacterial activities of $CHCl_3$ extract, AK_2 and Kanamycin standard.

Test Organisms	Diameter of zone of inhibition		
	A	B	C
Gram positive:			
1. Bacillus subtilis	13	18	19
2. Bacillus cereus	11	20	25
3. Bacillus megaterium	12	17	22
4. Staphylococcus aureus	13	15	22
5.Streptococcus-β-haemoliticus	10	16	20
6. Sarcina lutea	11	22	26
Gram negative:			
1. Shigella boydii	12	20	23
2. Shigella Shiga	10	17	20
3. Shigella sonnei	11	18	22
4. Shigella flexneriae	10	20	23
5. Shigella dysenteriae	11	21	22
6. Klebsiella species	13	22	25
7. Salmonella typhi A	11	21	26
8. Salmonella typhi B-56	12	19	22
9. Escherichia coli	11	16	21

A= $CHCl_3$ extract (500µg disk^{-1}) B= AK_2 (500µg disk^{-1})
C= Kanamycin standard (30µg disk^{-1})

The MIC value of AK_2 against six organisms were found to be 128 to 256µg ml^{-1} (Table 2). The cytotoxic activity of the $CHCl_3$ extract and AK_2 was determined by brine shrimp lethality bioassay and the results are presented in Table 3.

Table 2: The MIC values of the antibiotic AK_2 against six test organisms.

Test organisms	MIC (μg ml^{-1})
1. *Bacillus subtilis*	128
2. *Sarcina lutea*	256
3. *Staphylococcus aureus*	128
4. *Shigella dysenteriae*	128
5. *Escherichia coli*	128
6. . *Salmonella typhi B-56*	128

Table 3: Results of the brine shrimp lethality bioassay of $CHCl_3$ extract and antibiotic AK_2.

Test sample	Concentration (μg ml^{-1})	Log C	% mortality	LC_{50} (μg ml^{-1})
$CHCl_3$ extract	5	0.69	20	
	10	1.00	40	
	20	1.30	70	12.5
	40	1.60	90	
	80	1.90	100	
AK_2	5	0.69	10	
	10	1.00	30	
	20	1.30	60	15.8
	40	1.60	80	
	80	1.90	90	

The mortality was determined by counting survivors at the end of the exposure period (24hr) and the control mortality was adjusted by using Abbott's formula (Abbott, 1925). As shown in Table 3 the mortality rate of nauplii was increased with the increase of concentration of each sample. The LC_{50} was determined by extrapolation from graph (Logarithm of concentration versus percentage of mortality, Fig. 1) and the value were 12.5 and 15.8μg ml^{-1} for $CHCl_3$ extract and AK_2 respectively (Fig. 2).

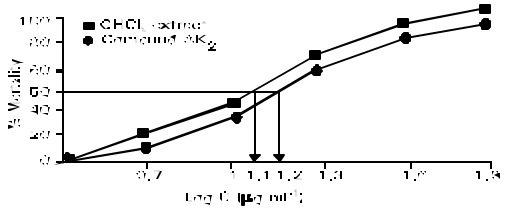

Fig. 2: Determination of LC_{50} of $CHCl_3$ extract and Pure compound AK_2 against brine shrimp nauplii.

From the anti bacterial results it is evident that the crude $CHCl_3$ extract showed moderate antibacterial activity. Whereas compound AK_2 showed significant antibacterial activity but was less potent than that of standard Kanamycin.

From the results of cytotoxic activity it may be concluded that the $CHCl_3$ extract and compound AK_2 were toxic to the brine shrimp nauplii and they are biologically and pharmacologically active. However, the $CHCl_3$ extract was comparatively more cytotoxic than compound AK_2. It may be due to the presence of any synergistic compound(s) in the crude $CHCl_3$ extract other than compound AK_2

The results of the this study demonstrate that the local *Streptomyces* species particularly its metabolite AK_2 possesses strong antibacterial and cytotoxic activity. This is in agreement with our previous finding on anti *shigella* activity of this species (Gafur *et al.*,1991).

In recent years the pathogenic organisms are gaining resistance to existing antimicrobial agents hence the search for new, safe and more effective antibiotics against these organisms is a pressing need. Thus the findings of this investigation and pervious investigation on other genus (Anisuzzaman *et al.*, 2001) would give valuable support to make clinical trial as well as toxicity studies of the isolated antibacterial and cytotoxic metabolites to get a more potent antibiotic.

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