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Research Paper

IDENTIFICATION OF ANTICANCER ACTINOMYCIN-D IN A NEWLY ISOLATED *STREPTOMYCES SP.* FROM SUDAN SOIL

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Abstract

An actinomycete strain, designated as AH 11.4, was isolated from the soil sample collected from Sudan. The new strain, (Streptomyces sp. AH 11.4), was taxonomically characterised on the basis of morphological, phenotypic characteristics, genotypic data and phylogenetic analysis. The pure bioactive compound was extracted and isolated from the culture supernatant and from the biomass using reverse phase HPLC-DAD. The compound was identified as actinomycin-D using 1H- and 13C-NMR, UV, and MS spectroscopy in comparison to reported data. Production of actinomycin D by strain AH 11.4 using starch as a carbon source yielded much higher level of actinomycin D (305 mg/l). Actinomycin-D produced by the identified Streptomyces strain showed significant anticancer effect against human cervical carcinoma cells and Glioma UG-87 cells. Growth inhibition of these cells was measured by the microculturetetrozolium (MTT) assay. Actinomycin D produced was assessed at different concentrations (1µg ml -1, 10µg ml -1, 20µg, and 30µg ml 1 and 40µg ml 1). It was able to cause death of 90% of the cancer cells in comparison to the control cells.

Key words: *Streptomyces sp*, Actinomycin-D, anti-cancer.

INTRODUCTION

The actinomycins are a family of chromopeptide lactone antibiotics that present antitumoral properties, being employed in the treatment of several human neoplasies (Vandana *et al* , 2008). They represent an important class of natural products that, despite being discovered more than 70 years ago, continue to be a focus of many research areas, especially in the biological and medicinal sciences. Structurally, they have a chromophorous group, identical in all actinomycins, and two pentapeptide chains with a variable composition of amino acids (Kurosawa *et al,* 2006)**.** Among the actinomycins, actinomycin D has been studied most extensively and is used for treatment of malignant tumors, such as Wilms' tumor (Green 1997)**,** and childhood rhabdomyosarcoma (Womer 1997). According to the World Health Organization (WHO) cancer is one of the leading causes of death worldwide, which accounted for 7.6 million deaths (around 13%) of the world's population in 2008. They have furthermore estimated that the worldwide deaths are likely to rise to over 11 million in 2030. Streptomycetes are known producers of industrial enzymes and medically important compounds, e.g. polyketides, tetracyclines , antitumor agents and the best known are antibiotics currently used worldwide as pharmaceutical and agrochemical products (Flärdh, 2003).. Various strains of *Streptomyces* and *Micromonospora* are reported to produce different forms of actinomycins, Actinomycin D is produced by a range of Streptomyces species as part of a mixture of actinomycins, (Shimizu *et al*, 2004: Kurosawa *et al,* 2006). The present contribution target the isolation and anticancer activity of actinomycin D from *Streptomyces sp.*.

MATERIAL AND METHODS

Isolation and identification of *Streptomyces sp*.

The producer microorganism, designated as AH 11.4, was isolated from the soil sample, collected from Sudan. Pure and active Streptomyces isolate was stored for long term as lyophils and in glycerol deeps and for short term (up to 6 months) at 4^{o} C on agar slopes. Morphological and cultural characteristics were studied using the ISP media recommended by Shirling and Gottlieb, 1966. In order to confirm the identified isolate, 16S rRNA gene analysis of the isolate was studied (Fourati *et al*., 2005: Abdelhalem[a] *et al.*, 2013).

Extraction and purification of actinomycin

The culture broth (1 L) was centrifuged at 6,000 rpm for 15 min to separate the supernatant and mycelial biomass. Activities against test organisms were monitored during the isolation, using the agar well diffusion method (Abdelhalem[b] *et al*., 2013). Mycelium and culture supernatants were beastly extracted with ethyl acetate. 1 gram crude Ethyl acetate extract was dried and resuspended in small volume of methanol and separated by solid phase extraction (SPE) on a Hypersil C18 column. further purification was achieved by reversed phase HPLC, using a Varian Prostar system containing of two solvent with filtered water in 0.05% Trifluoro acetic acid (TFA) and CH3CN delivery modules (210), DAD detector (335), autoinjector (410) and fraction collector (710). Further separation was done by preparative HPLC (Zorbax StableBond 4.6 x 250 mm 5 mm column 3ml/min, isocratic 80% MeOH/H2O). Fractions showing biological activity were identified in the chromatogram and the corresponding UV spectra analysed. MS data of the compound was obtained on a MAT 95 mass spectrometer in EI and DCI mode (Finnian). The compound mass fragments [M+1 , M+2] was compared with the relevant metabolite(s) and corrected with the reference compounds in the database. NMR analyses were performed using Varian Inova 300 MHz

spectrometer. Samples for H1 NMR and C13 NMR spectroscopy was dissolved in chloroform (CDCl3). NMR data for the compound was assigned by 2D NMR analysis and compare to literature data.

Antitumor activity of the actinomycin D

Various cell lines, such as cervical carcinoma cells and Glioma cells UG- 87, were used to evaluate the in-vitro antitumor effects of the actinomycin-D in the UCD Conway institute of biomolecular and biomedical research. Growth inhibitory effect of glioma cells (UG- 87) and hela (cervical) cells with various treatments was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT, Sigma Chemical Co) assay as described by (Qing Chen *et al,* 2003, Lu *et al.* 2005). Cells were grown in MEM medium and incubated at 37 ºC in a humidified atmosphere containing 5 % CO2. Tumor cells (1500 cell per well) in their exponential growth phase were transferred into 96 well plates, Then cells were exposed to different concentrations (1µg ml -1 , 10µg ml -1 , 20µg, 30µg ml -1 and 40µg ml -1) of the compounds for 24 h, and each treatment was tested in triplicate wells. At the end of exposure, 20 µL of MTT 5 g/L was added to each well and the plates were incubated at 37 ºC for 4 h. Incubation was carried out for 48 h incubation in 5% CO2 incubator. the medium was removed, rinsed with phosphate buffer solution and trypsin was added. The trypsinised cells were counted by mixing equal volumes of the cell suspension and trypan blue dye. Morphological changes and death in cells were evaluated by phase-contrast microscopy.

RESULTS AND DISCUSSION

Cultural characteristics of biologically active *Streptomyces* sp.

The morphological and cultural characteristics of the isolate was observed after 14 days incubation on the International Streptomyces Project (ISP) media and various other media and they showed considerable variations as in figure 1. The isolate was taxonomically characterized on the basis of morphological and physiological characteristics, phylogenetic analysis and genotypic data. these studies strongly suggested that the strain is a members of the genus Streptomyces. A complete 16s rRNA (1277 bp) sequence of the isolate was determined. The isolate was found to have similarity (97%) with *Streptomyces themocarbodoxydus* 173998. However, the phylogenetic analysis indicated that isolate AH 11.4 was quite different from those recognized Streptomyces species in the data base. Additionally the phylogenetic analysis demonstrated that this strain was on a different node to previously identified strains. It was clear from the combination of genotypic and phenotypic data that the isolate AH 11.4 should be classified as a new strain of the genus Streptomyces,. These closely related Streptomyces spp. are not reported earlier to produce actinomycins.

This streptomyces strain was deposited at the Industrial Microbiology Department (IMD) University College of Dublin (UCD) under the number (2730), and the 16S rRNA sequences of the strain was submitted to GeneBank database (accession numbers GU013556).

Fig 1. Morphological colonies of isolated biologicaly active *Streptomyces* spp. AH 11.4 in ISP4 after 14 days incubation at 30°C.

Purification, Identification and Structure Elucidation of Actinomycin D from *Streptomyces sp*. AH 11.4

Different methodologies were tested to find the most appropriate strategy to achieve rapid and efficiently the identification of the active substances contained in the ethyl acetate extracts. HPLC was the most successful strategy investigated and became the routine approach for identification of the active compounds. The correlation between peaks in the chromatogram and biological activity levels in the different HPLC fractions, allowed the identification of the active compound contained in the extracts. Preliminary HPLC-DAD analysis of this extract identified an absorbance spectrum characteristic of actinomycin D figure 2. This tentative identification was supported by the observation of strong ions in the ESI (+) MS spectra of the extracts corresponding to the presence of actinomycin D (m/z 1255, [M+H] +)

NMR analysis (COSY, HSQC, and HMBC) was conducted, which was used to assign the 1H and 13C NMR resonances of actinomycin D (Table 1). These assignments are in agreement with those given by Booth et al. (1976) and . Inbarand and Lapidot (1991). RF-HPLC chromatogram of the purified actinomycin-D produced by Streptomyces sp. AH 11.4. Figure 3 illustrated the Steps towards extraction of actinomycin-D from Streptomyces sp. AH 11.4.

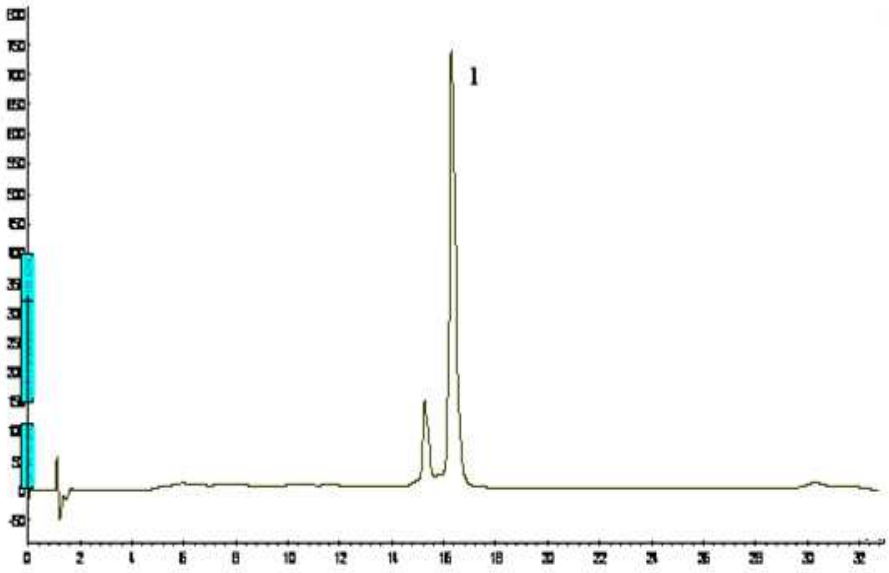

Fig 2. RP-HPLC-DAD chromatogram of purified actinomycin D isolated from *Streptomyces* sp. AH 11.4

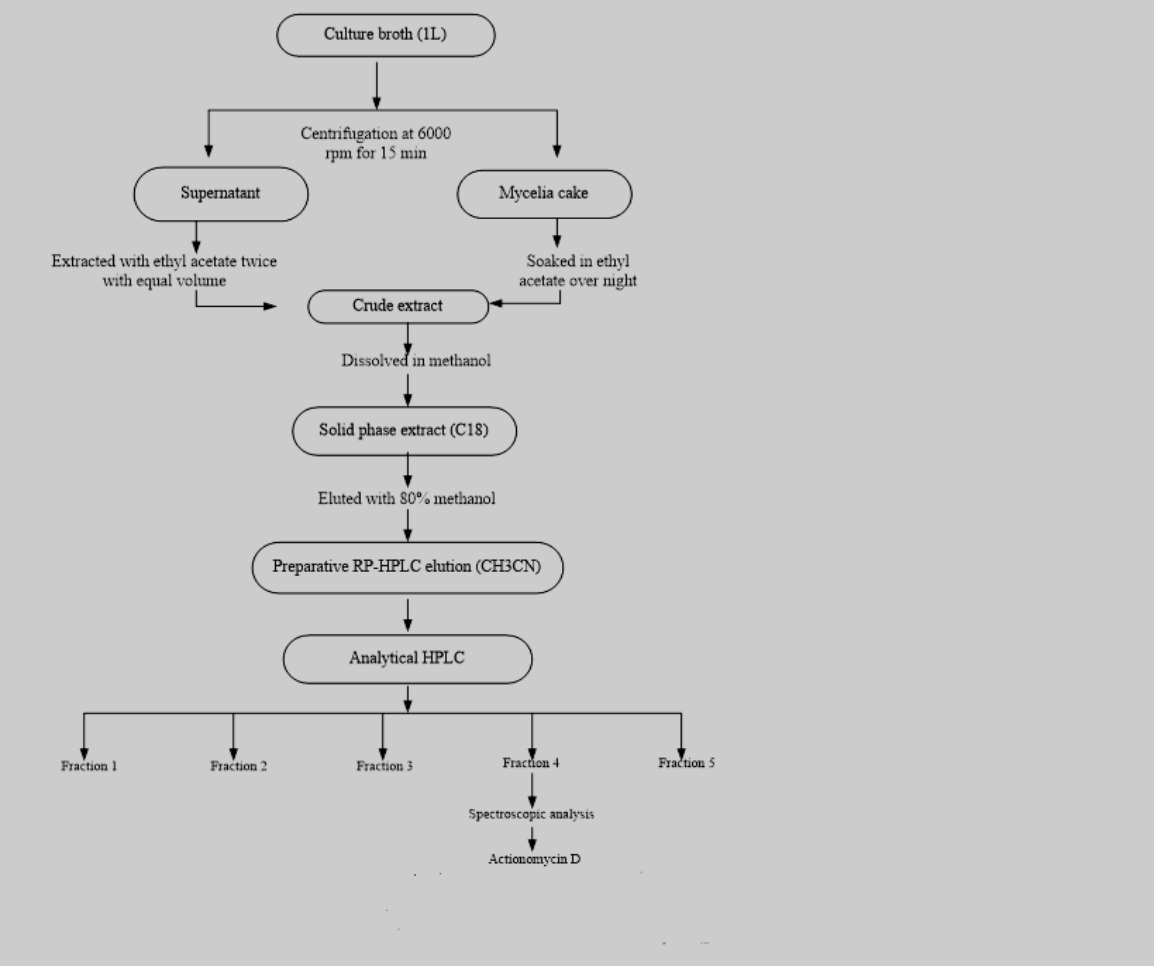

Fig 3. Steps towards extraction of actinomycin-D from Streptomyces sp. AH 11.4.

Antitumor activity of the actinomycin D

Activity of actinomycin D against human cervical carcinoma cells and Glioma cells UG-87 as antitumor was assessed in microtiter plate using MTT assay. Figure 4. showed that the actinomycin D in concentrations of 1µg ml -1, 10µg ml -1, 20µg ml -1, 30µg ml -1 and 40µg ml -1 showed significance activity to the human cervical carcinoma cells and Glioma cells UG- 87. Microscopically analysis of the isolated compound suggested antitumor response, because they caused 90% death of the treated cells in compression to the control cells. These results agreed with those of (Takusagawa et al., 2001) who found that the actinomycin D has been shown to have higher activity toward human leukemia cell lines such as HL-60 cells.

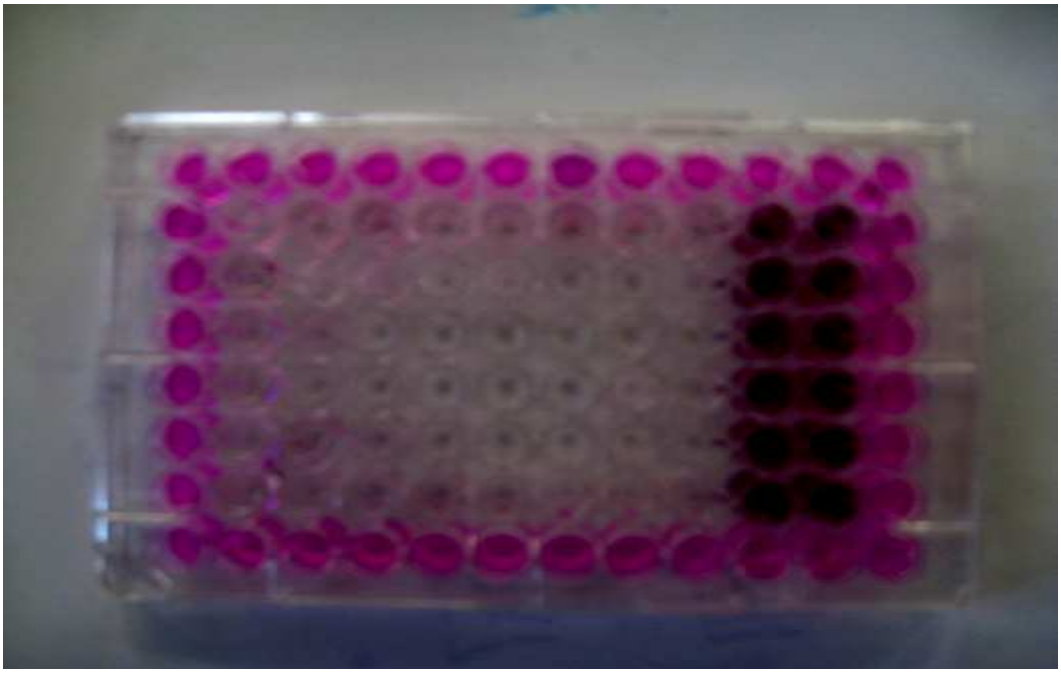

Fig 4. Antitumor activity of Actinomycin-D with various concentrations against human cervical carcinoma cells using Microtiter plate assay.

Table 1. ^{1}H and ^{13}C NMR data for actinomycin D (CDCl3, 400 MHz)

	α-ring		β-ring	
	C	H	C	H
Methyl-valine				
1	167.63		167.53	
	71.43	2.66 m	71.28	2.66 m
	26.83	2.65 m	26.80	2.64 m
	21.59	0.95 d (5.6)	21.70	0.97 d (6.1)
	19.07	0.73 d (5.7)	19.11	0.74 d (6.1)
NMe	39.32	2.93 s	39.20	2.89 s
Sarcosine				
1	166.48		166.26	
	51.40	4.78 d (17.5), 3.61 d (17.5)	51.35	4.71 d (17.5), 3.64 d (17.5)
NMe	34.91	2.88 s	34.84	2.88 s
Proline				
	173.58		173..79	
	56.15	6.03 d (9.8)	56.26	5.97 d (9.1)
	30.94	2.66 m 1.90 dd (11.7, 6.3)	31.31	2.95 m, 1.86 dd (11.7, 7.0)
	23.04	2.29 m, 2.05 m	22.88	2.26 m, 2.08 m
		3.97 ddd (2.11, 10.9, 7.1)		3.82 ddd (11.2,10.7, 7.0)
	47.33	3.71 m	47.60	3.72 m
D-Valine				
	173.26		173.29	
	58.90	3.52 dd (10.7, 5.6)	58.70	3.55 dd(7.0, 9.5)

3	31.52	2.21 m		31.83	2.15 m
4	18.98	1.13 d (6.3)		19.11	1.11 d (6.3)
5	19.24	0.88 d (7.1)		19.30	0.91 d (6.6)
NH					
Threonine					
1	168.35			168.91	
2	55.31	4.48 dd (6.8, 2.4)		54.89	4.60 dd (6.3, 2.4)
3	74.96	5.20 dd (6.1, 2.4)		75.06	5.16 dd (6.1, 2.4)
4	17.44	1.24 d(6.1)		17.86	1.25 d (6.1)
NH		7.19 d (6.8)			
Chromophore					
1	101.66		11	15.07	2.55 s
2	147.66		12	7.81	2.25 s
3	179.14		13	166.07	
4	113.47		14	166.26	
4a	145.01				
5a	140.45				
6	127.56				
7	130.21	7.36 d (8.0)			
8	125.88	7.65 d (8.0)			
9	132.53				
9a	129.09				
10a	145.88				

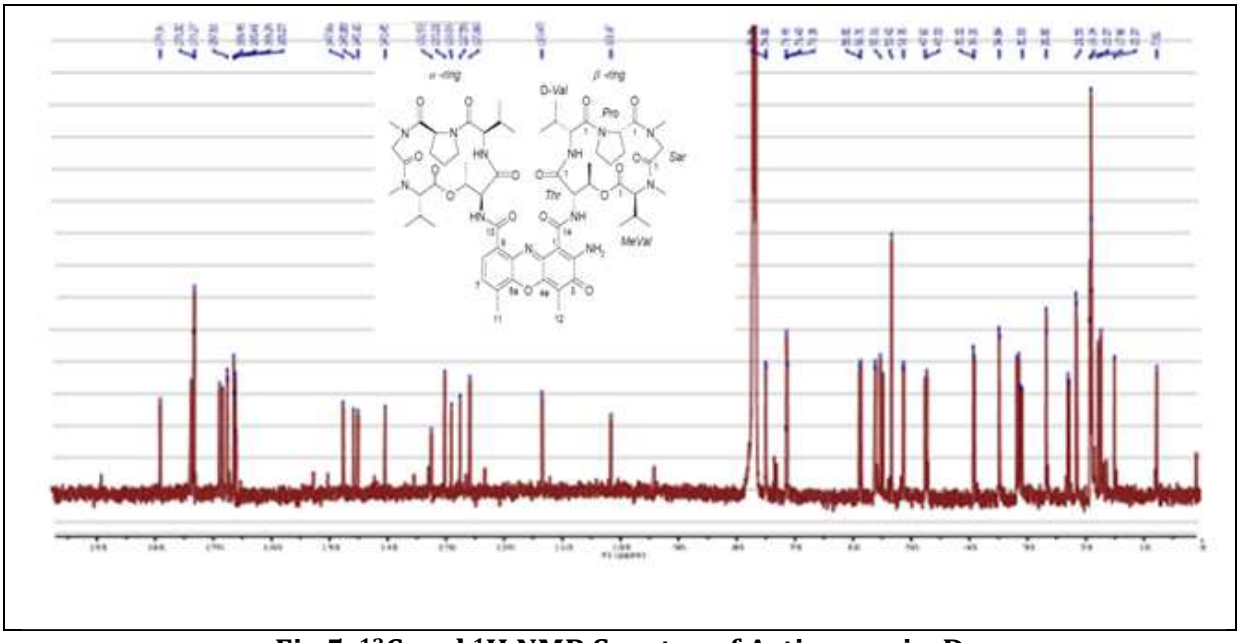

Fig 5. ^{13}C and ^{1}H NMR Spectra of Actinomycin-D

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