

Purification of The Antitumor Active Metabolite from *Streptomyces xylophagus* Ha.Ph-7 Crude Extract Isolated from Hamam Pharaoh Hot Spring

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ABSTRACT:

Aim: This study aimed at production, extraction, purification and characterization of active antitumor compound from marine actinomycete secondary metabolites crude extract that was selected as most potent crude secondary metabolites from the preliminary screening of total fifty one extract.

Material and methods: These tumor cell lines were breast cancer (MCF-7) (ATCC HTB-22) and Hepato-cellular carcinoma (HepG2) (ATCC 77400), colon cancer (Caco) (ATCC HTB-37) and cervix carcinoma (HeLa) (ATCC CRL-13011) cells. Purification process was done using silica gel column chromatography while purity was detected using thin layer chromatography (TLC). The active fraction was detected using morphological changes (cytotoxicity) by microscopic examination and anti-proliferative activity using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

Result: The result indicated that there is only one fraction responsible for antitumor activity. This active fraction has cytotoxic and anti-proliferative activity against HepG2, MCF7, Caco and HELA cells with IC₅₀=24.5, 20.1, 27.6 and 17.7 µg/ml respectively. The analysis of physico-chemical, elemental and spectroscopic analysis (UV, IR, H.NMR, Mass spectroscopy) indicated that; the active compound has the nature of anthracycline compound.

Conclusion: This purified compound has promising broad spectrum cytotoxicity and anti-proliferative activity in an in vitro system. The next research step shall include different optimization parameter that maximize productivity with long-term goal; discovery of an antitumor drug from actinomycetes native to Egyptian hot springs habitat.

Key words: Actinomycete extracts, antitumor, Cytotoxicity, Anti-infectivity, HepG2, Caco, MCF7, HELA.

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INTRODUCTION:

Actinobacteria represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria [1]. In addition, the isolation of marine actinomycetes has been a great source of new compounds and their isolation all around the globe, from shallow costal sediments to the deepest sediments from the Mariana Trench, demonstrates that actinomycetes are ubiquitous in marine sediments, but at lower numbers than in soil [2-14]. Progress has been made recently on drug discovery from actinomycetes by using high-throughput screening and fermentation, mining genomes for cryptic pathways, and combinatorial biosynthesis to generate new secondary metabolites related to existing pharmacophores [15]. Marine actinomycetes have attracted great attention since they have developed unique metabolic and physiological capabilities that not only ensure survival in extreme habitats, but also offer the potential to produce compounds with antitumor and other interesting pharmacological activities that would not be observed in terrestrial

microorganisms [16-20], perhaps because of their close relationships with marine eukaryotic organisms including mammals [2,9]. Polyketides are a large family of natural products produced by step-wise decarboxylative Claisen type condensation of acyl-CoA precursors, reactions catalyzed by polyketide synthases (PKSs). Three types of PKSs are known to date: type I PKSs are multifunctional enzymes organized into modules, type II PKSs are multienzyme complexes carrying out a single set of activities and type III PKSs, also known as chalcone synthase-like PKSs, are iteratively acting condensing enzymes [21]. In addition anthracyclines represent a wide family of compounds with indicator-like properties, red in acid and blue-violet in alkaline solution, that are produced by cultivation of microorganisms belonging to the genus *Streptomyces* [22] Anthracyclines are among the most effective and widely prescribed anticancer agents [23]. Anthracycline has three mechanisms of action: 1- Inhibits DNA and RNA synthesis by intercalating between

base pairs of the DNA/RNA strand, thus preventing the replication of rapidly-growing cancer cells. 2- Inhibits topoisomerase II enzyme, preventing the relaxing of supercoiled DNA and thus blocking DNA transcription and replication. 3- Creates iron-mediated free oxygen radicals that damage the DNA and cell membranes [24].

MATERIAL AND METHODS:

Production of Extracellular Bioactive Metabolites:

Spores from five to seven days old culture of the selected actinomycete isolates were used to inoculate 250 ml Erlenmeyer flask containing 100 ml of the fermentation medium (liquid starch-nitrate nutrient medium, pH = 7). The seeded flasks were incubated at 30 – 33 °C for 7 days at submerged conditions. After the incubation period, the fermentation broth was harvested and filtered through cotton wool then; the clear filtrate was exposed to the extraction process.

Extraction of Extracellular Bioactive Metabolites:

The collected filtrate (20.0 liters) of *Streptomyces xylophagus* Ha.Ph-7 was extracted with chloroform : Ethanol solvent system (2:1 v/v) in a separating funnel. The lower layer was separated and collected then the solvent was evaporated under reduced pressure using a rotary evaporator. The evaporation process was continued until deep brown viscous syrup (3.9 gm) was obtained.

Bioactivity-guided fractionation of the crude extract obtained from *Streptomyces xylophagus* Ha.Ph-7.

Fractionations of the crude extracts obtained from *Streptomyces xylophagus* Ha.Ph-7 using silica gel column chromatography.

Three grams of the crude extracts was dissolved in the least amount of the eluting solvent (Ethanol/chloroform mix) and applied to silica gel column (3.5 X 40cm), then eluted with a gradient solvent ratios (table:1)

Fifty ml fractions were collected then fractionated using TLC analysis. The fractions that have similar TLC profiles were pooled to give finally a total of 13 fractions (Table: 2).

Cell culture:

HepG2 cells (ATCC 77400) passage number 40, MCF-7 (MCF-7) (ATCC HTB-22) passage number 32, colon cancer (Caco)(ATCC HTB-37) passage number 25 and cervix carcinoma (HeLa)(ATCC CRL-

13011) passage number 29 were obtained from cell culture bank at the Holding Company for Biological Product and Vaccine (VACSERA) and maintained in our lab from passage number 40-51 for HepG2, 32-40 for MCF7, 25-39 for Caco and 29-43 for HELA. The medium used was Dulbecco's modified Eagle's medium (DMEM, GIBCO, USA). All cells were supplemented with 10% Foetal bovine serum (FBS) and antibiotics (100IU penicillin and 100IU streptomycin /ml) solution and maintained with 2% FBS and antibiotics solution and incubated at 37°C and 5% CO₂ [25].

Cytotoxicity and Anti-proliferative Assays:

Cells (Caco, HELA and MRC5) were seeded in 96-well plates at the density of 1000 cells/well in 100 µl medium except on column (8 wells) without cells as blank and incubated at 37°C for 24 hour in 5% CO₂ for the formation of confluent monolayer. The monolayer of cells in the plate was exposed to various dilutions of the secondary metabolite extract (100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 1µg) except another column (8 wells) seeded with cells only as positive control and incubated at 37°C for 24 hour in 5% CO₂. After 24 hour incubation the cytotoxic effect of different microbial extracts are visualized under inverted microscope before proceeding with MTT assay protocol. Then 20 µl of MTT reagent was added to each well and incubated for additional 4 hour. Then 180 µl of DMSO solution was added to each well to solubilize the formazan crystals. The cell viability was measured using MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) with MTT (5 mg/ml) and DMSO. This tetrazolium salt is metabolically reduced by viable cells to yield a purple insoluble Formozan product measured at 570nm spectrophotometrically [26]. The assay was performed in triplicate for each of the extracts. The mean of the cell viability values was compared to the control to determine the effect of the extract on cells and % cell viability form this equation:

$$\text{Survival rate \%} = \frac{A_{\text{sample}} - A_{\text{b}}}{A_{\text{c}} - A_{\text{b}}} \times 100$$

A_{c} = Negative control

A_{b} = Blank

Then the % of cell viability was plotted against concentration of different extract to calculate the IC₅₀ values of each extract required to kill 50% of cancer cells.

RESULTS AND DISCUSSION:

Bioactivity evaluation of the fractions obtained from the crude extract *Streptomyces xylophagus* Ha.Ph-7.

The obtained fractions from column chromatography were tested for cytotoxicity and anti-proliferative activity against HepG2, MCF7, Caco and HELA cell lines. Fractions that inhibit cell growth at concentration similar to that obtained with crude extracts survey are considered active (the fraction that exhibit activity at $IC_{50} < 30 \mu\text{g/ml}$) according to the American National Cancer Institute (NCI) guidelines in which $IC_{50} < 30 \mu\text{g mL}^{-1}$ in the preliminary assay is considered active [27]. The IC_{50} value shows the inhibition concentration at which only 50% of the cells are viable. It was reported that crude materials that require a drug concentration greater than $30 \mu\text{g mL}^{-1}$ to exhibit cytotoxicity were not considered as cytotoxic agents [28]. It was recommended the survey of actinomycete

extracts over a panel of cell lines Then antitumor activity is judged as the ability of the test substance to selectively kill (cytotoxic) or inhibit the growth (anti-proliferative) of certain cell lines, rather than general cytotoxicity to all or most cell lines of the panel [29]. Therefore, among all studies the most cytotoxic potential with an IC_{50} value $< 30 \mu\text{g mL}^{-1}$ is considered active and should be selected for the next step of research.

Antitumor activity of the fractions obtained from the crude extract of *Streptomyces xylophagus* Ha.Ph-7 against HepG2 cells.

It was found that the fraction (S-12) revealed cytotoxic activity against Hepatocellular carcinoma (HepG2) cell line with $IC_{50} = 24.5 \mu\text{g/ml}$ while other fractions failed to exhibit any IC_{50} value even at concentrations up to $30.0 \mu\text{g/ml}$, which is a value higher than those of the crude extract (figure:1).

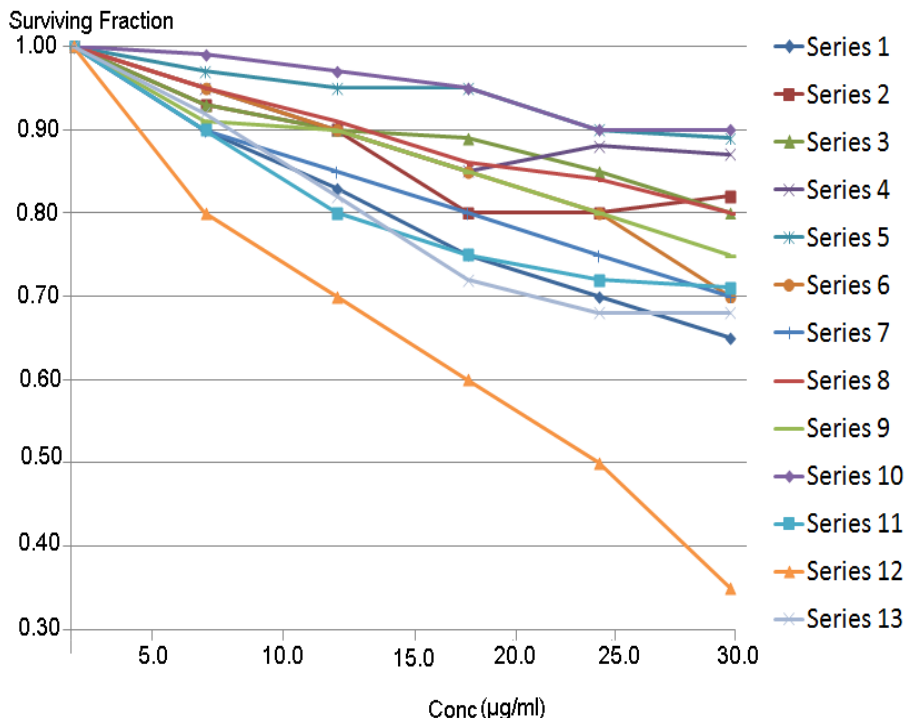


Figure (1): Cytotoxicity profile of the bioactive fractions of *Streptomyces xylophagus* Ha.Ph-7 against HepG2 cell line.

Antitumor activity of the fractions obtained from the crude extract of *Streptomyces xylophagus* Ha.Ph-7 against MCF7 cell lines.

It was found that the fraction (S-12) revealed cytotoxic activity against MCF7 cell line with IC₅₀= 20.1 µg/ml while other fractions failed to exhibit any IC₅₀ value even at concentrations up to 30.0 µg/ml, which is a value higher than those of the crude extract (figure: 2).

Antitumor activity of the fractions obtained from the crude extract of *Streptomyces xylophagus* Ha.Ph-7 against Caco cell lines.

It was found that the fraction (S-12) revealed cytotoxic activity against Caco cells with IC₅₀= 27.6 µg/ml while other fractions failed to exhibit any IC₅₀ value even at concentrations up to 30.0 µg/ml, which is a value higher than those of the crude extract (figure: 3).

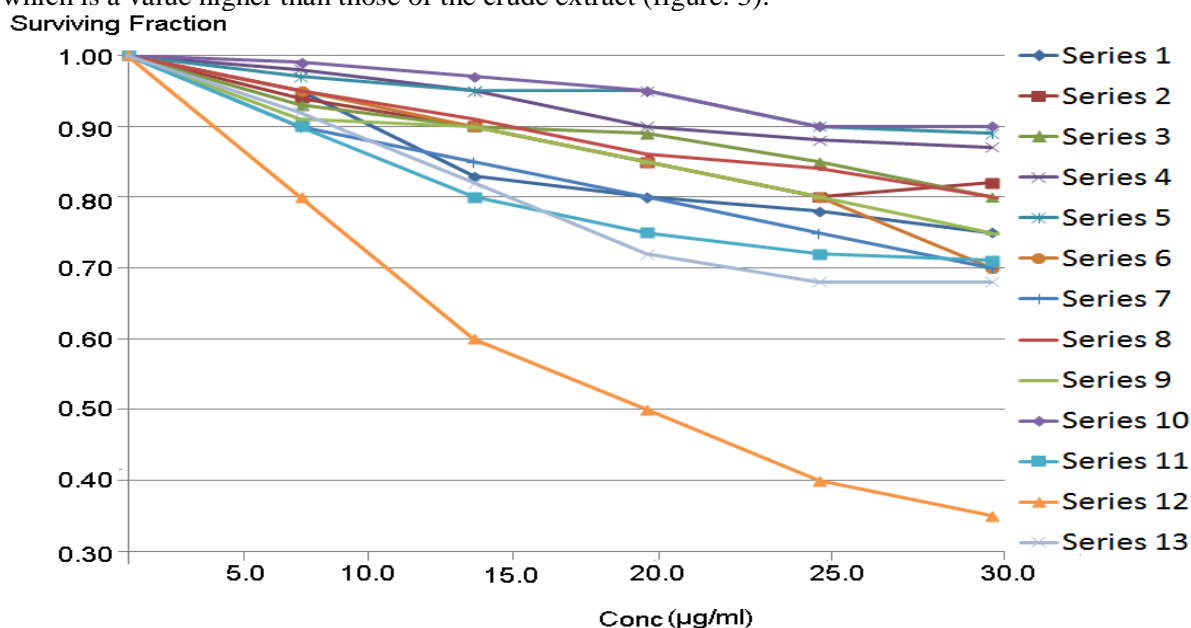


Figure (2): Cytotoxicity profile of the bioactive fractions of *Streptomyces xylophagus* Ha.Ph-7 against MCF7 cell line

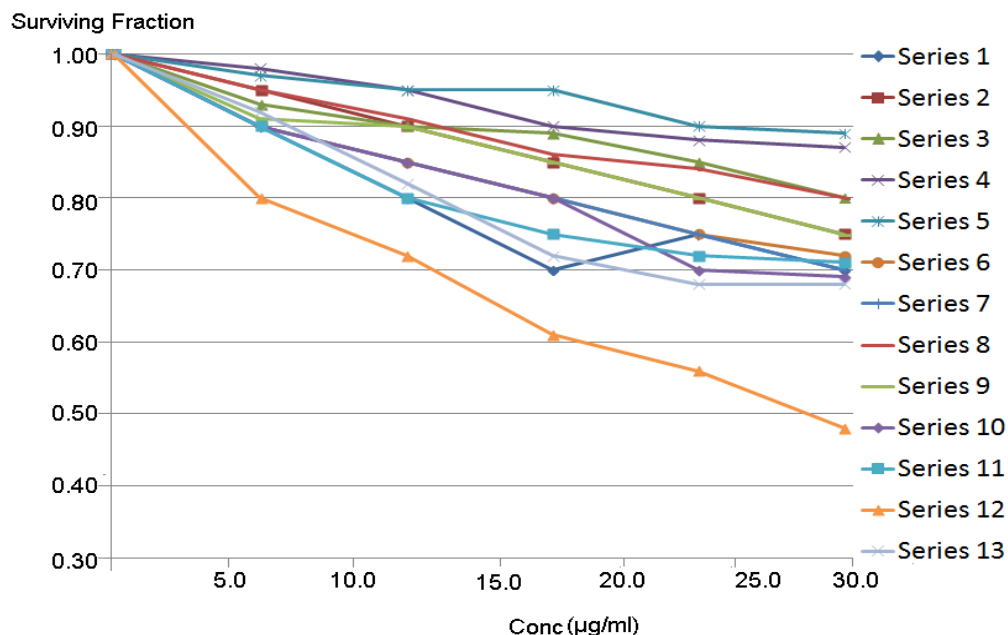


Figure (3): Cytotoxicity profile of the bioactive fractions of *Streptomyces xylophagus* Ha.Ph-7 against Caco cell line

Antitumor activity of the fractions obtained from the crude extract of *Streptomyces xylophagus* Ha.Ph-7 against HELA cell lines.

It was found that the fraction (S-12) revealed cytotoxic activity against HELA cells with $IC_{50} = 17.7 \mu\text{g/ml}$ while other fractions failed to exhibit any IC_{50} value even at concentrations up to $30.0 \mu\text{g/ml}$, which is a value higher than those of the crude extract (figure:4).

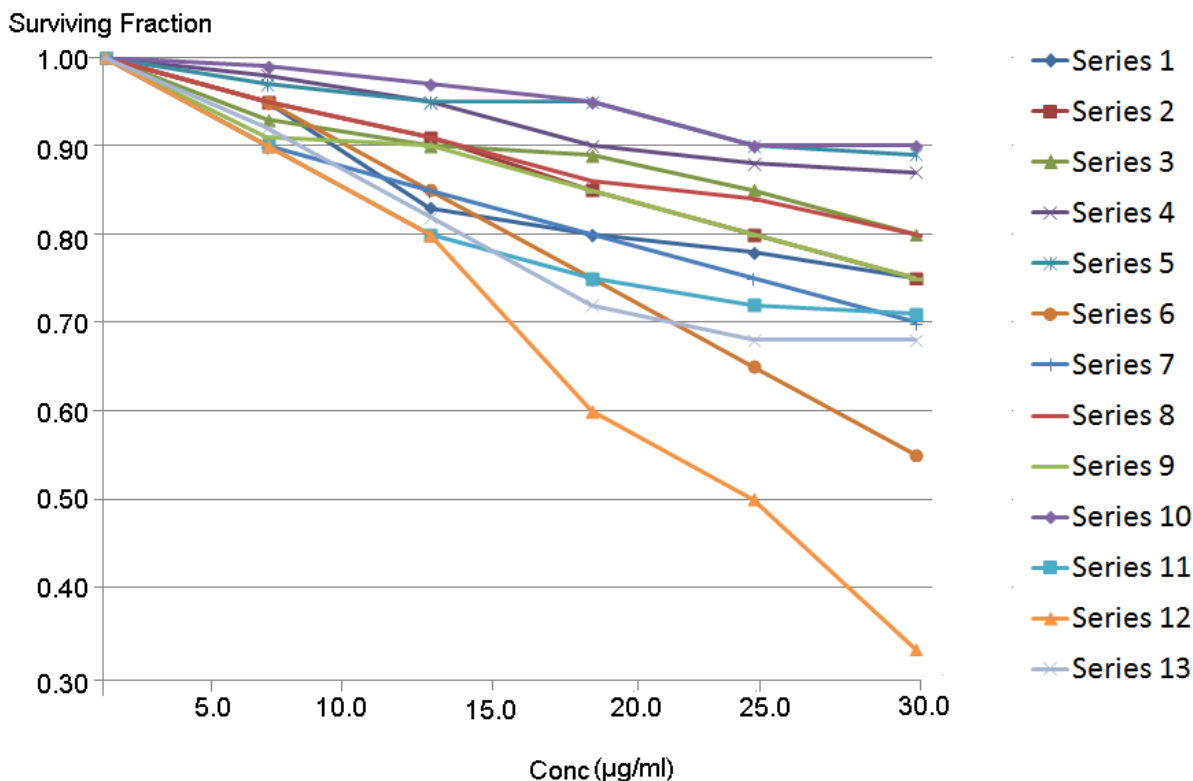


Figure (4): Cytotoxicity profile of the bioactive fractions of *Streptomyces xylophagus* Ha.Ph-7 against HELA cell line.

The TLC plate of the obtained compound (C-1) is illustrated in the following figure:



Figure (5): TLC plate of compound (C-1).

Identification of the purified active compound obtained from *Streptomyces xylophagus* Ha.Ph-7.

Physicochemical characteristics:

The purified compound was isolated as amorphous red powder with no characteristics odor, red in acidic and blue-violet in alkaline solution, melting point is 145-155°C. The compound is freely soluble in chloroform, ethyl acetate, ethyl alcohol and methanol and insoluble in petroleum ether, hexane and benzene and water.

Spectral characteristics:

Structural elucidation of the purified compound could be achieved by analysis of UV, IR, ¹HNMR and Mass spectrum.

The ultraviolet (UV) absorption spectrum of the active substance in methanol using Perkin-Elmer Lambda 15 UV/V spectrophotometer, exhibit maximum absorption peaks at 237nm (Figure: 6).

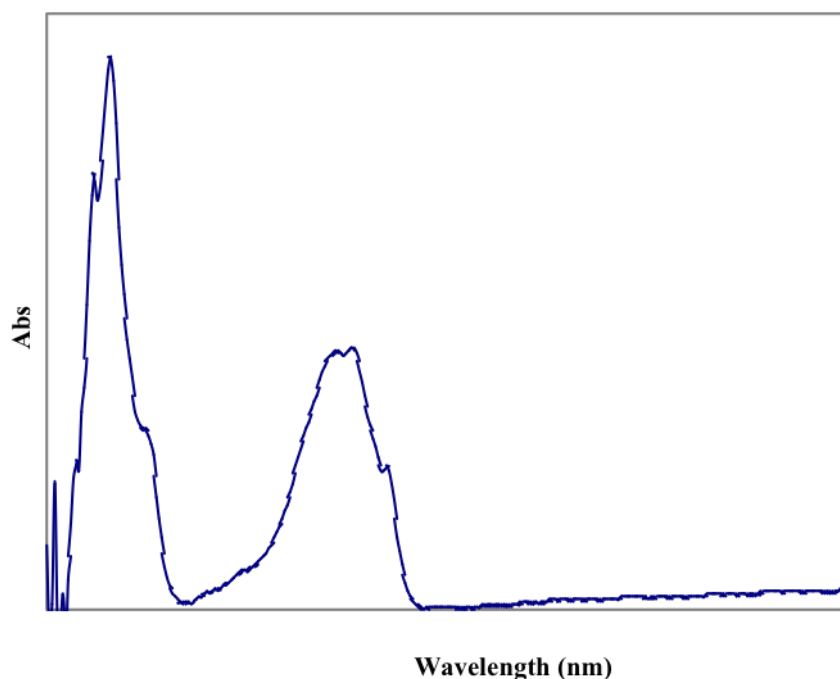


Figure (6): UV spectrum of the purified compound

The IR spectrum of the purified compound in (KBr) $\tilde{\nu}_{\text{max}}$ showed: 668, 1026, 1554, 1646, 2358, 3344, 3612, 3732 and 3802 cm^{-1} figure (7).

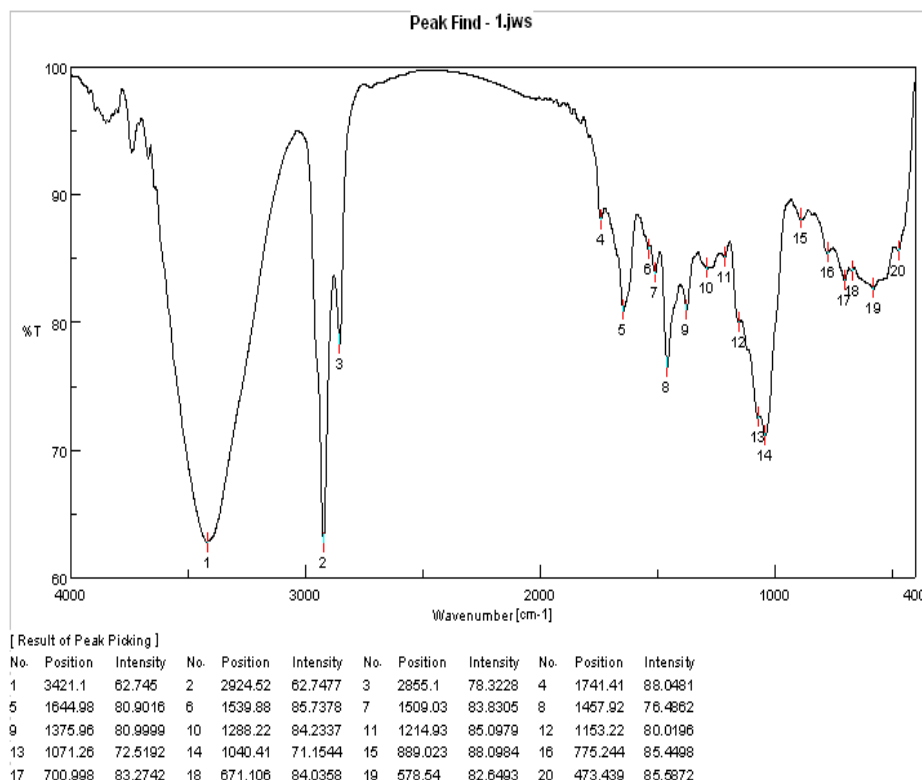
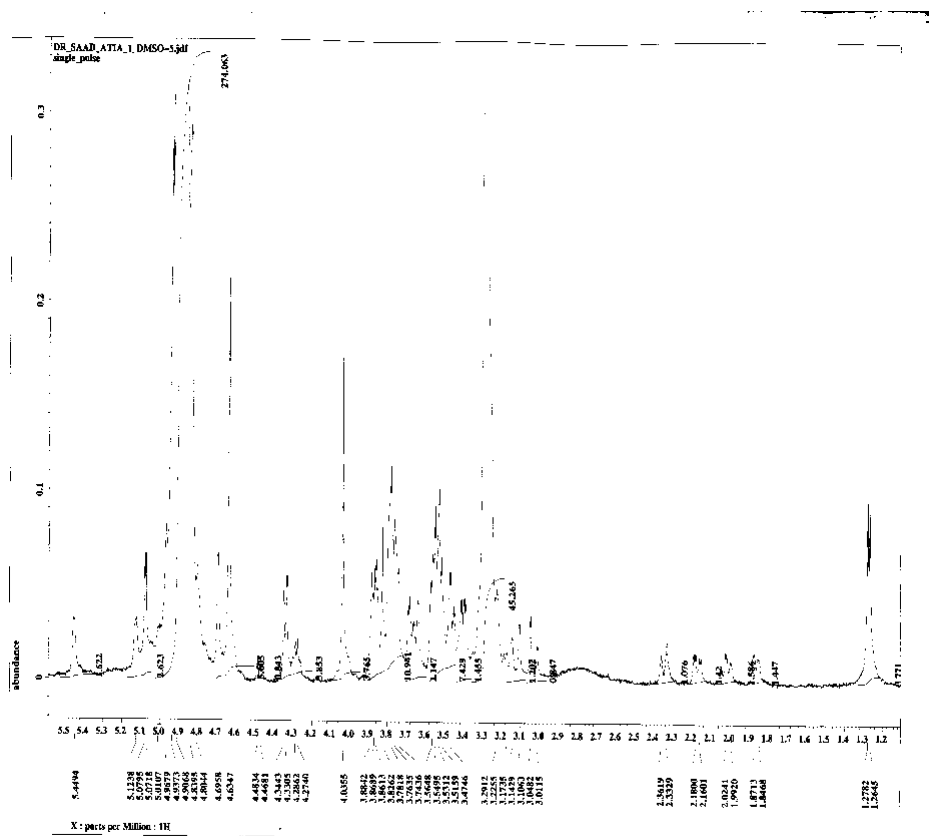


Figure (7): IR spectrum of compound (C – 1) in KBr.

¹HNMR spectrum of compound (C – 1) in CDCl₃ exhibited the following signals at δ :0.04-0.0144 (m, 3H, CH₃); 0.87-0.928 (m,2H, CH₂-CH₃CH₂CHOH); 1.2446 (s, 1H, CH-CH(CH₃)), 1.574 (d, 2H,CH₂-CH₂CH(CH₃)), 3.48 (s, 1H,OH), 3.63 (m, H,CH –CH(OH)CH₂), 7.04-7.28 (m, aromatic protons) figure (8).

The mass spectrum showed a molecular ion peak at m/z 384 (0.58%) which is corresponding to the molecular formula (C₂₁H₁₈O₇) figure: 9.



Figure(8): ¹H NMR spectrum of the compound (C – 1) in CDCl₃

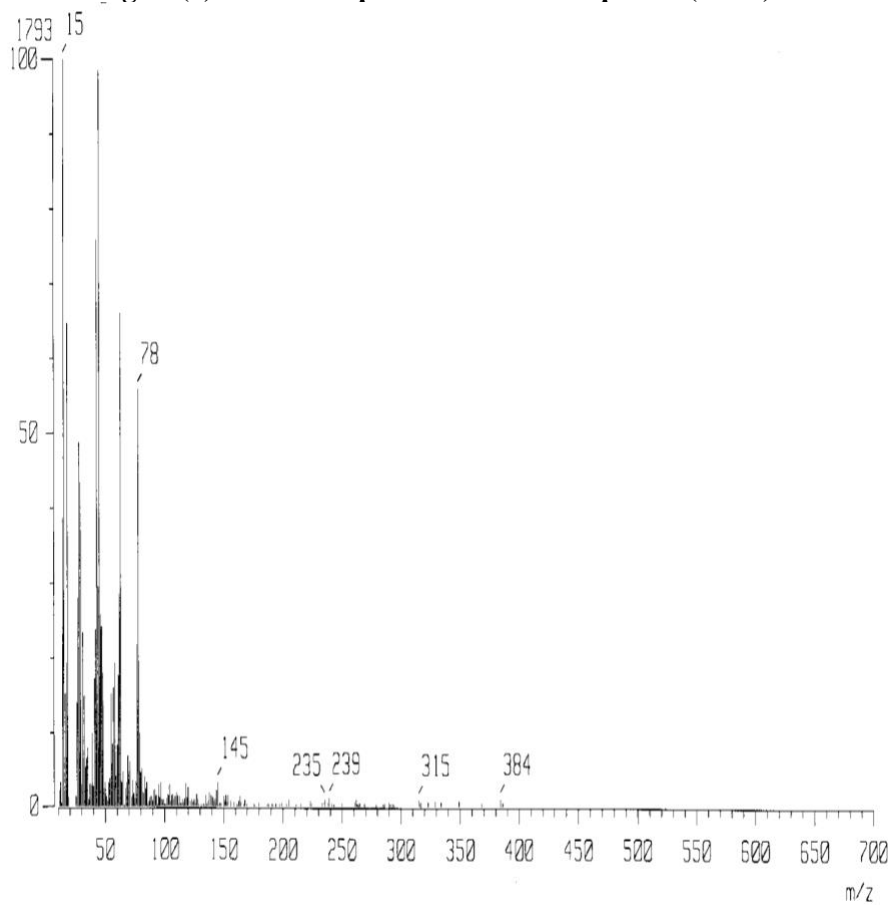
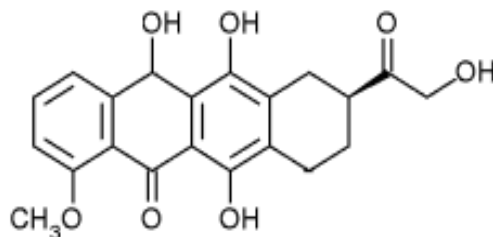


Figure (9): Mass spectrum of the purified compound

From the previous data it is clear that the suggested chemical structure of the purified compound is the following:



5,6,8,11-tetrahydroxy-8-hydroxyacetyl-1-methoxy-7,8,9,10-tetrahydrotetracene-12-dione

On the basis of comparative study of the recorded chemical composition and physical properties of the purified compound produced by *Streptomyces xylophagus* Ha.Ph-7 and by consulting the recommended identification keys of antibiotics such as [30] it could be stated that, the purified active compound is belonging to anthracyclines antibiotic group as a derivative of Daunomycin (Aglycone of daunorubicin, the prototypical anthracyclines) (Arcamone *et al.*, 1964 and Barbieri *et al.*, 1964).

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Table (1): Gradient elution of the crude extract obtained from *Streptomyces xylophagus* Ha.Ph-7

Solvent system	Ratio (v/v)	Volume of eluates (ml)
n-Hexane	100 %	200
n-Hexane : Ethyl acetate	100:0	200
	95:5	600
	90:10	400
	80:20	600
	70:30	200
	50:50	400
	30:70	400
	0:100	200
Methanol	100 %	200

Table (2): Fractions weight of the crude extract obtained from *Streptomyces xylophagus* Ha.Ph-7.

Fraction No.	Collected fractions	Weight (mg)	% Composition in dry extract
S-1	1-2	20	0.66 %
S-2	3-10	80	2.66 %
S-3	11-17	100	3.33 %
S-4	18-22	40	1.33 %
S-5	23-24	65	2.16 %
S-6	25-28	120	4.00 %
S-7	29-31	30	1.00 %
S-8	32-37	135	4.5 %
S-9	38-45	61	2.03 %
S-10	46-47	76	2.53 %
S-11	48-51	111	3.7 %
S-12	52-61	189	6.30 %
S-13	62-end	420	14 %

* S= *Streptomyces*.