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Characterization and Biological Activity of Daunomycinone Produced by *Actinomyces* Strain Isolated from Egyptian Soil

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ABSTRACT

The ongoing rise of microbial antibiotic resistance underscores the importance of discovering new antimicrobial agents. Thus, this research aims to isolate and identify an antimicrobial agent from *Actinomyces* species living in Egyptian soil and test for other biological activities. To address this objective, an *Actinomyces* isolate strain was obtained from Egyptian soil and subsequently identified using 16S rRNA sequence analysis method. We used a bioassay-directed fractionation to guide the isolation of a bioactive compound from the fermentation broth of the isolated active *Actinomyces* strain using the well-diffusion method. The bioactive compound was characterized, and tested on human tumor cell line viability using MTT assay, and on Raw 264.7 macrophage cells nitrite production ability using Griess reagent. Our results showed the isolation and identification of *Actinomyces vulturis* FHM51 strain from the Egyptian soil that possessed an antimicrobial activity. Furthermore, daunomycinone was isolated from the fermentation medium that showed promising antimicrobial activity with MIC for example 100 µg/mL for *Pseudomonas aeruginosa,* 120 µg/mL for *Salmonella* Typhimurium, and for multidrug-resistant pathogens: 2000 µg/mL for *Escherichia coli* 797 and *Candida albicans* 210, and 2500 µg/mL for methicillin-resistant *Staphylococcus aureus.* In addition, daunomycinone showed a selective cytotoxicity against human leukemia HL-60 cells when compared to the non-tumor fibroblasts BJ cells with selectivity index of 2.78. Finally, daunomycinone showed a promising anti-inflammatory effect by inhibiting lipopolysaccharide-mediated nitrite production of Raw 264.7 macrophage cells. Collectively, daunomycinone, isolated from *Actinomyces vulturis* strain FHM51, possesses promising antimicrobial, cytotoxic and anti-inflammatory properties.

Keywords: Daunomycinone, Antimicrobial activity, *Actinomyces* sp., Cytotoxicity, HL-60 cells, Raw 264.7 macrophage cells

1. Introduction

The genus *Actinomyces*, which represents the order Actinomycetales within the phylum Actinobacteria, also called actinomycetes, encompasses a diverse array of anaerobic or facultative anaerobic Gram-positive, non-spore forming, non-motile rods (Barka *et al.,* 2016). Actinomycetes are widely distributed in nature and play a crucial role in the biodegradation of organic matter (Elmallah *et al.,* 2020). Actinomycetes are rich sources of antibiotics and approximately two-thirds of all antibiotics available in the drug market were produced from them (De Simeis and Serra, 2021).

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Globally, it was estimated that bacterial resistance (BR) to antibiotics participated in around 5 million deaths in 2019. Several reasons were introduced to justify this resistance, however, the irrational usage of antibiotics, including the over usage and the misusage, remain the core reasons (Antimicrobial Resistance Collaborators, 2022). BR not only can worsen several diseases and surgeries such as cancers and caesarean sections but can also increase the financial burden of treatment of several diseases (Maarouf *et al.,* 2023). A similar situation is found in Egypt, where BR to antibiotics is an irritating problem that affects several Egyptian patients. *Escherichia coli*, and *Klebsiella* spp. were found to be the most resistant bacteria isolated from patients in Egyptian hospitals, which possess high levels of resistance to several antibiotics, namely, cefixime, ampicillin, and penicillin (Helmy *et al.,* 2023). Therefore, the search for new antibiotics is still in progress.

In the current study, we isolated an *Actinomyces* strain from the Egyptian soil. To isolate a promising bioactive compound from the fermentation media, we used the antimicrobial activity against several test microorganisms (including multidrug-resistant (MDR) pathogens) as our guiding element. The identified promising compound was purified and characterized using column chromatography, thin layer chromatography (TLC), ultraviolet (UV), infrared (IR), mass spectrum (MS), proton nuclear magnetic resonance (^{1}H NMR), and carbon-13 nuclear magnetic resonance (^{13}C NMR) analyses. Furthermore, we tested the effect of the promising compound on human promyelocytic HL-60 leukemia cell line viability in comparison to non-tumor skin fibroblasts BJ cell line viability. Finally, we tested the effect of the promising compound on lipopolysaccharide-mediated nitrite production of Raw 264.7 macrophage cells as a measure of its anti-inflammatory properties.

2. Materials and Methods

2.1. Soil samples collection

Five samples were collected from the soil of different sites in Egypt, namely, Giza (1 sample), Dakhlia (2 samples), and Assuit (2 samples) using soil borer with an open-end diameter of 25 cm and 10 cm depth, air-dried for 24 h and stored in sterile plastic bags (Dezfully and Ramanayaka, 2015).

2.2. Isolation of actinomycetes

Soil samples were 10-fold serially diluted with 1 g of soil dissolved in (10 mL) phosphate buffer solution and were inoculated on starch casein agar medium. The medium was aseptically distributed in sterile glass petri dishes containing streptomycin (30 mg/L) and amphotericin B (50 mg/L) to avoid contaminant growth and left to grow at 28±2℃ for 5 to 10 days. Thereafter, a streaking method was used to purify the selected actinomycetes colonies, and the purified strains were stored on starch casein slants and 25% glycerol stocks at -20°C (Sapkota *et al.,* 2020).

2.3. Antimicrobial activity of the isolate strains

Each isolate was cultured on starch casein medium broth and the supernatant was collected for antimicrobial activity testing using the agar well-diffusion method (Balouiri *et al.,* 2016). The plates, containing Müeller-Hinton agar for bacteria or Müeller-Hinton agar supplemented with 2% glucose for fungi, were swabbed separately with the test micro-organisms which include Gram-positive bacteria such as *Bacillus subtilis* subsp. spizizenii (ATCC 6633), *B. cereus* (ATCC 33018), and Methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 43300), Gram-negative bacteria such as *Pseudomonas aeruginosa* (ATCC 9027*), E. coli* 797*,* and *Salmonella* enteric subsp. enteric serovar Typhimurium (ATCC 14028), yeast such as *Candida albicans* 210 and *Aspergillus niger* (NRRL A-326) fungus (Alastruey-Izquierdo *et al.,* 2015). *E. coli* 797, *and C. albicans* 210 are MDR clinical strains and were obtained from Faculty of Medicine, Department of Chemical and Clinical Pathology, Cairo University, Egypt.

A sterile cork borer was used to make wells (6 mm diameter) in each agar plate and supernatant (20 µL) was put into each well against each test microorganism. Plates were incubated at 37°C for 24 h for bacteria, and at 26°C for 48 h for fungi, and the diameter of inhibition zones (mm) were determined. The isolate that showed a broad spectrum of antimicrobial activity against all microorganisms including MDR, was chosen for further study.

2.4. PCR and sequencing

The active bacterial isolate was selected, allowed to grow in Luria-Bertani broth (LB) for 24 h, washed 3 times using normal saline, and harvested by centrifugation at 12,000 xg for 5 min. Extraction of genomic DNA was carried out using Gene JET Genomic DNA purification kit (Thermo Scientific, Lithuania) according to the manufacturer's recommendations. PCR and sequencing were performed as mentioned previously (Motawe *et al.,* 2024).

Analysis of 16S rRNA sequences of the isolated active strain was performed using the BLAST(N) program, National Center of Biotechnology Information (NCBI) (Rockville Pike, Bethesda MD, USA). Multiple sequence alignments of other actinomycetes from the Genbank were compared with the isolated strain sequence. Construction of the phylogenetic tree was carried out by neighbor joining method using MEGA-X software (Altschul *et al.,* 1997).

2.5. Extraction and purification of the bioactive compound

The selected isolate was cultured in a starch casein broth medium (pH 7) at 30°C with shaking for 6 days. The broth (around 10 L) was filtered and the filtrate was shaken with an equal volume of ethyl acetate. The ethyl acetate fraction was separated and evaporated under vacuum at 40°C. The yield is a dark reddish-brown residue (650 mg) representing the crude bioactive compounds, that re-tested against the previously mentioned test microorganisms.

To purify the crude bioactive compounds, it was dissolved in ethyl acetate and fractionated using silica gel 60-column chromatography with a particle size of 0.063 mm to 0.2 mm (Merck, Darmstadt, Germany). Petroleum ether was used for elution followed by a petroleum ether:ethyl acetate mixture (95:5 v/v). Fractions were concentrated under vacuum and re-dissolved in petroleum ether. TLC sheet (Silica gel 60 F254, layer thickness 0.2 mm, E. Merck, Darmstadt, Germany) was used for fractions spotting using petroleum ether:ethyl acetate (60:20 v/v) as a solvent system till separating a purified bioactive antimicrobial compound (Agour *et al.,* 2022).

2.6. Antimicrobial activity of the purified and crude bioactive compounds

The minimum inhibitory concentration (MIC) is the lowest concentration needed to prevent any visible growth of the test microorganisms. Whereas, the minimum bactericidal concentration (MBC), is the least concentration that resulted in a minimum of 99.8% reduction in the microbial viable count after overnight incubation (Agour *et al.,* 2022).

MIC was determined using a 2-fold micro-dilution procedure (Jorgensen and Ferraro, 2009). The crude and purified compounds (10 mg/mL) or antibiotic standards were serially diluted in 96-well micro-titer plates containing 100 µL/well Müller-Hinton broth medium. Thereafter, each test microorganism in culture broth (10 µL) was adjusted to 0.5 McFarland standard turbidity with OD = 0.1, and added to the antimicrobial agents-containing wells with 10^6 cfu per mL final inoculum size for 24 h at 30°C. The lowest concentration of the crude/bioactive compound exhibited no apparent growth was identified as the MIC value for that microorganism. Thereafter, we cultured the broth dilutions that equal or higher than the MIC by streaking on agar plates and allowed to incubate for 24 to 48 h. The least concentration that showed no apparent growth was identified as MBC for that microorganism (Rodríguez-Melcón *et al.,* 2021).

2.7. Structure elucidation of the purified bioactive compound

The ultraviolet (UV) and Fourier transform infrared (FTIR) spectra were obtained using a SHIMADZU UV-2401PC UV-VIS recording spectrophotometer and a Jasco FTIR-6100 (Japan), respectively. The nuclear magnetic resonance (NMR) spectra, including proton (^{1}H) and carbon (^{13}C) NMR of the purified antimicrobial substances were measured using chloroform with a Joel JNM-EX270 FT-NMR system at the National Research Centre, Egypt. An SSQ700-Finnigan MAT with electron ionization (EI) energy of 70 eV and a scan speed of 0.5 sec was used to identify the MS of the purified bioactive compound.

2.8. Cell culture

Human acute promyeloblastic HL-60 leukemia, human skin fibroblasts non-tumor BJ, and Raw 264.7 murine monocyte/macrophage cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). HL-60 and Raw 264.7 cells were maintained in RPMI media (Biowest,

Nuaille, France), whereas BJ cells were maintained in DMEM-F12 media (Biowest, Nuaille, France). All media were supplemented with 100 IU/mL penicillin, 10% heat-inactivated Fetal bovine serum (FBS), and 100 µg/mL streptomycin. Cells were sub-cultured every 3-5 days using cell scrapper for Raw 264.7 cells, trypsin/EDTA (Invitrogen, Carlsbad, CA, USA) for BJ cells, and centrifugation for HL-60 as they are suspended cells.

2.9. Determination of cell viability

HL-60 leukemia cells ($5x10^4$ cell/well) were suspended in RPMI media containing 2% FBS with increasing concentrations of the bioactive compound in 96-well round-bottom cell culture plates. After 72 h incubation, the plate was centrifuged at 3000 xg for 20 min, media decanted and 40 µL of 4 mM MTT solution (Sigma-Aldrich, MO, USA) was added to each well for 2 h. Another centrifugation for the plate was performed, media were completely removed, and isopropanol (100 µL) was added to each well and incubated for 1h at 37°C. Similar procedure was performed for BJ cells, except that they are monolayer cells and do not need centrifugation. We used doxorubicin (100 µg/mL, Adricin®, EIMC United Pharma (EUP), Egypt) as a positive control, which possessed more than 90% cytotoxicity in both cell lines (El Gendy *et al.,* 2022a; El Gendy *et al.,* 2022b). A BIO-TEK Instrument EL 312e micro-plate reader (Winooski, VT) was used to measure the blue color formed by the viable cells at 570/690 nm. Cell viability was calculated as a percentage relative to control wells (vehicle) (El Gendy *et al.,* 2023).

2.10. Nitric oxide assay

RAW 264.7 macrophage cells were seeded in a 96-well plate at a density of $5x10^4$ cells per well and allowed for a 24 h incubation period at 37ºC and 5% CO_2. Thereafter, the medium was replaced with FBS-free medium and the cells were incubated with increasing concentrations of the bioactive compound for 1 h before the addition of lipopolysaccharide (LPS, 1 µg/mL, Sigma-Aldrich, MO, USA) as a cell stimulator for 24 h. Indomethacin (50 µg/mL; Liometacen® ampouls, El-Nile Co. for Pharmaceuticals, Egypt) was used as a positive control. Nitrite concentration was determined in the culture media using Griess reagent (Sigma-Aldrich, MO, USA). Briefly, an equal volume of cell culture medium and Griess reagent (50 µL each) were mixed in a new micro-titre plate, incubated for 10 min at 37ºC and the pink color was measured at 540 nm in a micro-plate reader (Maksimovic *et al.,* 2005). A standard curve of sodium nitrite ($NaNO_2$) was established to calculate the concentration of nitrite in the culture media.

2.11. Statistical analysis, median inhibitory concentration (IC_{50}), and selectivity index (SI) determination

One-way ANOVA followed by Student–Newman-Keuls post hoc test was used to statistically analyze viability curves. The median inhibitory concentration (IC_{50}) for the bioactive compound was determined on each cell line by establishing a figure (semilog), the best-fitted line was drawn, and the concentration corresponding to 50% viability was determined. Furthermore, the selectivity index (SI) that determines the safety of the bioactive compound was calculated by dividing the mean IC_{50} value of the bioactive compound on non-tumor fibroblasts BJ cells by its mean IC_{50} value on the HL-60 human leukemia cell line. SigmaStat 3.5 program for Windows, Systat Software Inc. (San Jose, CA) was used for performing all statistical analyses (El Gendy *et al.,* 2022a).

3. Results and Discussion

3.1. Isolation of the active actinomycetes

From five different soil samples collected from different areas in Egypt, 150 actinomycetes isolates were recovered and screened for their ability to produce antimicrobial compounds. Out of the 150 isolates, only 65 exhibited antimicrobial activities against the tested microorganisms. Table 1 shows the antimicrobial activity of the most active isolated actinomycetes against the test microorganisms. Isolate FHM51, as depicted in Fig. 1, was found to possess the highest antimicrobial activity and was selected for further investigations, as illustrated in Table 1. In agreement with our model, several antimicrobial compounds were identified and isolated previously from actinomycetes (Chaudhary *et al.,* 2013; Sengupta *et al.,* 2015).

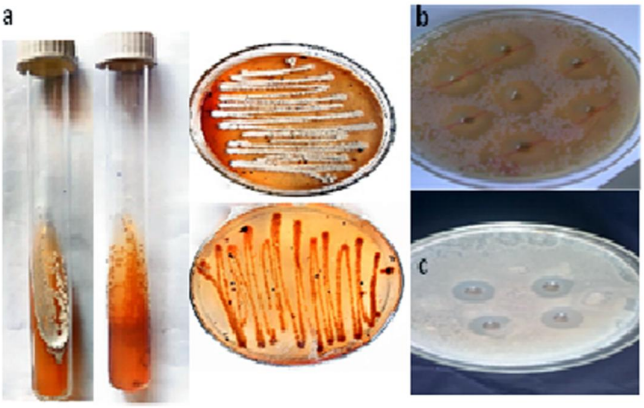


Fig. 1: Isolate FHM51 (a) and its antimicrobial activity against some MDR: *C. albicans* (b) and MRSA(c).

Table 1: Antimicrobial activity of isolated actinomycetes broth

Broth of different isolates of actinomycetes	Antibacterial activity (Inhibition zone, mm)						Antifungal activity (Inhibition zone, mm)	
	Gram +ve			Gram -ve				
	B. subtilis	***B. cereus***	**MRSA (MDR)**	***P. aeruginosa***	***E. coli 797* (MDR)**	***S.* Typhimurium**	***C. albicans 210* (MDR)**	***A. niger***
FHM23	30	30	20	25	-	-	-	-
FHM32	-	-	20	15	15	-	-	-
FHM33	-	-	-	20	-	10	15	20
FHM35	20	25	-	20	20	-	10	-
FHM51	30	30	30	25	30	20	35	25
FHM52	10	15	-	-	-	-	-	-
FHM56	-	-	-	20	-	10	-	20
FHM65	25	20	-	22	-	15	-	20

MRSA: Methicillin-resistant *Staphylococcus aureus*, (-): no activity.

3.2. Genetic identification of the most potent isolated strain FHM51

The 16S rRNA gene of the isolate showing the highest antimicrobial activity (isolate FHM51) was sequenced, and compared with known sequences in the GenBank database using (BLASTN) (http://www.blast.ncbi.nlm.nih.gov/Blast). A very close similarity, with 100% homology of the isolate FHM51 was shown to *Actinomyces* sp., as depicted in Fig. 2. According to DNA sequence analysis, the FHM51 isolate was identified as *Actinomyces vulturis* strain FHM51 with a given accession number PP758394 from the GenBank. The phylogenetic tree was constructed according to the neighbor joining method, as illustrated in Fig. 2.

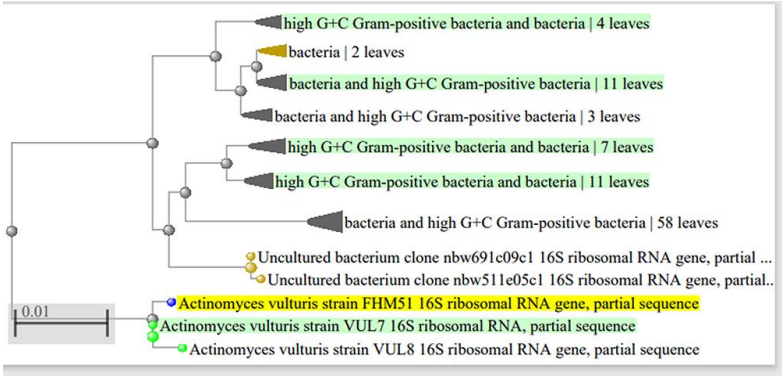


Fig. 2: Phylogenetic tree based on partial 16S rRNA sequences, the tree was constructed using the MEGA-X software for carrying out the neighbor joining method.

3.3. Antimicrobial activity of the crude bioactive compounds

The antimicrobial activity of the crude bioactive compounds was determined by estimating its MIC, which ranged from 55 µg/mL for *B. subtilis* to 200 µg/mL for *A. niger*, compared to the reference antibiotic MIC which ranged from 160 µg/mL for *B. subtilis* to 600 µg/mL for *S.* Typhimurium, as shown in Table 2. Regarding MDR pathogens, crude bioactive compounds' MIC values were between 2500 to 3000 µg/mL which showed resistance to the reference antibiotics, as seen in Table 2. MBC/ MIC ratio was used to discriminate bactericidal from bacteriostatic compounds. It was mentioned previously that compounds were considered bactericidal when MBC/MIC ratio was ≤4, bacteriostatic when the ratio was between 4 and 32, and bacteria-resistant when the ratio was above 32 (Makade *et al.,* 2024). According to this classification, the crude bioactive compounds can be considered as bactericidal as the MBC/MIC ratios were ≤4, as seen in Table 2.

Table 2: Antimicrobial activity of the crude bioactive compounds from *A. vulturis* strain FHM51

Microorganisms	MIC (µg mL^{-1})		MBC (µg mL^{-1})		MBC/ MIC ratio
	Crude bioactive compounds	Reference antibiotic	Crude bioactive compounds	Reference antibiotic	
B. cereus ATCC 33018	120	300	240	600	2
B. subtilis ATCC 6633	55	160	110	160	2
P. aeruginosa ATCC 9027	126	380	150	380	1.1
S. Typhimurium ATCC 14028	160	600	200	600	1.25
A. niger NRRL A-326	200	370	250	370	1.25
MDR microorganisms:					
E. coli 797	2500	c	10000	C	4
MRSA ATCC 43300	3000	c	10000	C	3.33
C. albicans 210	2500	c	5000	C	2

MBC: minimum bactericidal concentration, MIC: minimum inhibitory concentration, Reference antibiotic: metronidazole as antifungal and amoxycillin trihydrate as antibacterial, c: resistant to the following reference antibiotics (µg/desk): amphotericin b-20, augmentin (clavulanic acid-10 + amoxicillin-20), ciprofloxacin-5, gentamicin-10, levofloxacin-5, TS (sulfamethoxazole-23.75 + trimethoprim-125), voriconazole-1000.

3.4. Purification and antimicrobial activity of the bioactive compound

The most bioactive compound was purified using silica gel column chromatography and was given a name of compound R due to its red color. Compound R showed MIC values between 50 to 150 µg/mL in comparison to the reference antibiotic MIC values that ranged between 160 to 600 µg/mL, as shown in Table 3. For MDR pathogens, the bioactive compound R MIC values ranged between 2000–2500 µg/mL, which were resistant to several reference antibiotics, as seen in Table 3. According to a previous classification system, the bioactive compound R can be assigned as a good to moderately good antimicrobial agent. It has been previously reported that the antimicrobial compounds are recognized as "good" when the MIC is less than 1 mg/mL, and "moderately good" when they showed MIC between 1 to 4 mg/mL (Gibbons, 2004; Ríos and Recio, 2005). Moreover, according to Makada *et al.* (2024) classification, the pure bioactive compound R can be considered as bactericidal to all tested microorganisms (MBC/MIC ranged from 1.2 to 2.4), except for *E. coli* 797 that showed a bacteriostatic effect (MBC/MIC equal to 4.5), as seen in Table 3 (Makade *et al.,* 2024).

Table 3: Antimicrobial activity of the bioactive compound R isolated from *A. vulturis* strain FHM51

Microorganisms	MIC (µg mL^{-1})		MBC (µg mL^{-1})		MBC/ MIC ratio
	Bioactive compound R	Reference antibiotic	Bioactive compound R	Reference antibiotic	
B. cereus ATCC 33018	80	300	140	600	1.7
B. subtilis ATCC 6633	50	160	100	160	2
P. aeruginosa ATCC 9027	100	380	150	380	1.5
S. Typhimurium ATCC 14028	120	600	160	600	1.3
A. niger NRRL A-326	150	370	190	370	1.2
MDR microorganisms:					
E. coli 797	2000	c	9000	c	4.5
MRSA ATCC 43300	2500	c	6000	c	2.4
C. albicans 210	2000	c	4000	c	2

MBC: minimum bactericidal concentration, MIC: minimum inhibitory concentration, Reference antibiotic: metronidazole as antifungal and amoxycillin trihydrate as antibacterial, c: resistant to the following reference antibiotics (µg/desk): amphotericin b-20, augmentin (clavulanic acid-10 + amoxicillin-20), ciprofloxacin-5, gentamicin-10, levofloxacin-5, TS (sulfamethoxazole-23.75 + trimethoprim-125), voriconazole-1000.

3.5. Structure elucidation of the bioactive compound R

UV absorption of compound R was in the range of anthracyclines at 224 nm, 274 nm, 277 nm, 322 nm and 327 nm, as shown in Fig. 3. In addition, the IR of compound R gave characteristic bands at: 3441 cm^{-1} for (OH), 2958 cm^{-1}, 2926 cm^{-1}, and 2856 cm^{-1}, for (CH, CH_2, and CH_3, respectively), 1731 cm^{-1} for (C=O), 1461 cm^{-1} for (CH_3), 1383 cm^{-1}, and 1273 cm^{-1} for (C-OH, and CH_3), and 743 cm^{-1}, and 651 cm^{-1} for aromatic ring, as shown in Fig. 4.

^{1}H NMR and ^{13}C NMR of compound R are listed in Table 4 and shown in Figs. 5 and 6, respectively. The mass spectrum of compound R was illustrated in Fig. 7 and gave molecular ion peak at m/z 343 that was resulted from the removal of three H_2O and one H to give fragment with molecular formula $C_{21}H_{11}O_5$, as seen in Fig. 8B. The base peak was shown at m/z 148 with molecular formula $C_9H_8O_2$, as illustrated in Fig. 8C. The base peak resulted from the cleavage of ring B with the removal of O to give a fragment in ionic form.

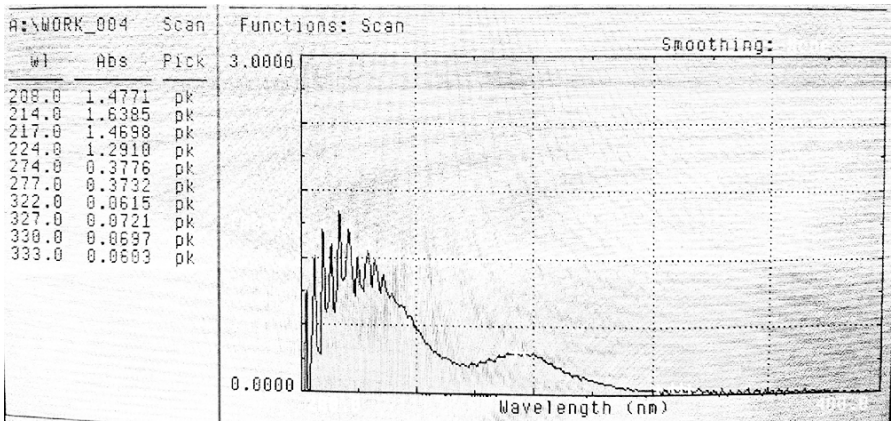
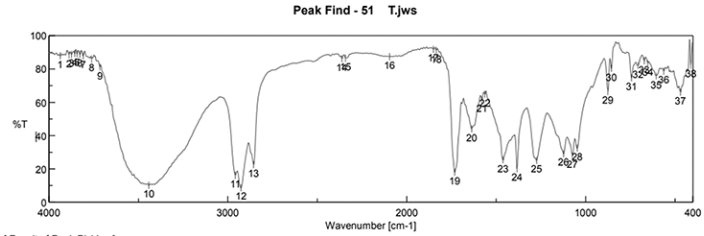


Fig. 3: UV spectrum of the bioactive compound R produced by *A. vulturis* strain FHM51.



[Result of Peak Picking]

No.	Position	Intensity	No.	Position	Intensity	No.	Position	Intensity
1	3936.97	87.8675	2	3887.79	88.4853	3	3874.29	88.6778
4	3855.97	89.2599	5	3843.43	89.1404	6	3827.04	88.8026
7	3809.69	87.7857	8	3761.47	85.918	9	3715.19	81.0379
10	3441.35	10.0973	11	2958.27	16.1536	12	2926.45	8.66634
13	2856.06	22.5335	14	2362.37	86.1362	15	2342.12	86.4013
16	2096.24	87.3419	17	1851.33	91.7266	18	1834.94	91.2138
19	1731.76	17.9901	20	1637.27	44.0435	21	1579.41	61.3712
22	1563.02	64.8085	23	1461.78	25.4095	24	1383.68	20.0308
25	1273.75	25.0493	26	1123.33	29.1358	27	1074.16	27.7371
28	1047.16	32.4601	29	875.524	66.4637	30	856.239	80.3177
31	743.424	74.6482	32	706.783	82.0864	33	672.071	84.8323
34	651.822	83.2307	35	605.539	75.7565	36	563.112	78.5582
37	469.582	65.9649	38	411.728	82.2306			

Fig. 4: IR spectrum of the bioactive compound R produced by *A. vulturis* strain FHM51.

Table 4: ^{1}H and ^{13}C NMR of compound R

C	δ H	δ ^{13}C
1	7.78	118
2	7.76	131
3	7.47	117
4	——	153
5	——	120
6	——	187
7	——	113
8	——	113
9	——	188
10	——	133
11	——	154
12	——	133
13	——	134
14	——	154
15	——	68
16	——	32
17	——	77
18	——	34
19	——	> 200
20	44.22	25
21	3.7	55
Ring D proton hump	0.8 - 2.49	——

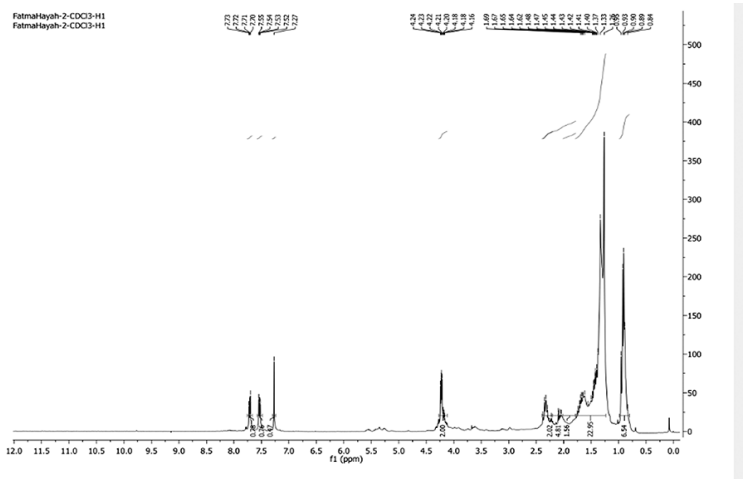


Fig. 5: ^{1}H NMR spectrum of the bioactive compound R produced by *A. vulturis* strain FHM51.

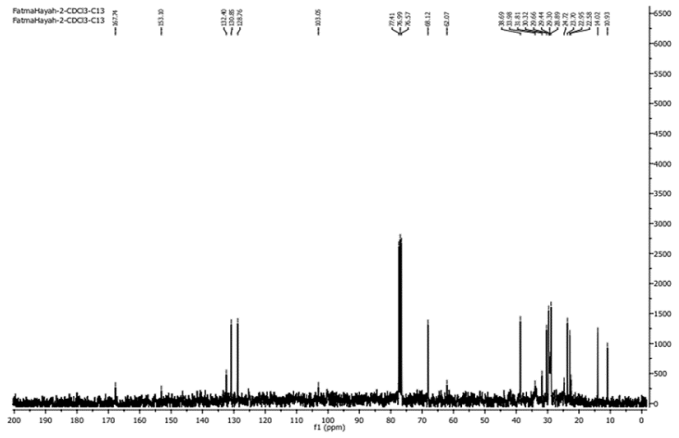


Fig. 6: ^{13}C NMR spectrum of the bioactive compound R produced by *A. vulturis* strain FHM51.

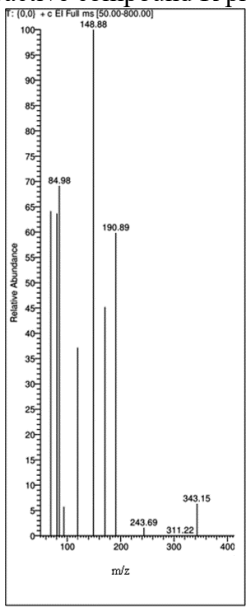


Fig. 7: Mass spectrum of the bioactive compound R produced by *A. vulturis* strain FHM51.

Compound R is related to anthracyclines and it gave negative results in Molisch's test indicating the absence of carbohydrates and that the compound is an aglycon and not a glycoside. From the mass spectral data in comparison with cited data, it was deduced to be daunomycinone, the aglycone of daunomycin, as seen in Fig. 8A, with a molecular formula of $C_{21}H_{18}O_8$. Elementary analysis of compound R gave 63.38 for carbon, 4.55 for hydrogen, and 32.13 for oxygen from which an empirical formula C_3H_3O was deduced.

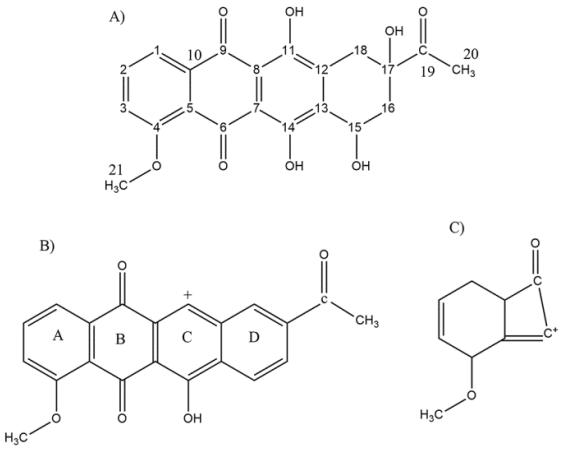


Fig. 8: A): Daunomycinone structure ($C_{21}H_{18}O_8$, compound R), B):Molecular ion peak of daunomycinone; m/z 343 ($C_{21}H_{11}O_5$), C): Base peak of daunomycinone; m/z 148 ($C_9H_8O_2$).

It was mentioned previously the ability of several Streptomyces strains such as *Streptomyces coeruleorubidus* JA 10092 and ISP 5145 to produce dihydrodaunomycinone and traces of daunomycin, and daunomycinone, whereas *S. peucetius* IMI 101 335 managed to produce daunomycin (Blumauerová *et al.,* 1977). However, according to the literature review, this is the first report to mention the production of daunomycinone from *Actinomyces* species. In agreement with our results, daunomycin, the aminoglycoside of daunomycinone, has been approved from Food and Drug Administration (FDA) as an adjuvant and potentiation therapy with the last-resort antibiotics (such as colistin) against MDR Gram-negative pathogens (Cai *et al.,* 2023).

3.6. Effect on cell viability

Daunomycinone significantly reduced HL-60 leukemia and non-tumor BJ cell lines viability at all tested concentrations with estimated IC_{50} of 18.6 and 51.7 µg/mL, respectively, as seen in Fig. 9. To determine the selectivity of daunomycinone to tumor cells, SI was calculated and it was found to be 2.78, which means that daunomycinone is more selective to leukemia HL-60 cells by 2.78 times than non-tumor BJ cells. In agreement with our results, it was mentioned previously the anti-leukemic properties of some daunomycinone derivatives using mouse lymphoblastic leukemia L1210 cells (Perchellet *et al.,* 2001). Moreover, daunomycin, the aminoglycoside of daunomycinone, has been approved and used in combination with cytarabine for treating acute myeloid leukemia (Strickland and Vey, 2022).

3.7. Effect on Raw 264.7 macrophage

In order to explore other biological activities of daunomycinone, we tested its effect on murine Raw 264.7 macrophage nitric oxide production ability (detected as nitrite concentration). As shown in Fig. 10, LPS significantly induced nitrite concentration by 7-fold. Moreover, pre-treatment of daunomycinone, significantly reduced LPS-mediated induction of nitrite concentration in a concentration-dependent fashion by 19.8%, 27.3%, 45.6%, and 58.1% with 12.5, 25, 50 and 100 µg/mL, respectively, as indicated in Fig.10. We used indomethacin (50 µg/mL) as a positive control and a known anti-inflammatory agent that reduced LPS-mediated nitrite concentration by 54.6%, as illustrated in Fig. 10. These findings may suggest that daunomycinone can modulate macrophage function and may be considered as anti-inflammatory agent.

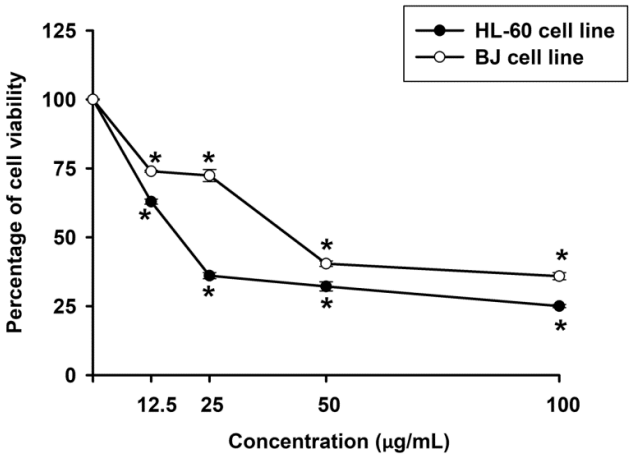


Fig. 9: Effect of daunomycinone isolated from *A. vulturis* strain FHM51 on human leukemia HL-60 and non-tumor human fibroblasts BJ cell viability. Results were presented as percent of vehicle-treated control, which was set at 100%, ± S.E.M. (n = 3). (*) $P < 0.05$ compared with vehicle-treated control.

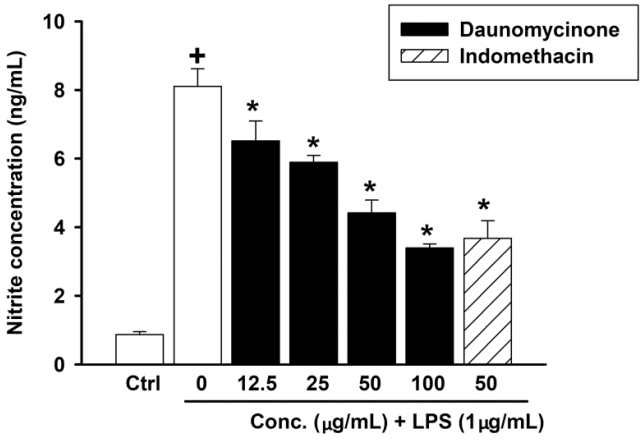


Fig. 10: Effect of daunomycinone isolated from *A. vulturis* strain FHM51 on murine Raw 264.7 macrophage cell line nitrite production ability. Data were expressed as average, ± S.E.M. (n = 4). (+) $P < 0.05$ compared with vehicle control, (*) $P < 0.05$ compared with LPS.

4. Conclusion

To the best of our knowledge this study reported, for the first time, the isolation of daunomycinone from *Actinomyces* species. The produced daunomycinone has a promising effect as an antimicrobial agent against fungi, Gram-positive and Gram-negative microorganisms including multidrug-resistant ones. Moreover, daunomycinone showed a selective cytotoxic impact for human leukemia HL-60 cells and affected the function of lipopolysaccharide-stimulated Raw 264.7 macrophage cells, suggesting anti-inflammatory properties.

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