



Novel aromatic polyketides from soil *Streptomyces* spp.: purification, characterization and bioactivity studies

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Abstract

Aromatic polyketides are important therapeutic compounds which include front line antibiotics and anticancer drugs. Since most of the aromatic polyketides are known to be produced by soil dwelling *Streptomyces*, 54 *Streptomyces* strains were isolated from the soil samples. Five isolates, R1, B1, R3, R5 and Y8 were found to be potent aromatic polyketide producers and were identified by 16S rRNA gene sequencing as *Streptomyces spectabilis*, *Streptomyces olivaceus*, *Streptomyces purpurascens*, *Streptomyces coeruleorubidus* and *Streptomyces lavendofoliae* respectively. Their sequences have been deposited in the GenBank under the accession numbers KF468818, KF681280, KF395224, KF527511 and KF681281 respectively. The *Streptomyces* strains were cultivated in the media following critically optimised culture conditions. The resulting broth extracts were fractionated on a silica gel column and preparative TLC to obtain pure compounds. The pure compounds were tested for bioactivity and the most potent compound from each isolate was identified by UV–Vis, IR and NMR spectroscopic methods. Isolated *S. spectabilis* (R1), yielded one potent compound identified as dihydrodaunomycin with an MIC of 4 µg/ml against *Bacillus cereus* and an IC₅₀ value of 24 µM against HeLa. *S. olivaceus* (B1), yielded a comparatively less potent compound, elloramycin. *S. purpurascens* (R3) yielded three compounds, rhodomycin, epelmocin and obelmocin. The most potent compound was rhodomycin with an MIC of 2 µg/ml against *B. cereus* and IC₅₀ of 15 µM against HeLa. *S. coeruleorubidus* (R5), yielded daunomycin showing an IC₅₀ of 10 µM and also exhibiting antimetastatic properties against HeLa. *S. lavendofoliae* (Y8), yielded a novel aclacinomycin analogue with IC₅₀ value of 2.9 µM and potent antimetastatic properties at 1 µM concentration against HeLa. The study focuses on the characterization of aromatic polyketides from soil *Streptomyces* spp., which can serve as potential candidates for development of chemotherapeutic drugs in future.

Keywords *Streptomyces* · Anthracyclines · Anticancer · Aromatic polyketides

Introduction

Streptomyces are aerobic, Gram-positive, filamentous, soil dwelling bacteria which belong to the order *Actinomycetales* within the class *Actinobacteria* (Anderson and Wellington 2001). These bacteria with exceptional metabolic diversity are a rich source of several useful bioactive natural products,

such as polyketides (Arifuzzaman et al. 2010; Holkar et al. 2013b). Polyketides are pharmacologically very important and are considered second to penicillins in terms of their importance as naturally derived medicines. Aromatic polyketides consist of a large group of structurally diverse natural products which include tetracyclines, anthracyclines (e.g., daunorubicin), aureolic acids (e.g., mithramycin), tetracenomycins, angucyclines (e.g., jadomycin, pradimicin), and benzisochromanequinones (e.g., actinorhodin and frenolicin). They exhibit a broad range of biological activities such as antibacterial, antifungal, antitumor and antiviral activities and afford some of the most common antibiotics and anticancer drugs currently in clinical use.

The commonly used anticancer drugs are: daunomycin and doxorubicin as preferred antineoplastic agents; adriamycin for treatment of breast cancer, Hodgkin's

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lymphoma, lung cancer, multiple myeloma, and recurring ovarian cancer; mithramycin for treating Paget's disease, chronic myelogenous leukemia and advanced testicular carcinoma; epirubicin, a less cardiotoxic doxorubicin analogue for treating sarcomas, breast and gastric cancer; idarubicin, for acute myeloid leukemia; and valrubicin, for the intravesical treatment of bladder cancer (Kummar et al. 2006; Kizek et al. 2012). However, a major problem with their clinical use, in addition to adverse common side effects like myelosuppression, nausea and vomiting, mouth ulcers, local aggressivity and alopecia, is their cardiotoxicity, gastrointestinal, hepatic, and bone marrow toxicity (Rahman et al. 2007). Similarly, the biggest problem hampering the use of the current antimicrobial drugs in use like tetracyclines, macrolides and aminoglycosides is the development of resistance to them leading to the emergence of MDR strains. Nevertheless, despite an extensive list of available anticancer and antimicrobial compounds, new compounds that are more effective, less toxic and possessing broader activity are still required.

Most known aromatic polyketides are produced by *Streptomyces* (Hertweck et al. 2007). A systematic screening program was undertaken with the aim of isolating *Streptomyces* species producing novel aromatic polyketides. Fifty-four *Streptomyces* strains were isolated from the soil samples collected from in and around Nagpur, India. Five isolates, R1, B1, R3, R5 and Y8 were found to be potent aromatic polyketide producers and were selected for further studies (Bundale et al. 2014). The microscopic, morphological, biochemical and physiological characterization strongly suggested that the isolates belong to the genus *Streptomyces*. Furthermore, these strains were identified by 16S rRNA gene sequencing as *Streptomyces spectabilis*, *Streptomyces olivaceus*, *Streptomyces purpurascens*, *Streptomyces coeruleorubidus* and *Streptomyces lavendofoliae* and their sequences were deposited in the GenBank under the accession numbers KF468818, KF681280, KF395224, KF527511 and KF681281 respectively.

The current study involves the purification and characterization of the bioactive aromatic polyketides from the crude antibiotic complexes of the five selected isolates and the evaluation of their antimicrobial and anticancer properties.

Materials and methods

Chemicals and media

All chemicals and solvents were of analytical grade and purchased from Merck, Germany and culture media from Himedia, Mumbai, India. Standard doxorubicin was obtained from Sigma-Aldrich (St. Louis, MO, USA).

Test organisms

The target strains used for screening antimicrobial activity were procured from microbial type culture collection and gene bank (IMTECH, Chandigarh, India) and were: *Bacillus cereus* MTCC 430, *Escherichia coli* MTCC 443, *Candida albicans* MTCC 227, and *Aspergillus niger* MTCC 282. The cell lines were purchased from NCCS, Pune, India.

Cultivation and organic extraction

For each of the selected isolates, sterile media optimized in an earlier study (Bundale et al. 2015) were used for growth in a rotary shaker incubator (REMI CIS-24 BL) at 130 rpm at 28 °C for 10 days. Crude antimicrobial compound was recovered from the mycelium as well as culture filtrate of each bioactive isolate by solvent extraction with ethyl acetate (1:1 v/v). The solvent was evaporated to dryness in a vacuum concentrator to obtain the crude cell and broth extracts which were stored at -20 °C until further use.

Purification of the bioactive components

The dried crude extract was dissolved in ethyl acetate and 100–500 µl was loaded over the silica gel column. A step-wise gradient of chloroform/methanol was applied and the fractions thus separated were collected.

Preparative thin layer chromatography with Silica gel plate 60 F₂₅₄ was used for the partial purification of antimicrobial products. The crude extracts were spotted and developed in different solvent systems. The solvent systems used were SS1: chloroform:petroleum ether:methanol (10:10:3), SS2: benzene:acetone:methanol (100:10:1), SS3: methanol:chloroform:acetic acid:water (80:20:2:0.2), SS4: chloroform:methanol:aq ammonia (25%) (85:14:1), SS5: chloroform:methanol (8:2) and SS6: chloroform:benzene:ethyl acetate:acetic acid (40:40:15:5), SS7: chloroform:methanol (9:1), SS8: chloroform:methanol:acetic acid:water (80:20:16:6), SS9: chloroform:methanol (15:1), SS10: chloroform:methanol (10:1) (Yoshimoto et al. 1980; Batel et al. 1990; Johdo et al. 1991; Saito et al. 1995; Dickens et al. 1997). The developed plates were air dried and the separated bands were detected by observations of the color of the bands. The TLC was repeated several times and the mean R_f of the bands was calculated. The fractions were physically separated from each other by scraping the bands from the plates, extracting with methanol, concentrating the extracts

and again subjecting each concentrate to TLC using the same solvent system, thereby confirming the purity of each fraction (Johdo et al. 1991; Kim et al. 1996).

Identification and structural elucidation

Qualitative determination of aglycone and sugar residues by TLC

To an aliquot of the purified fraction, 0.5 ml of 0.1 N HCl was added and was heated at 85 °C for 30 min in a water bath. The pigment aglycone thus obtained was extracted with chloroform. The chloroform layer was evaporated to dryness and the residue was chromatographed on a Silica gel plate 60 F₂₅₄ in SS9 solvent system. The identity of aglycones was determined by comparing their *R_f* values and colors on the TLC plate with that of the standards (Holkar et al. 2013b; Johdo et al. 1991).

The aqueous layer containing sugar components was neutralized by adding silver carbonate with a small amount of charcoal and centrifuged. The supernatant fluid was concentrated in vacuo and chromatographed on TLC Silica gel plate 60 F₂₅₄ in butanol:acetic acid:H₂O (4:1:1). The sugar spots were detected with *p*-anisaldehyde—H₂SO₄ (each 5%) in 90% ethanol spray reagent followed by heating at 90° (Yoshimoto et al. 1980; Saito et al. 1995; Johdo et al. 1991).

Spectral studies

The UV–visible absorption spectra (190–1100 nm) of the purified fractions were determined to identify the chromophores present in the metabolites by using a double beam Bio-spectrophotometer (BL-198, Elico Ltd.) (Silverstein et al. 2014). Furthermore, FT-IR spectrum of each active extract was obtained (as KBr discs) between 400 and 4000 cm⁻¹ on Perkin Elmer 2000 FT-IR spectrophotometer and plotted as intensity versus wave number (Augustine et al. 2005). ¹H NMR spectra of the purified bioactive compounds was measured using a Bruker AMX 300 Coupling constants (*J*) in Hz. The software, ChemDraw was used for predicting the ¹H NMR spectra of the suspected compounds and the predicted spectra were compared with that of the physically determined ¹H NMR spectra (Workman 2000).

Antimicrobial activity

The antimicrobial activity of the pure compounds was assessed by the agar well diffusion method using Mueller–Hinton agar for antibacterial (Roe et al. 2003) and Potato Dextrose Agar for anti-fungal assays.

15 µl of 1 mg/ml stock extracts were used for the tests. The diameter of the inhibition zones was determined after 24 h of incubation at 37 °C for bacteria and 28 °C for fungi.

The minimum inhibitory concentrations (MICs) of the bioactive compounds were determined via a microdilution method using sterile 24-well plates with tetracycline as a standard (Arthington-Skaggs et al. 2002).

MTT-based cytotoxicity assay

The cytotoxicity of bioactive fractions on established cell lines like HeLa cervical cell line and mouse fibroblast cell line L929 was determined in vitro (Mosmann 1983; Begde et al. 2011; Wang et al. 2015). Freshly passaged subconfluent HeLa cells were centrifuged and washed with HBSS. The cell count was adjusted to 2.5 × 10⁵ cells/ml by suspending the cells in fresh DMEM with 10% FBS. This cell suspension (200 µl) was then transferred to a 96-well TC plate for the assay and a monolayer was developed at 37 °C for 24 h. Thereafter, the spent medium was discarded and the adherent cells were exposed to a concentration gradient of each purified fraction supplemented in the medium to obtain a final concentration of 2.5, 5, 10, 15, 20, 25, 30 and 35 µM respectively. The plate was incubated at 37 °C in a humidified environment with 5% CO₂ in air for 48 h. After incubation the cells were washed and resuspended in fresh medium containing MTT and were further incubated for 3 h. The MTT formazan produced by the viable cells was extracted in DMSO and quantified at 570 nm in the Bio-Rad ELISA plate reader.

Anti-metastatic efficacy by scratch/in vitro wound healing assay

Anti-metastatic activity of the purified compounds, was analyzed by wound healing assay (Liang et al. 2007). HeLa cells were seeded in 24-well tissue culture plates and cultured in DMEM containing 10% FBS to confluent cell monolayers, which were then carefully wounded using sterile 200 µl micropipette tips and any cell debris was removed with PBS. The cells were then exposed to sub inhibitory concentration of the purified compounds dissolved in the growth medium containing 5% FBS for 24 h at 37 °C (Gebäck et al. 2009). The extent of cell migration and wound healing was determined by using T-scratch analysis software (<http://www.cselab.ethz.ch/software.html>).

Statistical analysis

For MIC, values were expressed as average of four independent replicates ± SD and for MTT based cytotoxicity assay as an average of eight replicates ± SD. Student's *t* test was performed using SYSTAT Software (Systat Software Inc., Chicago, IL, USA). *P* value ≤ 0.05 was considered significant unless otherwise mentioned. For in vitro wound

healing assay, values were expressed as mean of four replicates \pm SD.

Results

Five aromatic polyketide producing *Streptomyces* strains, R1, B1, R3, R5 and Y8 were selected on the basis of pre-screening results. The following sections describe the characterization of the most potent bioactive compounds produced by the respective organisms followed by bioactivity studies.

Characterization of the aromatic polyketides produced by isolated *Streptomyces* spp.

The antibiotic complexes from all five producer organisms were purified using silica gel adsorption column chromatography followed by preparative TLC. The purified fractions were named R1A, R1B, R1C, R1D, R1E and R1F for isolate R1. A similar numbering scheme was used for all bioactive fractions obtained from their respective organisms. The solvent system used, and the color, R_f 's and λ_{max} of the fractions obtained from each organism are given in the Table 1. On acid hydrolysis, followed by TLC analysis, the purified fractions separated into aglycones and corresponding sugar components, the details of which are summarized in Table 2.

Identification of compound R1D

Compound R1D, obtained as a red colored compound, on acid hydrolysis yielded the corresponding aglycone whose R_f was found to be 0.25 in the solvent system chloroform:methanol (20:1). The sugar on direct comparison of R_f on TLC with the standard was found to be daunosamine. Compound R1D resolved as one single sharp red band with an R_f of 0.63 in the solvent system SS8. The UV visible spectra showed prominent peaks at 292, 334, 472 and 537 nm (Fig. 1a). The IR (KBr) spectra showed prominent peaks at 3351, 2945, 2832, 1449, 1116 and 1028 (cm^{-1}) (Fig. 1c). The chemical shifts shown by the proton NMR of R1D were δ 4.4 (CH tetrahydronaphthlene), δ 1.99–2.4 (CH_2), δ 3.12–3.37 (CH_2), δ 5.0 (aromatic OH), δ 4.8 (CH_2), δ 3.6–3.8 (tetrahydropyran), δ 4.8 (CH), δ 1.21 (CH_3), δ 2.0 (NH_2), δ 3.73 (methyl) (Fig. 1d).

Identification of compound B1B

Compound B1B was obtained as a dark yellow compound which was insoluble in water and soluble in chloroform and acetone. B1B showed a sharp orange-yellow band with an R_f of 0.69 in SS7. On acid hydrolysis, compound B1B dissociated into an aglycone with an R_f of 0.45, which

is the reported R_f of elloramycinone. The sugar residue was found to be rhamnose on comparison with standard rhamnose. The IR (KBr) spectra showed prominent peaks at 3619, 3045, 1561, 1361, 1286 and 1182 cm^{-1} (Fig. 2c). The ^1H NMR of B1B is shown in Fig. 2d. The shifts seen in the aglycone part were δ 5.0 (H), δ 3.80 ($-\text{OCH}_3$), δ 3.14 ($-\text{OH}$), δ 8.01 (H), δ 7.15 (H), δ 2.90 ($-\text{CH}_3$) and in the sugar residue attached to the chromophore were at δ 3.77, δ 3.24, δ 3.59, δ 1.21 and δ 4.11.

Identification of compound R3E

Compound R3E was found to contain β -rhodomycinone (β -RMN) as the aglycone and L-rhodosamine as the sugar on the basis of R_f and UV-Vis spectra. Also the UV-Vis spectrum of R3E was found to have λ_{max} at 297, 492 and 561 nm (Fig. 3a). The IR (KBr) spectra showed characteristic peaks at 3394, 2292, 1736, 1577, 1490, 1458 and 1190 cm^{-1} (Fig. 3c). The chemical shifts shown by the proton NMR of R3E were δ 7.02– δ 7.38 (Benzene), δ 4.2 (CH tetrahydronaphthlene), δ 1.72–1.97 (CH_2), δ 5.0 (aromatic OH), δ 1.44 (CH_2), δ 1.66–1.95 (tetrahydropyran), δ 2.81–3.55 (tetrahydropyran), δ 4.8 (CH), δ 1.21 (CH_3), δ 2.27 (CH_3) and δ 3.73 (CH_3) (Fig. 3d).

Identification of the compound R5F

18.8 mg of the compound R5F was obtained, which was orange red in color and on treatment with 0.5% magnesium acetate changed color from orange-red to violet. Acid hydrolysis of this compound liberated the aglycone, daunomycinone and the sugar daunosamine.

The UV-Vis spectra of the standard daunomycin and R5F showed the same peaks at 292, 421, 472 and 517. A comparison of their spectra is shown in Fig. 4a. The IR (KBr) spectra of R5F showed prominent peaks at 3431, 2949, 2837, 1645, 1474, 1288, 1118, 1068 and 1018 (cm^{-1}) and that of the standard daunomycin showed peaks at 3351, 2982, 2945, 2863, 2832, 1655, 1452, 1208, 1116 and 1032 (cm^{-1}). From the similarity in the two IR spectra, it can be concluded that R5F is very similar to daunomycin (Fig. 4b).

Using the software ChemDraw, the NMR spectra of daunomycin was predicted. The spectra showed shifts at δ 7.06–7.44 (3 aromatic protons), δ 1.99 and δ 2.24 (CH_2 , 1,2,3,4 tetrahydronaphthalene), δ 3.12 and 3.37 (CH_2 , 1,2,3,4 tetrahydronaphthalene), δ 5 (δ (aromatic OH), δ 2.09 (CH_3), δ 3.13–3.86 (tetrahydropyran), δ 2 (NH_3), 1.21 and δ 3.73 (CH_3). These shifts could also be seen in the NMR spectra of compound R5F (Fig. 4d).

Table 1 *R_f* values and UV–Vis λ_{max} of fractions of the isolates


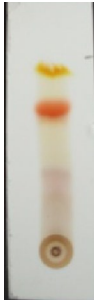
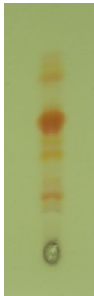


Isolates	Solvent system	Fractions	<i>R_f</i> of the fractions	Color		λ_{max}
Isolate R1	SS6	A	0.90	Light pink		277, 487, 532
		B	0.66	Yellow	222, 272	
		C	0.57	Orange	272, 540	
		D	0.34	Red	417, 535	
		E	0.30	Pink	273, 532	
		F	0.24	Violet	267, 532	
Isolate B2	SS7	A	0.77	Yellow		292, 416, 452
		B	0.69	Orange	292, 422, 503	
		C	0.55	Blue	245, 262	
		D	0.22	Brown	288, 419, 458	
Isolate R3	SS4	A	0.88	Red	284, 492, 522	
		B	0.80	Orange	297, 492	
		C	0.79	Reddish orange	237, 495, 522	
		D	0.68	Yellowish orange	287, 437	
		E	0.62	Orange	297, 492	
		F	0.48	Pink	297, 497	
		G	0.17	violet		

Table 1 (continued)

Isolates	Solvent system	Fractions	R _f of the fractions	Color		λ _{max}
Isolate R5	SS6	A	0.92	Purple		292, 492
		B	0.85	Yellow		270, 293, 490
		C	0.71	Pink		257, 292, 492
		D	0.53	Yellowish brown		257, 387
		E	0.38	Yellowish orange		242, 492, 525
		F	0.23	Orange		256, 419, 472, 517
Isolate Y8	SS10	A	0.47	Dark yellow	247, 379, 422, 445	
		B	0.23	Pale yellow	242, 377, 417, 437	

SS4—chloroform:methanol:aq ammonia (25%) (85:14:1), SS6—chloroform:benzene:ethyl acetate:acetic acid (40:40:15:5), SS7—chloroform:methanol (9:1), SS10—chloroform:methanol (10:1)

Identification of the compound Y8A

The dark yellow band with an R_f of 0.11 in chloroform:methanol (20:1) afforded 54 mg of the pure compound Y8A. Y8A on acid hydrolysis, yielded a yellow aglycone and two sugar moieties. The yellow aglycone on the basis of R_f and UV–Vis spectra was found to be aklavinone. The sugars on the basis of R_f and color reactions on TLC were found to be daunosamine and deoxyfucose. Thus this compound is a daunosaminyl deoxyfucosyl aklavinone, which may be a new aclacinomycin analogue. The UV–Vis spectra showed prominent peaks at 311, 423 and 444 nm (Fig. 5a). The IR (KBr) spectra exhibited prominent peaks at 3048, 2949, 2849, 2775, 1737, 1654, 1585 and 1004 cm⁻¹ (Fig. 5c). The ¹H NMR showed chemical shifts at δ 4.8, δ 3.3, δ 2.2, δ 1.8–2.0 (CH₂), δ 1.3 (CH₃), δ 2.0 (NH₂) as seen in aclacinomycins.

Table 3 summarizes the physicochemical properties of the most potent purified compounds obtained from all the five isolates.

Antimicrobial and antitumor activity

Antimicrobial activity

Compounds R1A and R1B did not exhibit any activity against *B. cereus*, whereas R1C–R1F showed good activity with zones of inhibition in the range of 4–8 mm. Though isolate R1 was found to possess antifungal activity in preliminary screening by cross streak method, subsequent secondary screening results with *C. albicans* and *A. niger* were negative. The MIC of R1C and R1D against *B. cereus* were found to be 10 and 4 μg/ml, respectively, while those for R1E and R1F were both 8 μg/ml. The pure compounds of isolate B1 failed to show any appreciable antifungal activity against *C. albicans* and *A. niger*. Also no antibacterial activity was observed against *B. cereus*. All the pure compounds obtained from isolate R3 were found to exhibit antibacterial activity against *B. cereus*, the most potent being R3A and R3E with zones of inhibition of 7 and 9 mm respectively.

Table 2 Preliminary identification of the components obtained from each isolate

Isolate	Fractions	Aglycones		Sugars			Compound predicted
		Rf ^a	Aglycone detected	Rf ^b	Color	Sugar detected	
Isolate R1	A	0.33	7-Deoxydihydrodaunomycinone	ND			ND
	B	0.70	7-Deoxyaklavinone	0.34	Sky blue	Daunosamine	Daunosaminy 7-deoxyaklavinone
	C	0.79	ϵ RMN	0.34	Sky blue	Daunosamine	Rhodomyacin D
				0.71		Rhodinose	
				0.56	Grayish blue	2-Deoxy-L-fucose	
	D	0.25	Dihydrodaunomycinone	0.34	Sky blue	Daunosamine	Di hydrodaunomycin
E	0.58	13-Deoxydaunomycinone	0.34	Sky blue	Daunosamine	13-Deoxydaunomycin	
			0.71		Rhodinose		
			0.56	Grayish blue	2-Deoxy-L-fucose		
F	0.43	β Iso RMN	0.34	Sky blue	Daunosamine	Antibiotic DCP-2	
Isolate B2	A	0.45	Elloramycinone	0.36	Brown	Rhamnose	ETME
	B	0.45	Elloramycinone	0.36	Brown	Rhamnose	Elloramycin
	C	0.43	Tetracenomycinone	–			Tetracenomycin C
	D	ND					ND
Isolate R3	A	0.27	α_2 -RMN	0.56	Grayish blue	2-Deoxy-L-fucose	α_2 -Rhodomycin II
	B	0.45	β RMN	0.56	Grayish blue	L-Rhodamine	Rhodomyacin
						2-Deoxy-L-fucose	
	C	0.79	ϵ RMN	0.56	Grayish blue	2-Deoxy-L-fucose	Epelmycin B
	D	0.67	Aklavinone	0.12	Sky blue	L-Rhodamine	Aclacinomycin A
				0.56	Grayish blue	2-Deoxy-L-fucose	
	E	0.45	β -RMN	0.12	Sky blue	L-Rhodamine	Rhodomyacin A or B
0.56				Grayish blue	2-Deoxy-L-fucose		
F	0.59	γ -Iso RMN	0.12	Sky blue	L-Rhodamine	Obelmycin	
G	0.27	α_2 -RMN	0.12	Sky blue	L-Rhodamine	Alldimycin B	
			0.56	Grayish blue	2-Deoxy-L-fucose		
Isolate R5	A	0.45	ϵ -RMN	0.34	Sky blue	Daunosamine	Rhodomyacin D
	B	0.90	ND	0.34	Sky blue	Daunosamine	ND
	C	0.73	7-Deoxyaklavinone	0.34	Sky blue	Daunosamine	7-Deoxyaklavin
	D	0.61	4- <i>o</i> -Methyl aklavinone	0.56	Grayish blue	2-Deoxy-L-fucose	ND
	E	0.57	10-Decarbomethoxy aklavinone	0.34	Sky blue	Daunosamine	10-Decarbomethoxyaklavin
	F	0.59	Daunomycinone	0.34	Sky blue	Daunosamine	Daunomycin
Isolate Y8	A	0.48	Aklavinone	0.34	Sky blue	Daunosamine	Aclacinomycin
	B	0.32	Deoxyaklavinone	0.56	Grayish blue	2-Deoxy-L-fucose	
0.34				Sky blue	Daunosamine	Deoxyaklavin	

Except R3E, which showed a very weak antibacterial activity against *E. coli*, no other compound showed any activity against *E. coli*. The MIC of R3A and R3F against *B. cereus* was observed to be > 20 $\mu\text{g/ml}$, whereas that of R3E was found to be an impressive 2 $\mu\text{g/ml}$. Of all the compounds obtained from isolate R5, R5F proved to be the most potent with a zone of inhibition of 8 mm against *B. cereus* and an MIC of 8 $\mu\text{g/ml}$. R5A and R5B were observed to have an MIC > 40 $\mu\text{g/ml}$. None of the compounds showed any activity against *E. coli*.

Y8A proved to be highly potent with a zone of inhibition of 18 mm against *B. cereus* whereas Y8B was much less

potent with a zone of a meagre 7 mm. However the MIC of both Y8A and Y8B was observed to be > 40 $\mu\text{g/ml}$. Neither of them showed any activity against *E. coli*. Y8A showed a huge zone of 20 mm against *C. albicans*.

In vitro antitumor activity

In vitro antitumor activity of the bioactive compounds was judged by MTT based cytotoxicity assay against an established human cervical cancer cell line, HeLa and compared with that of a normal established mouse fibroblast cell line L929.

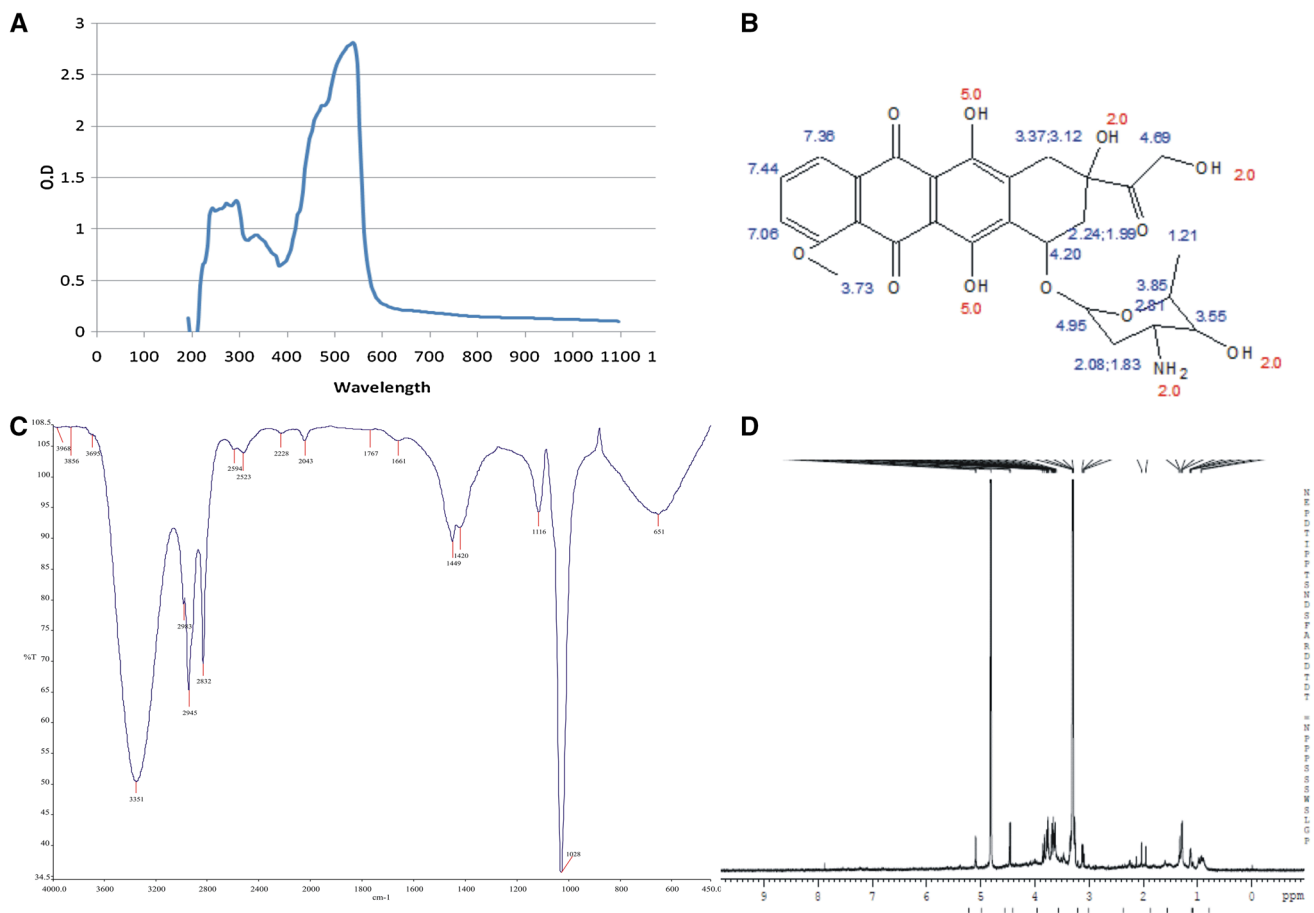


Fig. 1 Characterization of compound R1D obtained from isolate R1. **a** UV–Visible spectra of compound R1D. **b** Structure of dihydrodaunomycin. **c** IR spectra of compound R1D. **d** Proton NMR of compound R1D

The IC_{50} of R1C, R1D, R1E and R1F as determined from the graph (Fig. 6a) was ~ 5 , 8, 14 and 9.5 μM for L929. The IC_{50} of R1E and R1F was found to be 20 and 23 μM for the tumor cell line, HeLa (Fig. 6b). Similarly, the IC_{50} of R3A, R3E and R3F on HeLa cell line was determined from the graph to be 20, 12 and 25 μM (Fig. 6c).

Figure 6d shows the comparative effect of R5F and Y8B on HeLa. The IC_{50} of R5F and Y8B as determined from the graph was 10 and 25 μM respectively. Figure 6e shows the IC_{50} of the standard doxorubicin and Y8A. It was found to be ~ 3 and ~ 2.9 μM respectively for doxorubicin and Y8A.

In vitro wound scratch assay

Anti-metastatic activity of the isolated aromatic polyketides was tested in vitro by performing scratch assay on the tumor cell line HeLa. As shown in Fig. 7, the migration of HeLa cells as indicated by the ability to close the artificial wound, was significantly inhibited by the compounds Y8A, identified as an aclacinomycin analogue and R5F, identified as daunomycin, even at nontoxic concentrations. The open area

in the case of Y8A treated cells was 36.2% as compared to that of 16.1% of the untreated control. In the case of compound R5F, the inhibition was less with 49.8% open area as compared to that of 36.0% in untreated cells. Compound R3E, identified as rhodomycin, did not show any significant inhibition.

Discussion

Polyketides form the major class of medicinally used secondary metabolites of *Streptomyces* and related species. These compounds, which represent 20% of pharmaceutical drugs in the market, contain repeating ($-CH_2-CO-$) groups and possess antimicrobial, antifungal, antiparasitic, antitumor and antiviral activities (Donadio et al. 2010). *Streptomyces* are considered to be prolific producers of polyketides (Liu et al. 2012). The current study was undertaken with the aim of isolating novel aromatic polyketides from soil *Streptomyces* spp. These were purified and their antimicrobial and antitumor activities were assessed. The study also

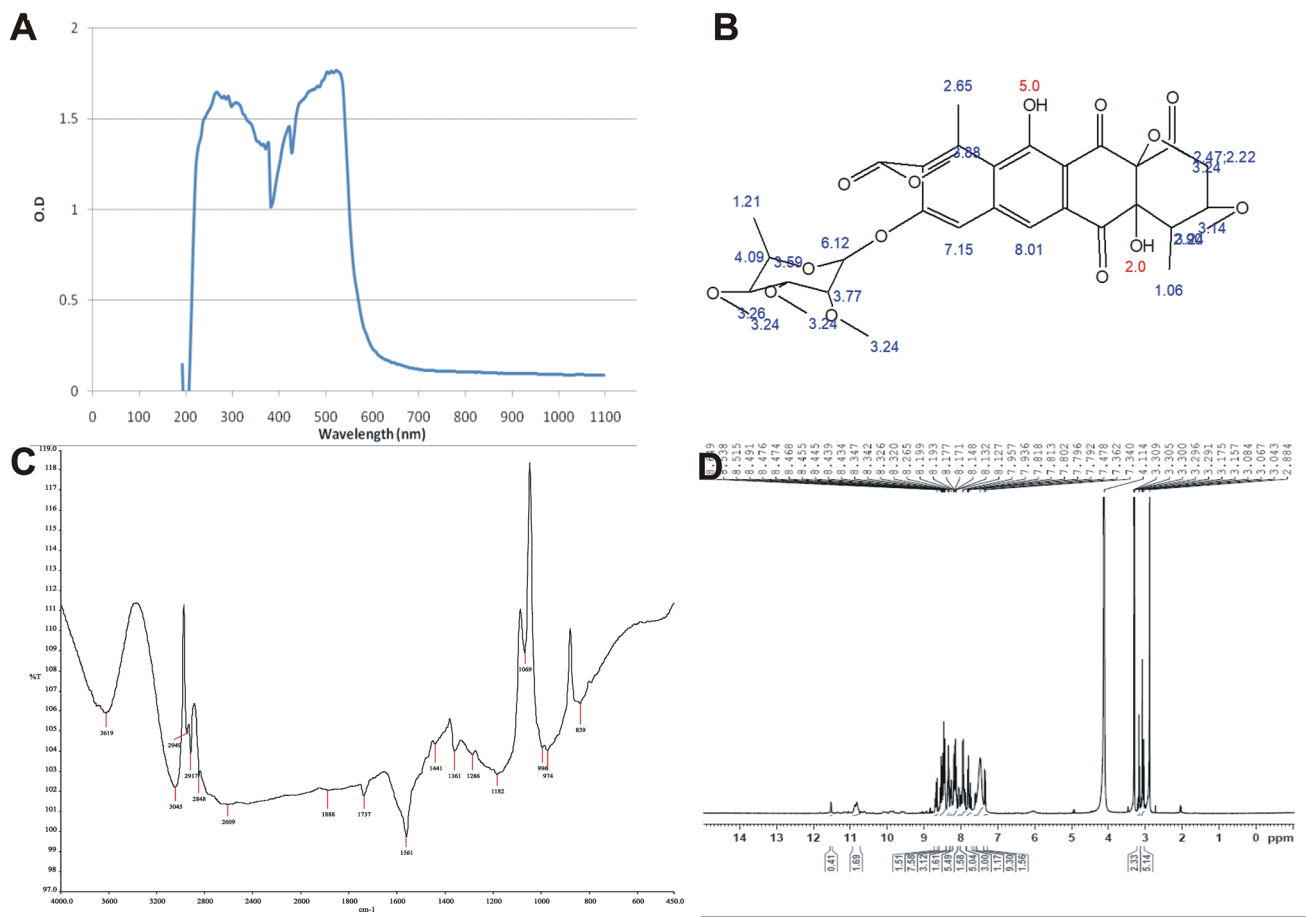


Fig. 2 Characterization of compound B1B obtained from isolate B1. **a** UV–Visible spectra of compound B1B. **b** Structure of elloramycin. **c** IR spectra of compound B1B. **d** Proton NMR of compound B1B

involved the structure elucidation of these aromatic polyketides. *Streptomyces* species usually produce the bioactive compounds as a complex, which can be separated on TLC to yield distinct bands. In this study, TLC with different solvent systems yielded 2–7 bands from the ethyl acetate extracts of each *Streptomyces* isolate. A bioactivity guided approach was used to select the most potent compound from each isolate. Although the structure of only the most potent bioactive compound from each isolate was elucidated, all the other fractions were also subjected to partial characterization to predict the compound identity (Table 2).

Isolate R1, identified as *S. spectabilis*, was grown for antibiotic production using the medium and culture conditions optimized in a previous study (Holkar et al. 2013a). The UV visible and IR spectra of the most potent compound, R1D, indicate that it is an aromatic polyketide, belonging to daunomycin group of anthracyclines (Dornberger et al. 1985; Bervanakis 2008). Acid hydrolysis of compound R1D yielded dihydrodaunomycinone and daunosamine indicating that the compound is perhaps dihydrodaunomycin. The chemical shift values shown by the proton NMR of R1D

were very similar to that of dihydrodaunomycin as previously reported (Arcamone et al. 1969; Yoshimoto et al. 1980). Thus UV–Vis, IR and NMR spectroscopy identified this compound as dihydrodaunomycin, which belongs to the anthracycline class of aromatic polyketides. This is the first report of dihydrodaunomycin, an anthracycline being produced by *S. spectabilis*. Earlier, other researchers have reported the production of aureolic acids and macrolides such as spectomycins and streptovaricins but not anthracyclines from this organism (Staley and Rinehart 1994). MIC of R1D was found to be much lower than the reported value of 12.5 µg/ml against *B. cereus*. However, IC₅₀ values obtained for R1D in the present study were much higher than those reported for dihydrodaunomycin for HeLa by other researchers (Arcamone et al. 1972). These results indicate that some substituent groups on R1D might be slightly different from conventional dihydrodaunomycin.

Isolate B1, identified as *Streptomyces olivaceus* was grown in Potato Dextrose Broth (PDB) and yielded an orange-yellow compound B1B as the major product. This compound did not show any colour change on treatment

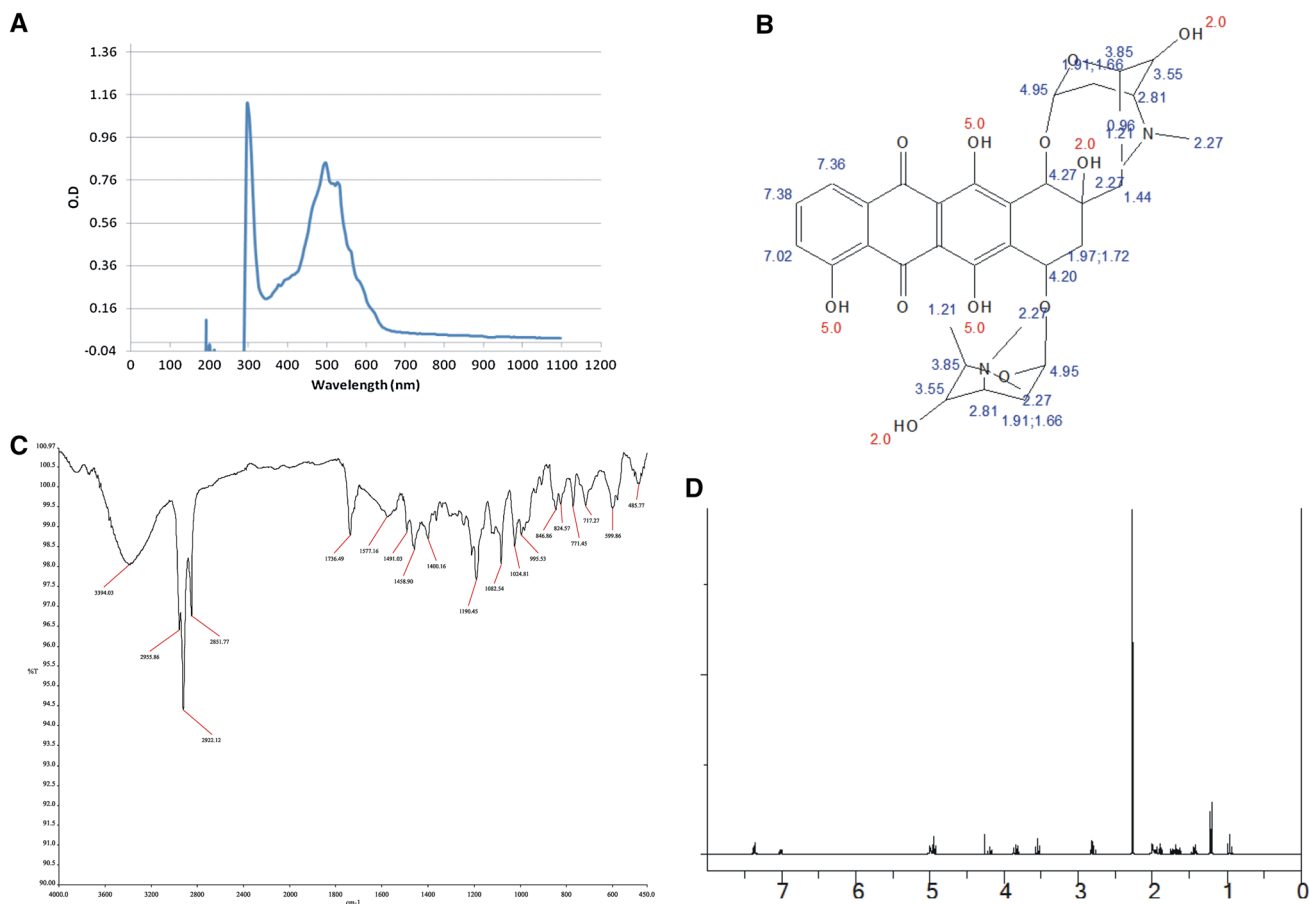


Fig. 3 Characterization of compound R3E obtained from isolate R3. **a** UV–Vis spectra of compound R3E. **b** Structure of rhodomycin. **c** IR spectra of compound R3E. **d** Proton NMR of compound R3E

with 0.5% magnesium acetate indicating that it is not a peri-hydroxy quinone (Laatsch and Fotso 2008; Holkar et al. 2013b). Acid hydrolysis followed by TLC analysis indicated the presence of the aglycone, elloramycinone and the sugar rhamnose in its structure. Further, the IR (KBr) spectra were similar to that reported for elloramycin (Drautz et al. 1985). The shifts in ^1H NMR of B1B in the aglycone, elloramycinone and the shifts shown by the sugar residue attached to the chromophore, rhamnose correspond to that reported by Drautz. Hence the conclusion that compound B1B is rhamnosyl–elloramycinone, that is elloramycin is inescapable. Elloramycin is an aromatic polyketide belonging to the class of tetracenomycins. It is a phenolic glycoside, a glycoside type rare among the secondary metabolites of *Streptomyces*. Elloramycin is the first secondary metabolite of microbes with its sugar permethylated (Drautz et al. 1985). Isolate B1, in preliminary screening was found to be only antifungal, hence only its antifungal activities were pursued. But, when purified compounds were tested against *C. albicans* and *A. niger* by agar well diffusion method, hardly any activity was observed. IC_{50} of compound B1B against HeLa cells was

observed to be 30 μM but was not found to be cytotoxic to L929 in the tested range. B1B thus showed promise of being an effective anticancer agent. Notably however, Elloramycin has been reported by Drautz to be weakly active against Gram-positive bacteria, especially Streptomycetes but not against *Bacillus subtilis*, *Micrococcus luteus* and *Mucor michei*. He also reported that the in vitro test against L-1210 leukemia cells showed 100 times weaker activity than doxorubicin which was attributed to the high grade of sugar methylation and the lipophilic character of the molecule. Therefore more careful investigations need to be planned to predict conclusions about the chemotherapeutic potential of B1B.

Isolate R3, identified as *S. purpurascens*, was grown for antibiotic production using the medium and culture conditions optimized in a previous study (Holkar et al. 2013b). The UV visible light absorption spectra of all the purified fractions of isolate R3, exhibited characteristic peaks of anthracyclines in the range of 297, 492–497, 522–526 and 557–562 nm (except R3D). The FT-IR spectra of all purified fractions indicated the presence of

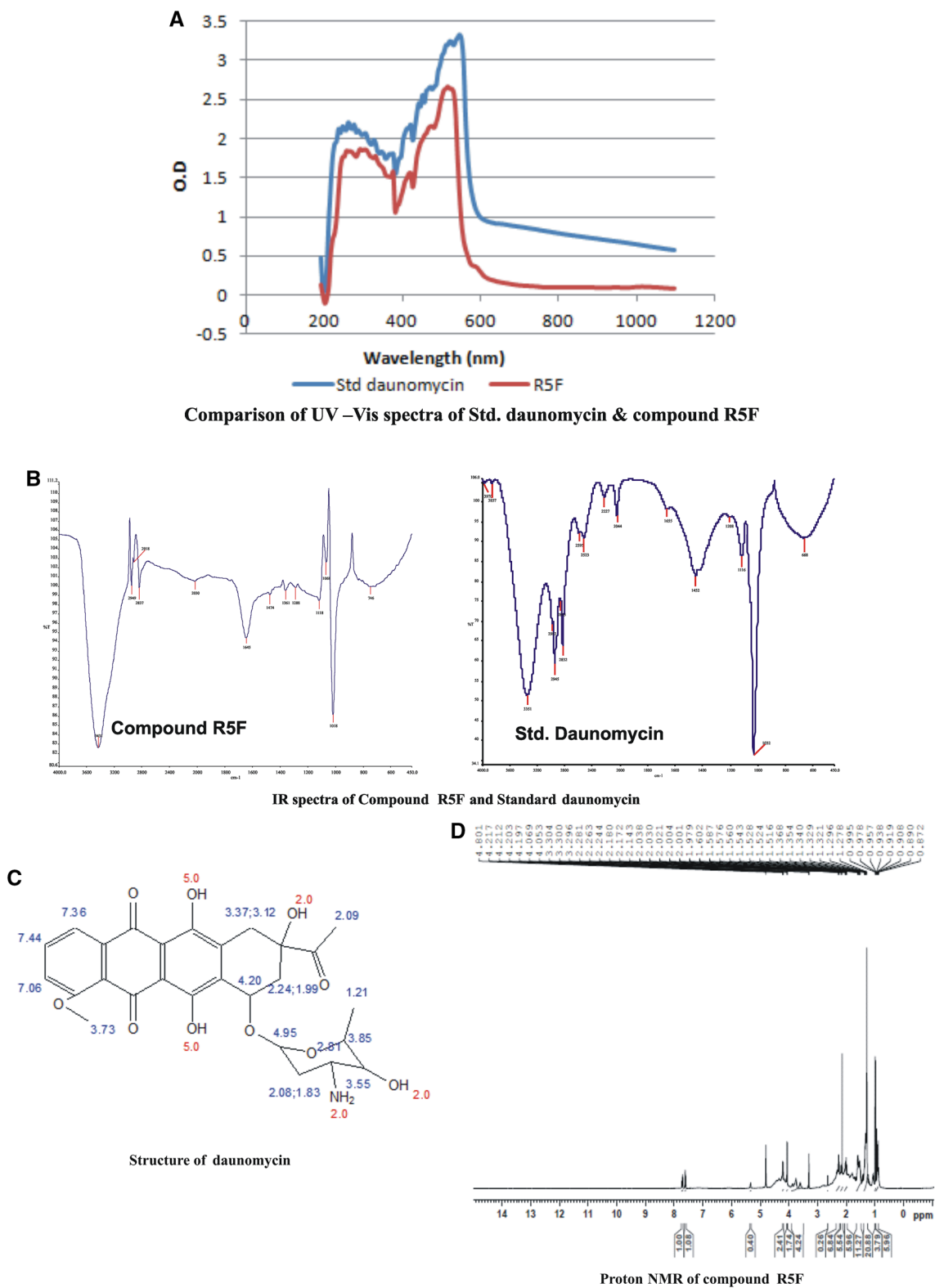


Fig. 4 Characterization of compound R5F obtained from isolate R5. **a** Comparison of UV-Vis spectra of standard daunomycin and compound R5F. **b** IR spectra of compound R5F and standard daunomycin. **c** Structure of daunomycin. **d** Proton NMR of compound R5F

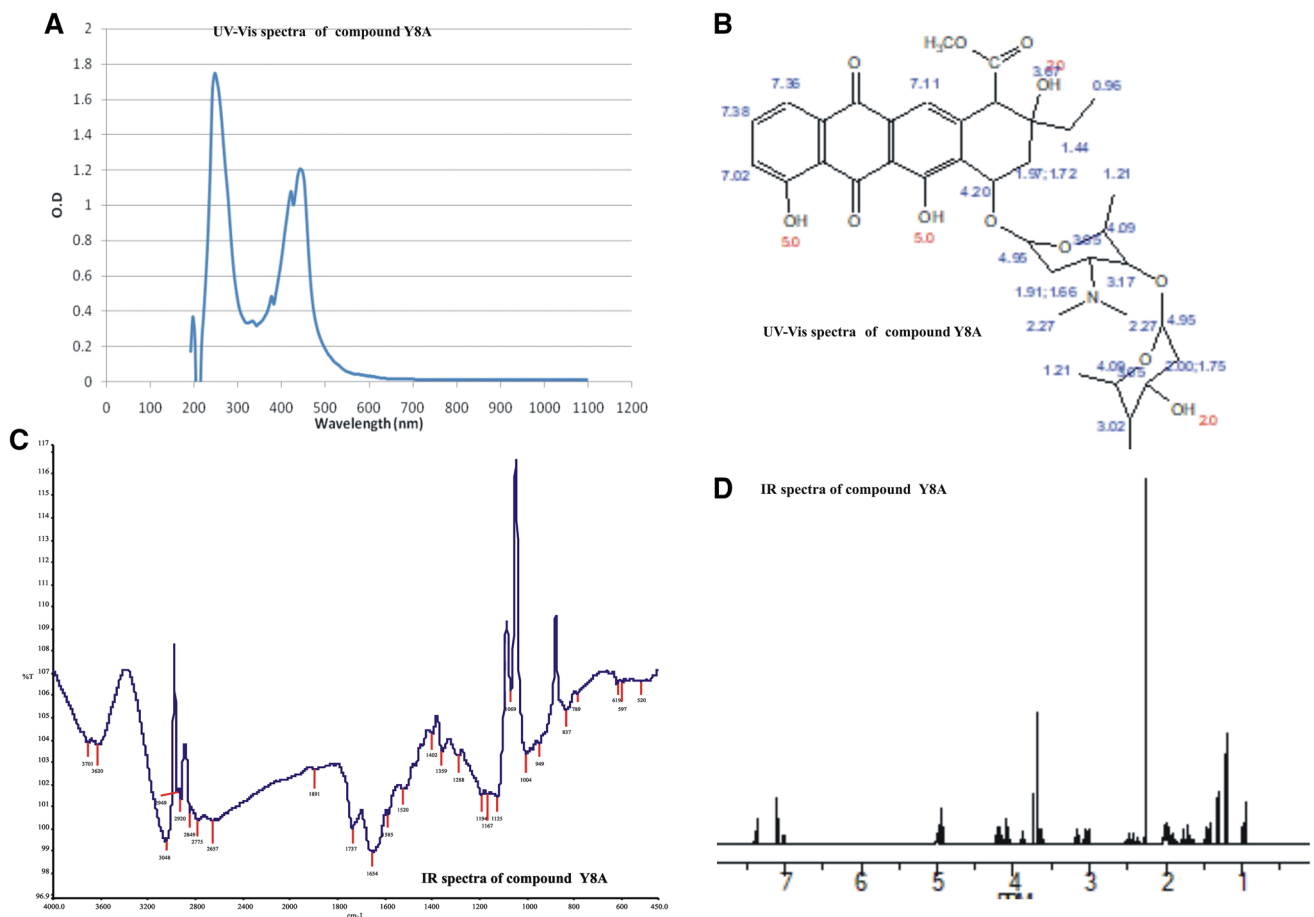


Fig. 5 Characterization of compound Y8A obtained from isolate Y8. **a** UV–Visible spectra of compound Y8A. **b** Structure of aclacinomycin. **c** IR spectra of compound Y8A. **d** Proton NMR of compound Y8A

Table 3 Physicochemical properties of the potent compounds

Properties	Compound				
	R1D	B1B	R3E	R5F	Y8A
R _f	0.34(SS6)	0.69 (SS7)	0.62 (SS4)	0.00 (SS6)	0.47 (SS10)
UV–Vis λ _{max} (nm)	417, 535	292, 422, 503	297, 492	256, 419, 472, 517	247, 379, 417, 437
IR max	3351, 2945, 2832, 1149, 1116, 1028	3619, 3045, 1561, 1361, 1286, 1182	3394, 2292, 1736, 1577, 1458, 1190	3431, 2949, 2837, 1645, 1474, 1118	3048, 2949, 2775, 1737, 1654, 1585
NMR	δ 4.4, δ 1.99–2.4, δ 3.12–3.37, δ 5, δ 4.8, δ 3.6–3.8, δ 4.8, δ 1.21, δ 3.73	δ 5, δ 3.8, δ 3.14, δ 8.01, δ 7.15, δ 2.9, δ 3.77, δ 1.21, δ 4.11, δ 3.59	δ 7.02–7.38, δ 4.2, δ 1.72–δ 1.97, δ 5, δ 1.44, δ 1.66–δ 1.95, δ 2.81–δ 3.55, δ 4.8	δ 7.06–δ 7.44, δ 1.99, δ 2.24, δ 3.12, δ 3.37, δ 5, δ 2.09, δ 1.21	δ 4.8, δ 3.3, δ 2.2, δ 1.8–2, δ 1.3, δ 2
Compound identified as	Dihydrodaunomycin	Elloramycin	Rhodomycin A	Daunomycin	Aclacinomycin

hydroxyl group ($3400\text{--}3300\text{ cm}^{-1}$) a ketonic group or ester carbonyl (1740 cm^{-1}) and a hydrogen bonded carbonyl (1600 cm^{-1}) which is characteristic of anthracyclines. However, an orange coloured compound R3E was found to be the most potent compound with an MIC of $2\text{ }\mu\text{g/ml}$ against *B. cereus*.

The R_f and UV–Vis spectra of the aglycone of R3E was found to be similar to that of β-RMN. Also the UV–Vis spectra of compound R3E matched that of S-583AII, which is reported to be a rhodomycin (Shoji et al. 1968). The IR and ^1H NMR spectra confirmed the identity of compound R3E as rhodomycin in the present work.

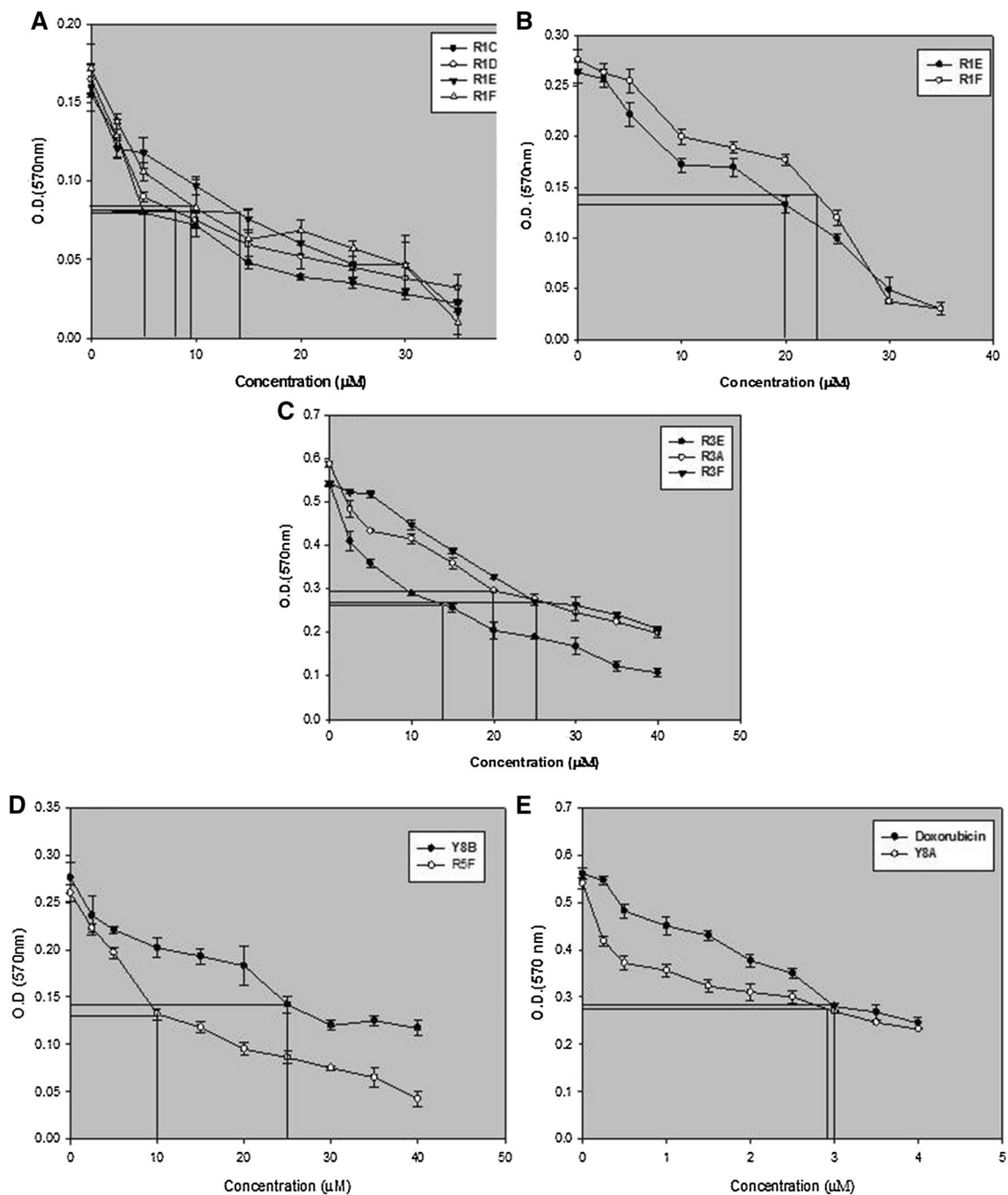


Fig. 6 **a** MTT based cytotoxicity assay of R1C, R1D, R1E and R1F on L929, IC_{50} was found to be 5, 8, 14 and 9.5 μ M. **b** MTT based cytotoxicity assay of R1E and R1F on HeLa, IC_{50} was found to be 20 and 23 μ M. **c** MTT based cytotoxicity assay of R3A, R3E and R3F against HeLa, IC_{50} was found to be 20, 12 and 25 μ M. **d** MTT based

cytotoxicity assay of Y8B and R5F against HeLa, IC_{50} was found to be 25 and 10 μ M. **e** MTT based cytotoxicity assay of standard doxorubicin and Y8A against HeLa, IC_{50} was found to be 3 and 2.9 μ M. Each point represents mean of three independent observations \pm SD

Rhodomycin group of anthracyclines are characterized by having β -RMN as the aglycone and was the first anthracycline to be identified. The sugar moiety attached to C-7 or C-10 or both of the aglycone always contains one or more rhodosamines (Fujiwara et al. 1985). Rhodomycin B

containing one rhodosamine and rhodomycin A containing two rhodosamines has been isolated previously from *S. purpurascens* (Saito et al. 1995).

In the present work, the MIC of R3E was found to be comparable to the reported value of 0.5–1 μ g/ml (Shoji et al.

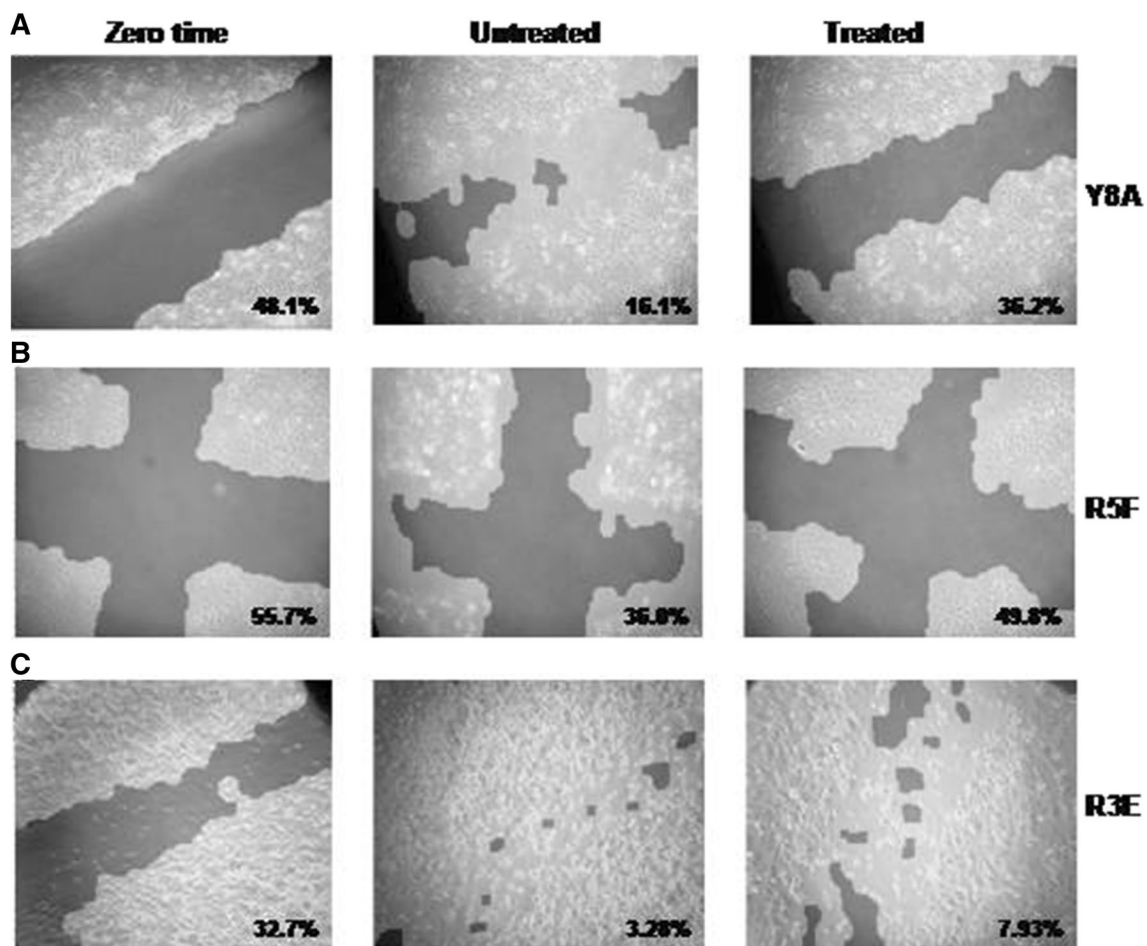


Fig. 7 Anti-metastatic activity by in vitro wound scratch assay. Cell migration of tumor cells (HeLa) in presence and absence of aromatic polyketides was compared. Analysis of cell migration was done by using T Scratch analysis software. Confluent cell cultures were scratched and immediately imaged at $\times 5$ magnification (0 h). HeLa cells were incubated in medium alone (24 h untreated control) or aro-

matic polyketides, Y8A-1, R5F-3 and R3E-4 μM (24 h treated) for 24 h before re-imaging. The automated analysis was performed using a disk size of 7, default threshold 0.25 and erosion size 2. Percentage of open image area represented as mean \pm standard deviation of 4 replicates (** $P \leq 0.05$)

1968). However, the IC_{50} value was higher than the reported value of $1 \mu\text{g/ml}$ (Saito et al. 1995).

Since the UV–Vis spectra and metabolic profile of both cell and broth extracts of isolate R5 (identified as *S. coeruleorubidus*) were similar, the two were mixed resulting in 185 mg of crude antibiotic complex. This crude complex when subjected to TLC using various solvent systems gave separation in SS6. Five well separated bands were obtained and were named R5A, R5B, R5C, R5D and R5E. A polar compound which did not migrate remained as a circular spot. This spot was scraped and run in SS3. It resolved as a single band indicating that it was a pure compound. This compound was named R5F, which on treatment with 0.5% magnesium acetate, changed color from orange-red to violet indicating the presence of perihydroxy quinone in the structure as seen in anthracyclines. The UV–Vis spectra of compound R5F showed peaks in

the UV region (233, 252, 292 nm) and in the visible region (421, 472, 517 nm) which are characteristic of a dihydroxyanthraquinone as seen in daunomycin (Bouma et al. 1986). The UV–Vis spectra of standard daunomycin also showed similar peaks. Also R5F showed an R_f of 0.75 in the same solvent system as has been reported for daunomycin (Dickens et al. 1997). Further the IR spectra of standard daunomycin and R5F were superimposable with similar peaks. The ^1H NMR spectra of R5F conclusively confirmed its identity as daunomycin. Daunomycin has been isolated previously from *Streptomyces peucetius*, *Streptomyces griseus*, *Streptomyces insignis* and other *Streptomyces* species and variously named by different researchers as rubomycin C, mitocromin D and leukaeomycin C. It has also been obtained from cultures of *S. coeruleorubidus* as rubidomycin (Arcamone et al. 1969). The antitumor activity of daunomycins, first established

using tumor bearing mice, was confirmed in clinical trials showing its powerful activity against acute leukemia. Daunomycin thus became the first anthracycline endowed with clinical usefulness. In the present work, Compound R5F was found to inhibit the proliferation of HeLa cells with an IC_{50} value of 10 μ M which was slightly higher than that shown by standard daunomycin (3 μ M) under similar experimental conditions. In vitro wound healing assay revealed the antimetastatic properties of compound R5F.

Isolate Y8, identified as *S. lavendofoliae* yielded an aklavinone glycoside which could be an aclacinomycin (Kitamura et al. 1981). Also an R_f of 0.12 in SS9 and 0.47 in SS10 was observed. These R_f 's when compared with literature suggested that the compound belongs to the aclacinomycin class of anthracyclines. The UV–Vis spectra and IR spectra also validated the fact that it could be an aclacinomycin. The chemical shifts in the 1H NMR at δ 4.8 and δ 3.3 could be due to the proton at C-1 and equatorial proton at C-4 of the 2-deoxyfucose. The characteristic chemical shift around δ 2.2 in the NMR spectra of the compound Y8A suggests the presence of an amino sugar moiety in its structure (Oki et al. 1979). This amino sugar may be daunosamine as indicated on the basis of the shifts at δ 1.8–2.0 (CH₂), δ 1.3 (CH₃), δ 2.0 (NH₂) as reported by Yoshimoto (Yoshimoto et al. 1980). Thus compound Y8A may be an aclacinomycin analogue. Aclacinomycins, which are a type of anthracyclines, are aklavinone glycosides with antitumor activity and are known to be produced by *Streptomyces galilaeus*. This group of yellow anthracyclines has the widest variety of sugar substituents, mainly rhodosamine, deoxyfucose and cinerulose, and to a lesser extent amicitose, aculose and rhodinosine (Fujiwara et al. 1985). Daunosamine and monomethyl daunosamine along with deoxyfucose and cinerulose have also been reported as sugars of aclacinomycin analogues produced by a mutant strain of *S. galilaeus* (Oki et al. 1979). Compound Y8A isolated in this work, was found to contain daunosamine and deoxyfucose but not cinerulose. Thus compound Y8A is perhaps a new aclacinomycin analogue which we isolated from the culture broth of Y8. Aclacinomycins are mainly produced by *Streptomyces galilaeus* or its mutants. However, in this study, Y8A was found to be produced by our isolate identified as *S. lavendofoliae*. There are only two reports before this where aclacinomycins have been reported to be produced by *S. lavendofoliae* (Kim et al. 1995a, b).

Compound Y8A was found to have an IC_{50} value of 2.9 μ M against HeLa cells. However, it was found to be cytotoxic to the normal cells, L929 at a much higher concentration as compared to that of aclacinomycin A and aclacinomycin B whose reported values are 0.12 and 0.24 μ g/ml. Compound Y8A was also found to exhibit antimetastatic activity wherein the migration of HeLa cells was inhibited even at non toxic concentration (1 μ M). Thus compound

Y8A has antiproliferative and anti invasive characteristics required in an antimetastatic antitumor compound.

Although screening programs do not always result in the discovery of new compounds, this study yielded a few novel aromatic polyketides which can be taken up in a drug development program wherein they may be chemically modified to further increase their activity. These can be subsequently evaluated in clinical trials rendering valuable chemotherapeutic compounds in future.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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