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Assessment of Resistomycin, as an Anticancer Compound Isolated and Characterized from *Streptomyces aurantiacus* AAA5

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A new actinomycete strain, isolated from humus soils in the Western Ghats, was found to be an efficient pigment producer. The strain, designated AAA5, was identified as a putative *Streptomyces aurantiacus* strain based on cultural properties, morphology, carbon source utilization, and analysis of the 16S rRNA gene. The strain produced a reddish-brown pigmented compound during the secondary metabolites phase. A yellow compound was derived from the extracted pigment and was identified as the quinone-related antibiotic resistomycin based on ultraviolet–visible spectrophotometry, fourier transform infrared spectroscopy, liquid chromatography and mass spectroscopy, and nuclear magnetic resonance analyses. The AAA5 strain was found to produce large quantities of resistomycin (52.5 mg/L). It showed potent cytotoxic activity against cell lines viz. HepG2 (hepatic carcinoma) and HeLa (cervical carcinoma) *in vitro*, with growth inhibition (GI_{50}) of 0.006 and 0.005 μg/ml, respectively. The strain also exhibited broad antimicrobial activities against both Gram-positive and Gram-negative bacteria. Therefore, AAA5 may have great potential as an industrial resistomycin-producing strain.

***Keywords*:** *Streptomyces aurantiacus*, resistomycin, cytotoxic compound, antibacterial activity

Cancer has emerged as a major public health threat worldwide. World population growth and aging imply that the cancer burden will increase in the future (Lin *et al*., 2009). Although many anticancer drugs have been developed, a limited number of these are currently utilized for treatment due to the toxic effects of such drugs on normal cells. Moreover, intrinsic or acquired multi-drug resistance is often a major hurdle to successful anticancer therapy. To overcome these limitations, it is necessary to isolate potent anticancer drugs. Most antitumor metabolites that have been identified are produced by microorganisms. Many drug resistant microbes have been identified, prompting extensive searching for additional alternate antibiotics. Out of 22,500 biologically active compounds that have been extracted thus far from microbes, 45% are produced by actinobacteria, 38% by fungi, and 17% by unicellular bacteria (Demain and Sanchez, 2009). Actinobacteria, particularly species of the *Streptomyces* genus, have long been recognized as prolific sources of useful bioactive metabolites. These bacteria have provided more than half of the naturally occurring antibiotics discovered to date and continue to be a source of new bioactive metabolites (Berdy, 2005). Bioactive metabolites have a wide range of useful biological activities, such as antifungal, antitumor, antibacterial, immunosuppressive and insecticidal properties, and enzyme inhibition (Solanki *et al*., 2008). However, some of the metabolites are cytotoxic and can include chemical structures such as macrolides, α-pyrones, lactones, indoles, terpenes, and quinones (Dharmaraj, 2010).

Quinones are compounds that have a fully conjugated cyclic dione structure, and are common constituents of biologically relevant molecules. *Streptomyces* species are particularly rich in highly biologically active quinones. Resistomycin is a quinone-related antibiotic, which has a unique structure and exhibits bactericidal and vasoconstrictive activity. It inhibits RNA and protein synthesis, but has no effect on DNA synthesis (Arora, 1985). Among other activities, resistomycin has been proposed to be a modulator of apoptosis (Shiono *et al*., 2002). The biosynthesis gene cluster of resistomycin has been isolated from and characterized for *S. resistomycificus* and involves a type II polyketide synthase (Jakobi and Hertweck, 2004). 1-hydroxy-1-norresistomycin and resistoflavin showed cytotoxic activity against human gastric adenocarcinoma HMO2 and hepatic carcinoma HepG2 cell lines (Gorajana *et al*., 2005, 2007). Resistomycin has also been isolated from *Streptomyces* sp. B4842 from mud sediments at a coastal site in Mauritius, Indian Ocean (Kock *et al*., 2005). To our surprise, no studies focusing on resistomycin production by actinomycetes from terrestrial ecosystems have been reported. Hence, we explored a resistomycin-producing *Streptomyces* isolate (strain AAA5) from the Western Ghats, the largest and most biologically diverse area of India. The taxonomy, fermentation, physico chemical properties, structure, and biological activities of resistomycin isolated from the strain were determined.

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Materials and Methods

Isolation

During systematic screening for pigmented actinomycetes, a reddish-brown pigmented strain (AAA5) was isolated from the Annaikatti hills, Western Ghats, India, a previously unexplored region. The soil sample was serially diluted and plated on starch casein agar (composition: soluble starch 1.0%, casein 0.03%, KNO_3 0.2%, NaCl 0.2%, K_2HPO_4 0.005%, $CaCO_3$ 0.002%, $FeSO_4 \cdot 7H_2O$ 0.001%, and agar 2.0%). The sample was incubated at 30°C for 7 days to allow sporulation and pigmentation, and was subsequently preserved in 20% glycerol at -80°C.

Cultural and morphological properties

The strain was characterized morphologically and physiologically based on the International *Streptomyces* Project guidelines (ISP; Shirling and Gottlieb, 1966, 1972). After the culture had been grown at 30°C for 8 days on oatmeal agar (ISP3), the morphology of the aerial hyphae, substrate mycelia, and spore arrangements was determined by light microscopy and scanning electron microscopy. Additional culture characteristics were also determined. The color of the aerial mass, substrate mycelium, and pigment production were examined in yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salts-starch agar (ISP4), glycerol asparagine agar (ISP5), and tyrosine agar (ISP7) (Shirling and Gottlieb, 1966). The utilization of carbon sources and melanoid production was determined for the strain according to the method of Gottlieb (1961). In addition, physiological and biochemical characteristics were determined based on the method described by Cappuccino and Sherman (2004).

16S rRNA sequence and phylogenetic analysis

Extraction of genomic DNA and amplification of the 16S rRNA gene from strain AAA5 was carried out as described by Lin *et al.* (2009). The amplified product was sequenced, and the sequence obtained was compared with similar sequences retrieved from the GenBank nucleotide sequences database. A distance matrix tree was constructed using the neighbor-joining method (Saitou and Nei, 1987), and the topology of the phylogenetic tree was built by bootstrap analysis (Felsenstein, 1985) using the PHYLIP package (version 3.69).

Nucleotide sequence accession number

The nucleotide sequence of the 16S rRNA gene reported in this article was submitted to GenBank.

Production, extraction, and quantification of pigment

Spores of the AAA5 strain, stored in 20% glycerol at -80°C, were spread on starch casein agar plates and cultured at 30°C for 7-10 days to allow good sporulation. Vegetative inoculum was prepared by inoculating 2 loops of spores from the plate culture in Erlenmeyer flasks, containing 50 ml of seed medium (starch-casein broth), followed by incubation at 30°C for 2 days on a rotary shaker (220 rpm). The culture was transferred as 5% (v/v) inoculum into 100 ml aliquots of the production medium (modified starch-casein broth with 1% soybean meal) contained in 20×250 ml Erlenmeyer flasks. The aliquots were then incubated on a reciprocating shaker (220 rpm) at 30°C for 8 days. The culture broth (2 L) was centrifuged at 10,000 rpm for 20 min at 10°C; the crude bioactive compound was recovered from the culture filtrate by solvent extraction with ethyl acetate. The mycelial biomass was extracted with 70% methanol. The extract was filtered and concentrated. The condensed solution was mixed with the supernatant and was extracted with an equal volume of ethyl acetate. The mixture was shaken overnight, and was then allowed to stand for 60 min for separation of the aqueous phase. The remaining organic phase (crude extract) was used for further analysis.

Analytical LC-MS of crude extract

Liquid chromatography and mass spectroscopy (LC-MS) analysis was performed on a high mass accuracy Agilent Time of flight (TOF) mass spectrometer, which was interfaced to an Agilent 1100 series HPLC system equipped with a diode array detector (DAD). Mobile phases were methanol-water (80:20) in channel A and dichloromethane in channel B. Chromatographic separation was performed with a Zorbax RP C_8 4.6×150 mm column operated with a flow rate of 1 ml/min. The following gradient was used: 0% B for 0-2 min, 0%-50% B from 2 to 20 min. The analyte was ionized using a chemical ionization source (APCI) with the following settings: 325°C dry temperature, 350°C vaporizer temperature, 50 psi nebulizer pressure, and 5.0 L/min dry gas. Reference ions for calibration of mass axis were tee-ed into the mobile phase after the column separation. This enabled routine accurate mass determination with over 3 ppm accuracy.

Quantification of the extract

LC-MS analysis confirmed that there was no noise present for the absorbance measurement of the crude extract. The concentration of the compound was determined by measuring the absorbance of crude extract at 457 nm using an ultraviolet (UV)-visible (vis) spectrophotometer (Shimadzhu 1601), and was calculated according to the following formula: Pigmented compound (mg/L) = ADV_1/Extinction coefficient*V_2 (Wen *et al*., 1993), where A is the absorbance of methanol extract solution at 457 nm, D is the dilution ratio, V_1 is the volume of methanol added, and V_2 is the volume of fermentative liquid.

Purification of pigmented compound

The organic phase, which was separated during pigment extraction, was dried over anhydrous sodium sulfate and was concentrated under reduced pressure to yield 6.2 g as crude extract. The ethyl acetate extract was subjected to silica gel (60-120 mesh) column chromatography, and was eluted with a linear gradient of hexane and ethyl acetate to yield 4 fractions.

Chemical characterization

Chemical characterization of the pure active compound was performed through UV-vis spectra, infrared (IR), and nuclear magnetic resonance (NMR) analyses. UV-analysis was carried out using a Shimadzu UV-vis scanning spectrophotometer (UV-2100 PC). Scanning was performed using a wavelength range between 200 and 700 nm. The sample was maintained in a vacuum desiccator over KOH pellets for 48 h, followed by IR-spectral analysis with 1 mg of the sample in a fourier transform infrared spectroscope (FITR; FT/IR-420 Jasco, USA). NMR spectroscopy was performed for the compound dissolved in deuterated dimethyl sulfoxide (DMSO), and the spectra were recorded on a Bruker Avance 600 MHz instrument fitted with an inverse triple-resonance CryoProbe (TCI).

Biological activity

Antimicrobial assay: Antimicrobial profiles of the bioactive compound were tested in terms of the minimum inhibitory concentration (MIC) against various pathogens by using the disc diffusion method

Table 1. Cultural characteristics of strain AAA5

Culture media	Growth	Aerial mycelium	Substrate mycelium	Pigment production
Yeast extract-malt extract-agar (ISP2)	Good	Grey	Reddish brown	Brown
Oatmeal-agar (ISP3)	Good	Greyish white	Reddish brown	Reddish brown
Inorganic salts-starch agar (ISP4)	Good	Grey	Dark brown	Reddish brown
Glycerol-asparagines-agar (ISP5)	Poor	Dull white	Yellowish brown	-
Tyrosine-agar (ISP7)	Poor	Dull grey	Brownish grey	Melanin
Malt extract agar	Moderate	White	Brown	-
Maltose tryptone agar	Poor	Reddish grey	Pale yellow	-
Nutrient agar	Poor	Pale grey	Pale yellowish to brown	-
Sabouraud's agar	Moderate	Pale grey	Brownish grey	-
Bennet's agar	Good	Dark grey	Dark brown	Brown

(Kavitha *et al*., 2009). The pathogen cultures were procured from PSG Hospitals, Coimbatore, India. Different concentrations ranging from 0 to 1000 μg/mL were employed for the antimicrobial assays. The lowest concentration of the bioactive metabolite, which exhibited antimicrobial activity against the test organisms, was considered to be the MIC of the compound.

Anticancer property: The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay (Lin *et al*., 2009) was employed to evaluate the anti-tumor effect of the purified compound on HepG2 (hepatic carcinoma) and HeLa (cervical human carcinoma) cells *in vitro*. The absorbance was read on an enzyme linked immunosorbent assay (ELISA) reader at 544 nm, and the growth-inhibitory ratio was calculated using the formula (1−A/B)×100%, where A and B correspond to the mean absorbance of the treated and control wells, respectively. 5-Fluorouracil (5-FU) was used as a reference compound for the positive control.

Results

Taxonomy of the strain

Out of the 37 pigmented actinomycetes isolated from the Western Ghats, strain AAA5 was selected for further studies due to its strong pigmentation. The cultural characteristics of strain AAA5 are presented in Table 1. The color of the aerial mycelium appeared grey while that of the mycelium substrate was yellow and produced a reddish-brown pigment.

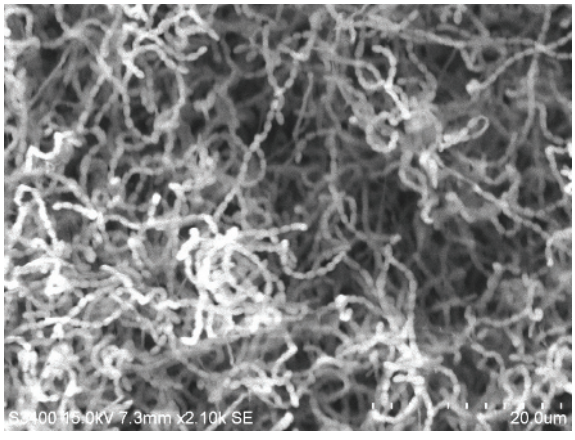

Fig. 1. Scanning electron microscope of strain AAA5.

A well-developed irregularly branched vegetative mycelium was observed. No fragmentation of the mycelium substrate hyphae was seen. The aerial mycelium branched monopodially and formed chains of arthrospores with numerous spores per chain. The spore chains were flexible, and spores were oval to cylindrical shaped with smooth surfaces. The scanning electron micrograph of spore morphology is shown in Fig. 1. Table 2 displays the physiological and biochemical characteristics of strain AAA5. They were determined to identify the genus of the organism. Melanoid pigment production was observed on ISP7 medium-hydrolyzed starch, cellulose, and casein–and was found to be catalase and urease positive.

Phylogenetic analysis

A 1,443 bp 16S rRNA gene sequence was determined for the strain. A BLAST search of the GenBank database showed sequence similarity to many species of the genus *Streptomyces*. Specifically, it showed about 99% similarity with *Streptomyces*

Table 2. Physiological and biochemical characteristics of strain AAA5

Characteristics	Strain AAA5		
Melanin production	+	Utilization of:	
H2S production	-	Adonitol	−
Hydrolysis of:		Arabinose	+
Starch	+	Cellobiose	+
Cellulose	+	Dextrose	+
Casein	+	Fructose	+
Gelatin	-	Galactose	+
Lipid	-	Inositol	−
Indole production	-	Inulin	−
Methyl red	-	Lactose	−
Voges Proskauer	-	Maltose	+
Citrate utilization	+	Mannitol	+
Nitrate reduction	-	Mannose	+
Urease	+	Mellibiose	+
Catalase	+	Raffinose	+
Oxidase	-	Rhamnose	+
Triple sugar iron	alk/alk	Sorbitol	−
Tolerance to NaCl	Up to 6%	Sucrose	−
Temperature optimum	37°C	Trehalose	+
pH optimum	7-8	Xylose	+

+, positive; -, negative; alk, alkaline.

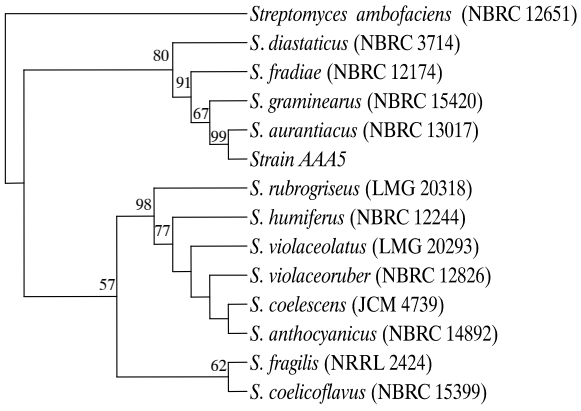

Fig. 2. Phylogenetic tree based on 16S rRNA gene sequence showing the relationship between strain AAA5 and species belonging to the genus *Streptomyces* and was constructed using the neighbour-joining method. Bootstrapping values >50 are not mentioned.

aurantiacus, about 98% similarity with *S. fradiae*, and <98% similarity with other *Streptomyces* species. A phylogenetic tree based on the 16S rRNA gene sequences of members of the genus *Streptomyces* was constructed according to the neighbor-joining method of Saitou and Nei (1987) using the PHYLIP package (Fig. 2). This relationship was supported by a high bootstrap value of 99% in the resulting phylogenetic tree. The sequence was submitted to GenBank (accession number JF802081).

Extraction and quantification

Fermentation of the strain was carried out at 30°C for 8 days with shaking conditions at 220 rpm/min. Pigment production was observed on day 2 after incubation and pigments continued to accumulate throughout the fermentation period. However, the pigment yield reached to its highest level on day 6. Accordingly, the fermented broth was harvested on the sixth day for further extraction and purification. Culture filtrate and biomass were separated by centrifugation and were extracted with ethyl acetate. This procedure yielded 6.2 g of crude extract for 2 L of fermented broth. The crude extract was subjected to LC-MS analysis. The total ion chromatogram (TIC) and contour plot derived from the diode array detection (DAD) are displayed in Fig. 3 (A and B, respectively). The crude extract was dominated by 2 pigments (pigment 1 eluted at 15.6 min and pigment 2 at 20.2 min). The UV-vis scans of the 2 pigments are shown in Fig. 3C and D. Pigment 1 displayed several peak maxima and the dominating mass/charge in the corresponding mass spectrum was 377.1016. The Dictionary of Natural Products (http://www.chemnetbase.com/) was searched using the accurate mass of 376.0944 with a 5 ppm window. The search returned 10 hits, and resistomycin was the most likely identity based on the corresponding UV/vis absorbance information. Pigment 2 had only 1 peak maxima, and the profile was similar to

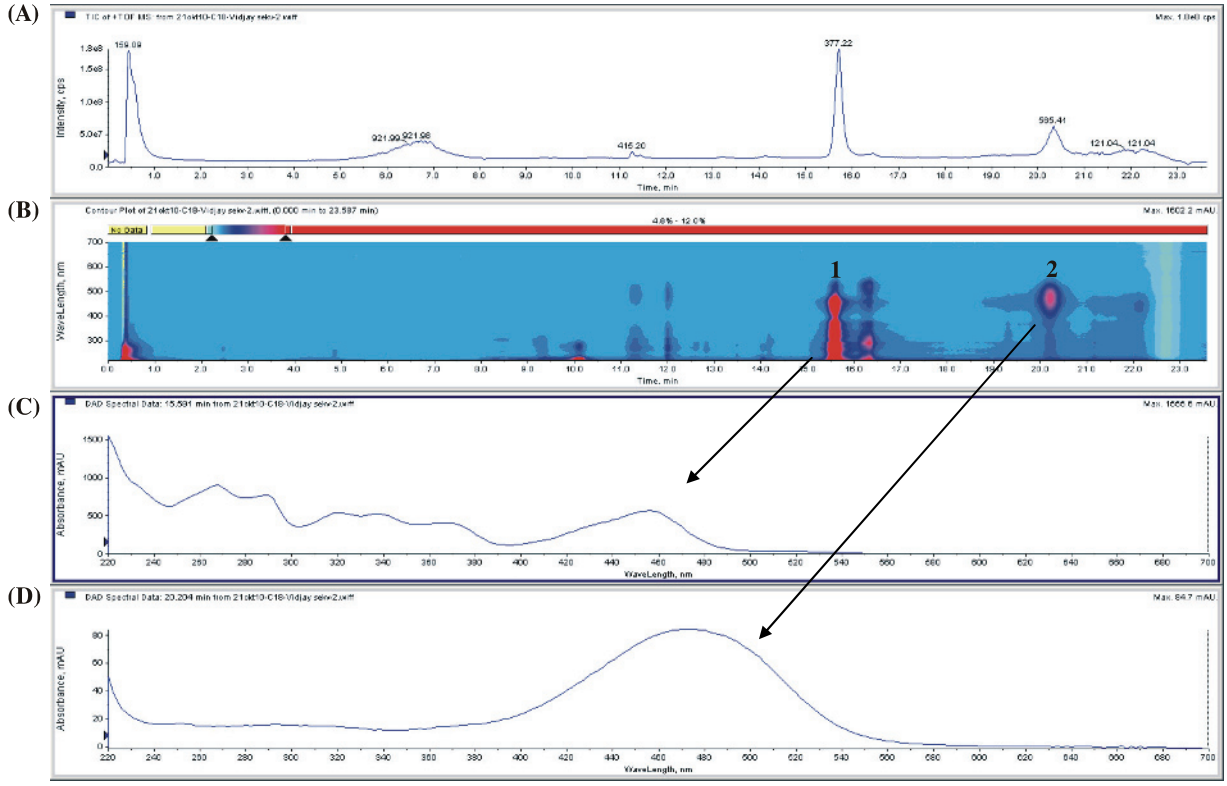

Fig. 3. LC-MS analyses of crude extract of *S. aurantiacus* AAA5. (A) Total Ion Chromatogram, (B) Contour plot from diode array detection, (C and D) UV/Vis absorbance profile of pigment 1 and 2, respectively.

Table 3. Physico-chemical properties of resistomycin

Physical nature	Yellow solid
Molecular formula	$C_{22}H_{16}O_6$
Molecular weight	377.10
TLC (R_f) (5% ethyl acetate in n-hexane)	0.63
Melting point (°C)	319
Solubility:	
Soluble in	chloroform, ethanol, dimethyl sulphoxide
Insoluble in	petroleum ether, water
ESI MS (m/z)	377.1 ([M^+], 100)
CH analysis, % (measured, calculated)	C (70.16, 70.20); H (4.32, 4.29)
λ_{max}, nm (ε, dm^3/mol·cm), in ethanol	267 (22536), 290 (18917), 319 (12719), 338 (12473), 369 (9758), 457 (14029)
ν_{max} (KBr), cm^{-1c}	3426 (OH), 2963 (C-H), 1639 (C=O), 1461 (C=C), 1096 (C-O), 1025 (C-O)

the absorbance spectra of several carotenoids. The dominating mass/charge in the corresponding mass spectrum was 565.4045, and this mass differed by 0.9 ppm from the theoretical mass/charge of several carotenoids with brutto formula $C_{40}H_{53}O_2$. The production of resistomycin increased with cell growth, reaching a maximum concentration of 52.5 mg/L, which was quantified by the absorbance.

Purification and characterization

The crude extract was subjected to silica gel column chromatography and eluted with a linear gradient of ethyl acetate and hexane to purify pigment 1. Four fractions were obtained, and fraction II yielded 98.6 mg when eluted with hexane and ethyl acetate (7:3). The fraction II compound was highly active and was obtained in a large quantity. The remaining 3 fractions were not active and were obtained in small quantities; therefore, we were only interested in fraction II, which was further characterized. The UV-visible spectrum of the purified yellow compound showed 6 peaks. The λ_{max} values and their corresponding ε values are shown in Table 3 along with additional physic-chemical properties of the compound. Transitions from $n\rightarrow\pi^*$ and $\pi\rightarrow\pi^*$ are the factors responsible for the observed peaks in the electronic spectra of the isolated compound. The IR spectrum of the compound showed a strong and broad band at 3426 cm^{-1}, which indicates the presence of 1 or more -OH groups. The band at 2963 cm^{-1} indicates C-H stretching of a CH_3 group. The presence of a carbonyl group was confirmed by the presence of a sharp and strong band at 1639 cm^{-1}. The $(M+H)^+$ peak (m/z=377.1016) observed in the APCI mass spectra differs by 0.9 ppm from the theoretical protonated ion species of resistomycin [$(M+H)^+$ 377.1019]. Furthermore, CH analysis of the compound was consistent with the calculated values for resistomycin. The structure of the purified compound was further confirmed using various NMR spectroscopy techniques. In addition to directly observed ^{1}H-NMR (600 MHz, DMSO-d6): δ_H=1.56 (6H, s, Me-1), 2.89 (3H, s, Me-9), 6.34 (1H, s, H-4), 7.01 (1H, s, H-8), 7.23 (1H, s, H-11), 11.83 (1H, s, OH-10), 13.99 (1H, s, OH-5), 14.34 (1H, s, OH-3), 14.50 (1H, s, OH-7), and ^{13}C-NMR (150 MHz, DMSO-d6): δ_C = 26.0 (Me-9), 28.8 (Me-1), 46.3 (C-1), 100.6 (C-4), 102.9 (C-2a), 106.0 (C-5a), 106.7 (C-6a), 107.6 (C-11b), 109.9 (C-11), 114.4 (C-9a), 119.7 (C-8), 128.8 (C-9b), 139.9

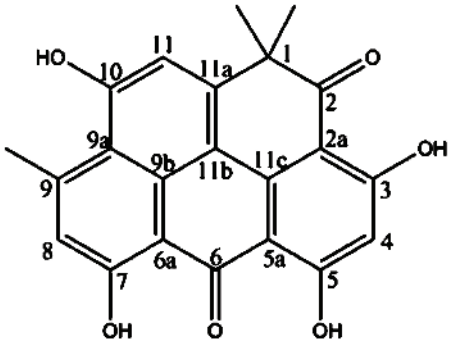

Fig. 4. Structure of resistomycin.

Table 4. Antimicrobial activities of resistomycin produced by *S. auranticus* AAA5

Test organism	MIC(μg/ml)		
	Resistomycin	Cefradine	SMS
Bacillus subtilis	25±0.34	8±0.03	-
Staphylococcus aureus	13±0.03	5±0.12	4±0.34
S. epidermis	8±0.05	4±0.43	-
Enterococcus faecalis	5±0.03	5±0.17	7±0.45
Klebsiella pneumoniae	16±0.05	10±0.22	-
Shigella sp.	45±0.16	6±0.12	9±0.21
Proteus vulgaris	70±0.12	-	12±0.33
Escherichia coli	42±0.23	-	-
Pseudomonas aeruginosa	34±0.34	17±0.14	-
Salmonella typhii	15±0.11	-	13.4±0.56

Cefradine, Gram-positive bacterial control reference; SMS (Streptomycin sulfate), Gram-negative bacterial control reference; -, No antimicrobial activity; MIC, minimum inhibitory concentration; Data are mean±SD

Table 5. *In vitro* sensitivity of cancer cell lines to resistomycin

Concentration (μg/ml)	HePG2		HeLa	
	Compound[a]	5-FU[a]	Compound[a]	5-FU[a]
5	97.24	97.49	100	100
5	81.17	84.24	94.91	98.42
0.5	73.42	75.88	74.75	79.42
0.05	64.25	-14.71	54.32	59.41
0.005	32.89	-6.02	39.96	30.14
0.0005	28.61	-2.12	21.24	12.16

5-FU, positive control
ND, Not done
[a]Data shown as percentage inhibition rate
Experiments were carried out in triplicates.

Table 6 Cytotoxic activities of resistomycin

Compound	Cell line HeLa			Cell line HePG2		
	GI_{50}	TGI	LC_{50}	GI_{50}	TGI	LC_{50}
Resistomycin	0.006	0.009	0.013	0.005	0.008	0.010

All the concentration is in (μg/ml); Experiments were carried out in triplicates.

(C-11c), 152.2 (C-9), 152.7 (C-11a), 162.7 (C-10), 168.2 (C-7), 169.9 (C-5), 170.5 (C-3), 184.8 (C-6), 205.1 (C-2) profiles, 2D NOESY, HSQC, and HMBC experiments were used to assign chemical shifts. Spectra were calibrated from the residual DMSO signal for ^{1}H (2.50 ppm) and the DMSO-d6 signal for ^{13}C (39.51 ppm). The structure of resistomycin was shown in Fig. 4.

Biological activities of resistomycin

The antimicrobial profiles in terms of MIC and cytotoxic activity of the resistomycin compounds are shown in Tables 4 and 5, respectively. The compound showed good antimicrobial activity against both Gram-positive and Gram-negative pathogens. Specifically, the compound exhibited strong antimicrobial activity against *Enterococcus faecalis* and *Staphylococcus epidermis* and moderate activities against *Staphylococcus aureus*, *Salmonella typhii*, *Klebsiella pneumoniae*, and *Bacillus subtilis*. The cytotoxicities were assessed based on their effects upon the growth of tumor cells. For each concentration tested, the GI_{50} (drug concentration causing 50% growth inhibition), TGI values (drug concentration causing 100% growth inhibition), and LC_{50} (minimum concentration that reduces the initial cell number to half) were determined (Table 6). The GI_{50} values were found to be lower in the HeLa (0.005 μg/ml) cell line compared to HepG2 (0.006 μg/ml).

Discussion

During a screening program for pigment producers, an actinomycetes strain (AAA5) was identified, which showed strong anti-tumor activity against human hepatoma and cervical carcinoma cell lines *in vitro*. It exhibited good growth on ISP2, ISP3, ISP4, and Bennet's agar. It has been reported that strains with a smooth surface are mostly in the grey color series. The strain utilized most of the sugars that were provided, indicating a wide pattern of carbon assimilation. These results were in close agreement with the findings of Williams *et al.* (1983). The tests used in this study are indispensable tools for the classification of *Actinobacteria* (Kampfer *et al*., 1991). The biochemical characterization revealed that the strain can hydrolyze starch, casein, and cellulose, but that it cannot hydrolyze lipids or gelatin. The strain utilizes arabinose, cellulose, dextrose, fructose, galactose, mellibiose, mannitol, raffinose, and rhamnose. The optimum pH and temperature for growth of the strain were 7.0-8.0 and 37°C, respectively. The strain was able to tolerate NaCl concentrations of up to 6%. It showed a negative result in the Indole production, Methyl Red, Voges Proskaeur, and Citrate Utilization (IMViC) test and did not produce hydrogen sulphide. The AAA5 strain showed 99% similarity to *S. aurantiacus* based on 16S rRNA gene sequences. The morphological and biochemical characteristics also reflected those of *Streptomyces* genera.

S. aurantiacus has previously been reported to produce only pamamycin-621 and aurantimycin D (Grafe *et al*., 1993; Schlegel *et al*., 1995). We have described for the first time that resistomycin, a quinone-related antibiotic, is produced by an *S. aurantiacus* strain.

The quantification of resistomycin from the crude extract was determined with a molar extinction coefficient of 15400. To make the production of antibiotics feasible, it is necessary to optimize the production conditions. Changes in the nature, type, and concentration of carbon and nitrogen sources or in the mineral element components of the culture medium greatly affect antibiotic synthesis in *Streptomycetes*. The utilization of starch by the strain for growth and the production of bioactive metabolites suggests the presence of an active uptake system for starch as substrate as previously reported for other *Streptomyces* sp. Lower concentrations of mineral ions were previously shown to support antibiotic production. The AAA5 strain also yielded good resistomycin production in starch-casein medium, which contains low levels of mineral ions. The positive action of mineral ions may be related to their roles in the formation of peptidases (El-Gendy *et al*., 2008; Dharmaraj *et al*., 2009). In order to identify suitable fermentation conditions for mycelia and to promote bioactive metabolite production on fermentative medium, it is necessary to determine the effects of fermentation duration on biomass and metabolite production. We have demonstrated that biomass production reaches a maximum after 6 days of incubation, and that mycelia production decreases beyond this fermentation period. This may be due to nutrient depletion during prolonged fermentation periods. However, resistomycin production was found to rise with an increase in biomass production, but reached maximum production only at the stationary phase of the sixth day. It has been found that synthesis of the secondary metabolites occurs after growth has ceased.

Purification of the compound was carried out in a silica column, and the compound was chemically characterized by ^{1}H NMR and ^{13}C NMR. The spectral data were compatible with resistomycin, as recorded by Kock *et al.* (2005) and Gorajana *et al.* (2006).

From the results obtained, it appears that the antibacterial action of the extracts is typically more pronounced on Gram-positive than on Gram-negative bacteria. These results are consistent with previous screenings of *Streptomycetes* isolates, which showed strong activity against Gram-positive bacteria (Saadoun *et al*., 1998) and low activity against Gram-negative test microorganisms (Saadoun and Gharaibeh, 2003; Sahin, 2005). The reason for the discrepancy in sensitivity is based on the morphological differences between Gram-positive and Gram-negative microorganisms. Whilst Gram-negative bacteria have an outer lipopolysaccharide membrane that makes the cell wall impermeable to lipophilic solutes, Gram-positive bacteria are more susceptible as they have a more permeable outer peptidoglycan layer (Yilmaz *et al*., 2008).

At the lowest concentration of 5×10^{-4} μg/ml resistomycin, the rate of inhibition was 21.24% and 28.61% for HeLa and HepG2 cells, respectively. In contrast, for 5-FU (the positive control), the rate of inhibition was less than zero at the same concentration level, indicating that our compound has potent cytotoxic effects. Purification of the antimicrobial substance shows that a single active component is responsible for the broad-spectrum antibacterial activity as well as for the cyto-

toxic activity against carcinoma cell lines. There are reports of similar, single compounds possessing such activity, which are produced by microorganisms belonging to the *Streptomyces* genus (Saha *et al*., 2005). The GI_{50} values were found to be minimum in HeLa (0.006 µg/ml) and HepG2 (0.005 µg/ml) cell line. There was no significant difference found between the two cell lines. Thus, our compound was found to be more potent in killing cervical carcinoma and liver carcinoma cells. Furthermore, the compound was found to have stronger inhibitory activity against cancer cells at the lowest concentrations. Similarly, Goragina *et al*. (2007) reported cytotoxicity in HMO2 and HepG2 cell lines with GI_{50} values of 0.007 and 0.010 µg/ml of resistoflavin respectively purified from *Streptomyces* strain B8005.

Conclusion

We have identified a putative *S. aurantiacus* strain (AAA5) from a terrestrial ecosystem based on morphological and physiological characterization and on 16S rRNA sequence analysis. The strain produces a reddish-brown pigment, and yields a bioactive yellow compound identified as resistomycin. The strain displays important biological activity against Gram-positive and Gram-negative bacterial pathogens. It also exhibits cytotoxic activity against human hepatic carcinoma and cervical carcinoma cell lines *in vitro*. Further studies focusing on the mechanisms of resistomycin cytotoxicity in human cancer cell lines are in progress. This study highlights potential strategies for the development of new antitumor compounds with improved therapeutic properties, and the utility of combinatorial biosynthesis approaches.

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