




Novel actinomycin group compound from newly isolated *Streptomyces* sp. RAB12: isolation, characterization, and evaluation of antimicrobial potential

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Abstract

Streptomyces sp. RAB12 having potential to produce novel actinomycin group compounds was isolated from soil samples collected from CSIR-Indian Institute of Chemical Technology, Hyderabad, India, garden premises using International Streptomyces Project (ISP) protocols. The 16S rRNA sequence of the strain RAB12 exhibited identity with *Streptomyces* sp. 13647M and the sequence was deposited in NCBI under the accession number KY 203650 while the strain RAB12 was deposited in The Microbial Type Culture Collection and Gene Bank (MTCC) with accession number MTCC 12747. Cell-free extract of this novel strain revealed two bioactive principles viz., RSP 01 and RSP 02. HR-MS analysis indicated a molecular mass of 1269.61 and 1270.63 m/z g/mol for RSP 01 and RSP 02, respectively. Proton ¹H, ¹³C NMR, 2D NMR and mass spectroscopy analysis revealed a similar fingerprint to that actinomycin D except for a peak at δ H3.59 J (¹H NMR) and δ 208.88 (¹³C NMR) for RSP 01 compound suggesting the presence of keto carbonyl at 5-oxo position on the proline moiety which is absent in actinomycin D. Purified RSP 02 depicted a similarity with RSP 01 except a peak in the ¹H proton NMR at δ H 3.81 J. HR-ESI mass spectra confirmed the molecular formulae for RSP 01 and RSP 02 as C₆₂H₈₄N₁₂O₁₇ and C₆₂H₈₆N₁₂O₁₇, respectively. Antimicrobial activity profile revealed higher antimicrobial activity against bacterial strains (*Pseudomonas aeruginosa*, *Micrococcus luteus*, *Staphylococcus aureus*, *Salmonella typhi*, and *Bacillus subtilis*) and *Candida albicans* compared to standard actinomycin D. MIC and MBC for RSP 01 were observed to be 0.0039 and 0.0078 (μg/ml) against *C. albicans*, while for actinomycin D, it was found to be 0.031 and 0.62 (μg/ml), respectively indicating a tenfold higher potency. Thus, these RSP 01 and RSP 02 compounds from *Streptomyces* sp. RAB12 may be promising candidates for industrial and clinical applications.

Keywords Actinomycin · MIC · MBC · NMR and mass spectroscopy

Introduction

Novel antibiotic discovery is gaining more prominence with an increase in pathogenic microbial multi-drug resistance in the twentieth century for better public health achievements (Xiong et al. 2012). Among all antibiotic-producing members, the genus, *Streptomyces*, is known as a rich source of bioactive compounds and accounts for the production of two thirds of the commercially available antibiotics (Bentley et al. 2002). One of the estimates suggested that more than 100,000 compounds were known to be synthesized by this genus and only a tiny fraction of which were unearthed so far (Milind et al. 2001). Therefore, this genus has been continuously explored for a wider variety of new antibiotics (Berdy 2005).

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Considering the above, Milind group developed a mathematical model to understand the potential of antibiotic production of *Streptomyces* genus (Milind et al. 2001).

Actinomycins, a family of chromopeptide lactones, are produced by various species of *Streptomyces*. The *Streptomyces* genus is a Gram-positive bacteria which shows a filamentous form and grows in various environments. Among several antibiotics produced by this genus, actinomycins are prominent. More than 20 naturally occurring actinomycins were isolated and observed to have commonality of two pentapeptidolactone moieties with actinoyl chromophore (Brockmann 1960); however, they differ in positional and/or functional groups. Among them, actinomycin D has been extensively studied and used clinically as an anticancer drug especially in the treatment of infantile kidney tumors, childhood rhabdomyosarcoma, and several other malignant tumors (Farber et al. 2002; Womer 1997).

In general, antibiotic production is species specific. These secondary metabolites are useful to the microbial strains to compete with other microorganisms for survival or to have a symbiotic relationship between strains and plants, as the antibiotic protects the plant against pathogens, and plant exudates allow the development of *Streptomyces* (Bosso et al. 2010). In fact, it was also reported in the literature that antibiotics originated as signal molecules and are able to induce changes in the expression of some genes (Chater et al. 2010). In addition, many fermentation factors are observed to influence the antibiotic synthesis as the microbes respond differently and employ different biochemical networks in different nutritional environments. Considering the prevailing multi-drug resistance, the imperative role of new antibiotics to prevent the microbial diseases, and untrapped nature of *Streptomyces* potential for actinomycins, the authors isolated a microbial strain having potential to produce a novel actinomycin variant, structurally elucidated and evaluated the antimicrobial potential in the present investigation. The isolated chromopeptides showed strong antibacterial activities with tenfold higher potency compared to standard actinomycin D.

Materials and methods

Sample collection

Three soil samples were collected from rhizosphere arena of the plant, *Wedelia trilobata*, present in the CSIR-Indian Institute of Chemical Technology, Hyderabad, premises. The collected samples were transferred to sterile bags and kept at 4 °C until use. Collected soil samples were used for screening and isolation of different actinomycetes strains.

Screening and isolation of actinomycetes

Five grams of soil was taken and added to 50 mL of sterile distilled water in a 250 mL Erlenmeyer flask. The flask was kept for shaking in an orbital shaker at 37 °C. After 60 min the supernatant was collected and subjected to serial dilutions from 10^{-1} to 10^{-8} . From each dilution, 1.0 mL of sample was taken and it was placed on glycerol asparagine agar medium (ISP5 medium) by spread plate technique and incubated at room temperature for 14 days. Developed microbial colonies were carefully picked and were sub-cultured on glycerol asparagine agar slants. The slants were maintained at 4 °C.

Preliminary screening for antibiotics

The isolates were initially screened for antimicrobial activity by agar well diffusion method (Zhang et al. 2013; Kim et al. 2003). Initially, the isolated cultures were grown in glycerol asparagine medium. After 7 days of incubation, the supernatant was collected from each isolate and tested initially for antimicrobial activity against bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans*). The plates were then incubated at 37 °C for 24 h. The isolates which show activity against tested organisms were collected and maintained. Among the collected isolates, the potential isolate designated as RAB12 was selected for further studies.

Identification of organism

Growth and morphology of the selected potential strain (RAB12) was studied on different media (ISP1 to ISP7) whose composition was given in supplementary data (Table S1). The physiological growth performance of the isolated strain (RAB12) at different temperatures ranging from 25 to 45 °C with a variation of 5 °C, pH tolerance ranging from 5 to 10 with a variation of 1.0 pH unit, and NaCl concentration ranging from 0.05 to 0.25% with a variation of 0.5% was studied by growing them at respective conditions, individually. Biochemical characterization based on specific tests like Gram staining, spore staining, motility, indole production, MR-VP test, gelatin hydrolysis, citrate utilization, growth on triple sugar iron agar, oxidase, catalase, nitrate reduction, urease production, and starch hydrolysis were analyzed according to Bergey's manual of bacteriology (2012). Isolated strain ability to ferment different sugars as sole carbon source such as dextrose, sucrose, fructose, arabinose, rhamnose, ribose, mannose, inositol, salicin and mannitol was studied (Taddei et al. 2005; Kurosawa et al. 2006). Molecular characterization of the strain based on ribotyping of 16S rRNA was performed at Xcelris genomics Ltd., Ahmadabad, India.

Phylogenetic analysis

Nucleotide sequence obtained from 16S rRNA of RAB12 was compared with those maintained in the GenBank Database through NCBI Blast (<http://www.ncbi.nlm.nih.gov>). Multiple alignments of nucleotide sequences was done using ClustalW software program. Data analysis was performed on a bootstrapped dataset containing 1000 replicates. A phylogenetic tree was constructed to determine the genetic relationship between aligned strains using the neighbor-joining method utilizing MEGA software (Saitou and Nei 1987; Tamura et al. 2011).

Isolation and purification of antimicrobial compounds

The cell-free extract of RAB12 was collected by centrifugation of fermented broth for 20 min at 17,000g and used for isolation of active compound using the solvent (methanol and chloroform in the ratio of 5:95) extraction method. The obtained compound was analyzed by thin-layer chromatography using the same solvent (methanol and chloroform solvent at a ratio of 5:95). The separated compounds were collected and further purified by preparative thin-layer chromatography using the same solvent as the mobile phase. Each compound band was extracted and checked for its purity and antimicrobial property and used for its characterization (Gaurav and Nissreen 2013).

Chemical characterization of isolated compounds

Melting points for purified compounds were determined using an Electro thermal melting point apparatus at 298 K. Thin-layer chromatography was performed on silica gel 60 F254 plates (Merck, 0.2 mm). Purity of the extracted compounds was analyzed by using HPLC fitted with the X Bridge C18 (50 X 2.1 mm) column and at a flow rate of 0.4 mg/ml ammonium acetate buffer (1 mM; pH 10.5). The elemental compositions of the compounds were determined by Vario micro elemental analyzer using helium and argon as carrier gases. Infrared (IR) spectrum of the active compound was investigated using KBr pellets with Thermo Nicolet Nexus 670 spectrophotometer. High-resolution mass spectra (HR-MS) were recorded by ion trap method and mass/charge (m/z) ratios were reported as values in atomic mass units. Mass spectrum of the compound was recorded by using AUTOSPEC-M, Micro mass, UK. $^1\text{H-NMR}$ spectra (600 MHz), $^{13}\text{C-NMR}$ spectra (150 MHz), and other hetero-nuclear experimental data were obtained in CDCl_3 at 25 °C on Bruker Avance-600 MHz with chemical shifts (δ , ppm) reported relative to TMS (0.0 ppm) and CHCl_3 (77.00 ppm) as an internal reference. Data reported as chemical shift (ppm) (δ), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, b = broad, m = multiplet), integration, coupling constant (Hz).

The antioxidant activity

The antioxidant activity of RSP 01 and RSP 02 was determined by 2, 2, Diphenyl-2-Picryl hydrazyl (DPPH) scavenging assay according to Jemimah et al. (2015). Initially, the selected compounds along with ascorbic acid (as standard) were diluted to get 50, 100, 150, 200 and 250 $\mu\text{g/mL}$ concentrations. The volume of each tube was made up to 3 ml of methanol and to it 150 μl of 0.002% DPPH dissolved in methanol solution (a freshly prepared) was added. The samples were incubated in dark at 37 °C for 20 min and the color intensity change was measured at 515 nm (UV-Vis spectrophotometer, Elico-India). The data expressed as the percent decrease in the absorbance compared to the control i.e. ascorbic acid. The percentage inhibition was calculated and reported.

Antimicrobial activity

Initially TLC-based bioautography was performed for identification of bioactive property of purified compounds on agar plates against pathogens (Choma and Edyta 2011). Subsequently, extracted compounds were analyzed for bioactivity by agar well diffusion method. Nutrient agar and Czapek Dox agar plates were prepared and spread with the 18 h grown selected microbial cultures on plates. Wells were made in every plate with a diameter of 10 mm using sterile cork borer. From prepared stock solution (1 mg/ml) of each compound, 100 μl of each compound were added into the well using micropipette and allowed to diffuse at -20 °C for half-an-hour. Plates were incubated at 37 °C for 18 h for bacterial and 30 °C for 48 h for fungal growth. The zone of inhibition was measured in millimeters (mm). The values reported were the average of five experiments.

Minimum inhibitory concentration and minimum bactericidal concentration

The compounds showing better antimicrobial activity were selected for the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) studies against the selective strains (*S. aureus* (MTCC 3160), *B. subtilis* (MTCC 736), *E. coli* (MTCC 40), *P. aeruginosa* (MTCC 1034) and *C. albicans* (MTCC 1637)). Selected compounds were serially diluted to get concentrations ranging from 500 to 1.9 $\mu\text{g/ml}$ and one tube without drug served as control. All the tubes were inoculated with 1 ml of respective cultures having an OD of 0.2 at 540 nm (McFarland standard) and the tubes were incubated at 37 °C for 12 to 16 h. The turbidity of each tube was measured with respect to the control tube. MIC value was defined as the lowest concentration of compound at which growth is completely inhibited. After incubation, the culture from each tube was plated in nutrient

agar to evaluate the MBC concentration. The concentration at which the cells are completely dead was defined as MBC.

Nucleotide sequence accession number

16S rRNA Genome sequence was deposited in the National Centre for Biotechnology Information (NCBI) with accession Sequence ID KY 203650.

Results

Isolation and identification of an antimicrobial compound producing strain

More than 18 numbers of isolates from different soil samples were isolated, purified, and screened for bioactivity against bacterial strains such as *S. aureus*, *B. subtilis*, *E. coli*, *P. aeruginosa*, and yeast strain, *C. albicans*. Among them, only four isolates showed bioactivity against the above test strains. Since one of isolates designated as RAB12, revealed a higher activity (20 mm) against tested bacterial strains, hence this strain was selected for further studies.

Morphology of the selected strain (RAB12) was studied by growing them in different media recommended by the International Streptomyces Project (ISP) and also MTCC-93 media, individually. The areal mycelia grew abundantly in glycerol asparagine agar (ISP5) and MTCC-93 media, while growth is moderate in tryptone yeast extract agar (ISP1) and malt extract agar (ISP2) whereas the growth is poor in inorganic starch agar (ISP4) and peptone yeast extract iron agar (ISP6) media and the data on growth characteristics of RAB12 strain in different media were given in supplementary data (Table S2).

Further characterization of strain RAB12 was performed by evaluating various biochemical tests and noticed that tests such as catalase, methyl red, and starch hydrolysis were shown positive while other tests like urease, gelatine hydrolysis, indole, Voges–Proskauer, citrate, and nitrate reduction were observed to be negative. To understand, its metabolic profile especially for utilization of different sugars (arabinose, ribose, mannitol, dextrose, fructose, sucrose, galactose, rhamnose, mannose, inositol, and salicin) as sole carbon sources was analyzed individually and the growth analysis indicated that upon metabolizing these sugars, no acid is produced by this strain (Table S3). This suggested that this strain has limited anaerobic fermentation ability. The above growth data of isolate RAB12 denote that the isolate belongs to the *Streptomyces* genus.

The function of the 16S rRNA gene over time has not changed; suggesting that random sequence changes are a more accurate measure of time (evolution) hence is used for informatics purpose (Patel 2001). More recently, it is suggested

that 16S rRNA gene can be used as a reliable molecular clock because 16S rRNA sequences from distantly related bacterial lineages are shown to have similar functionalities (Tsukuda et al. 2017). Fundamentally, 16S rRNA gene sequence is universal in all bacteria, comprising of 1550 bp long with a hyper variable and conserved regions. Woese (1987) reported that phylogenetic relationships of bacteria could be determined by comparing a stable part of the genetic code i.e. 16S rRNA region whereas the hyper variable regions of 16S rRNA gene sequences provide species-specific signature sequences thus adopted globally for bacterial identification. Considering above, isolated strain was further identified by constructing phylogenetic tree (Fig. 1) based on its 16S rRNA gene sequence and genetic identity of present isolated strain, RAB12, also indicated that this isolate belongs to the genus *Streptomyces* sp. Its 16S rRNA gene sequence showed high similarity (99%) with *Streptomyces* sp. 13,647 M. This strain RAB12 was deposited in The Microbial Type Culture Collection and Gene Bank (MTCC) with accession number MTCC 12747.

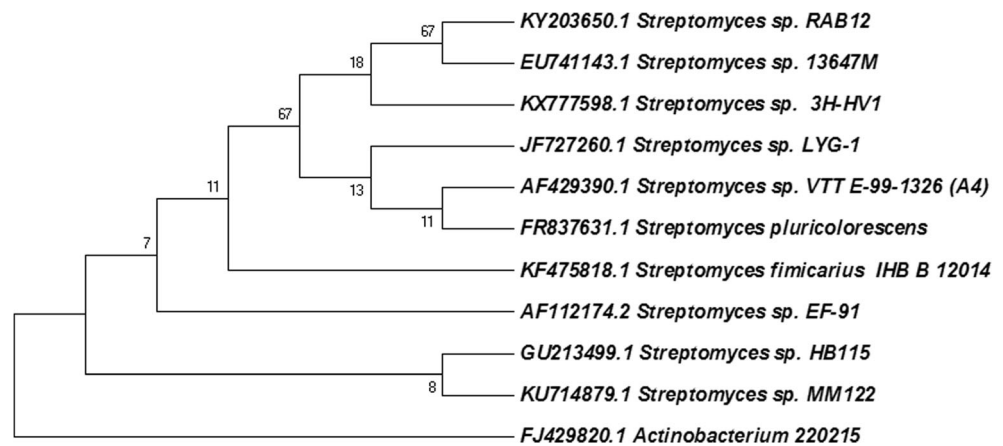
Isolation and purification of antimicrobial compounds produced by *Streptomyces* sp. RAB12

To evaluate the antimicrobial activity of bioactive compounds from isolated strain, 10 L of fermented broth was centrifuged and the obtained supernatant was subjected to liquid–liquid extraction in a separating funnel by using equal quantity of ethyl acetate (a ratio of 1:1). Upon evaporation of solvent containing bioactive compounds resulted in 2.0 g of solid compound. Analysis of this ethyl acetate extract by thin-layer chromatography revealed two major bands and five minor bands. TLC-based bioautography analysis revealed antibacterial activity for two major and two minor bands (data not shown). In view of the above, further concentration was focused on two major compounds where the first band was coded as RSP 01 and the next major compound was coded as RSP 02. Extraction of these two bands yielded a 300- and 175-mg compound, respectively, which were further analyzed using HPLC to detect purity of both compounds and noticed that these two compounds are 97% pure (Fig. S1). These two purified compounds (RSP 01 and RSP 02) were subjected to structural analysis.

Structural characterization of compounds

The isolated two compounds (RSP 01 and RSP 02) appeared as orange amorphous powders. Melting points of RSP 01 and RSP 02 compounds were observed to be 244 and 280 °C, respectively. Solubility analysis indicated that both these compounds were freely soluble in methanol, chloroform, ethanol, acetone, dichloromethane, and dimethyl sulphoxide and partially soluble in water. The elemental analysis of both RSP 01

Fig. 1 Phylogenetic tree of isolated *Streptomyces* sp. RAB12



and RSP 02 compounds revealed carbon 58.54%, nitrogen 8.69%, hydrogen 6.47%, oxygen 21.3%, and nitrogen 13.30% suggesting both these compounds are almost similar in their molecular formulae however may differ in structural architecture. FT-IR analysis of the compounds suggested that compounds denoted functional group peaks OH (3424), CH (2962), CH₂ (2927), C=O (1746), C=O, C=O (1687), C=O (1673), C=O (1478), C–H (1296), and C–O (1120) at cm⁻¹ for RSP 01. In the case of RSP 02, except the C=O (1746 at cm⁻¹) functional group peak, the rest of the functional group peaks were retained as seen in FT-IR analysis (Fig. S2).

Further analysis by high-resolution mass spectrometry (HR-MS) and the HR electrospray ionization (HR-ESI) mass spectrum of RSP 01 and RSP 02 depicted the [M + H]⁺ ion at *m/z* 1269.6156 and *m/z* 1271.6313, respectively, revealing the

molecular formulae of C₆₂H₈₄N₁₂O₁₇ for RSP 01 (Fig. 2) and C₆₂H₈₆N₁₂O₁₇ for RSP 02 (Fig. S3). Comparative evaluation of present data with Pubchem data suggested that these two molecules may belong to the actinomycin family.

NMR spectra of RSP 01 and RSP 02 molecules revealed that these two molecules contain amino acids similar to that of the actinomycin D molecule however differ at proline moiety. In case of RSP 01, the noticed peak in the ¹H proton NMR at δH 3.59 and δ 208.88 ppm in the ¹³C NMR indicated for the presence of keto carbonyl at 5-oxo position on the proline moiety which is absent in actinomycin D (Table 1). In case of RSP 02 molecule, except a peak in the ¹H proton NMR at δH 3.81 ppm (assigned to OH group), the rest of the spectra of ¹H proton (Fig. S4) and ¹³C NMR (Fig. S5) were similar to

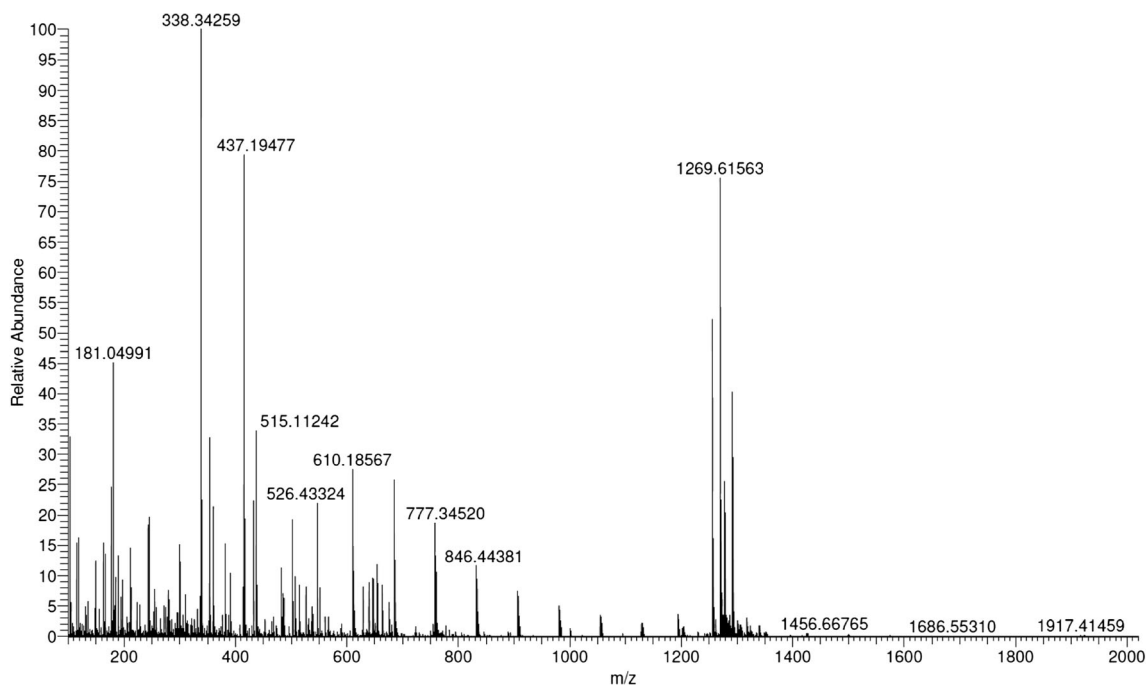


Fig. 2 HR-MS spectra of RSP 01 compound

Table 1 ^1H and ^{13}C NMR spectra of RSP 01 compound

α -ring	Pos.	δC	$\delta\text{H } J$ [Hz]	β -ring	pos	δC	$\delta\text{H } J$ [Hz]
Thr	1	168.98	–	Thr	1	168.62	–
	2	55.09	4.53 m		2	54.73	4.63 m
	3	74.92	5.22 m		3	74.80	5.95 dt (5.0, 2.5)
	4	17.29	1.25 d (6.0)		4	17.73	1.26 d (6.1)
	NH	–	6.03 d (5.5)		NH	–	7.35
D-Val	1	173.20	–	D-Val	1	173.14	–
	2	58.71	3.56 dd (10.0, 5.0)		2	58.89	3.74 dd (7.0, 9.5)
	3	31.77	0.95, 2.21 m		3	31.50	0.97, m
	4	19.26	0.741.13 d (6.3)		4	19.23	0.74 d (6.3)
	5	19.06	0.88 d (7.1)		5	19.20	8.26 d (5.5)
HMPro	NH	–	7.74 d (5.0)	HMPro	1	208.87	–
	1	173.66			2	173.23	
	2	56.44	6.03 m		3	56.24	6.01 d (9.1)
	3	47.56	3.71 m		4	47.31	2.95 m 2.8 m
	4	34.93	4.71 m 1.29 dd		5	34.86	3.77 ddd (11.2, 10.7, 7.0)
Methyl-valine	5	22.99	1.50 d 2.68 m	Methyl-valine	1	–	2.90 dd (7.0, 9.5)
	1	167.6	–		2	167.54	1.26 q (7.0)
	2	71.27	2.92 d (9.0)		3	71.10	2.67 m
	3	26.91	2.68 m		<i>N</i> -Me	26.87	
	4	21.66	0.95 d (6.5)			39.12	
Sar	5	19.06	0.74 d (6.5)	Sar	1	166.45	–
	<i>N</i> -Me	39.19	2.92 s		2	51.31	3.65 d (17.5) 4.53 d (017.5)
	1	166.51	–		<i>N</i> -Me	–	2.60 s
	2	51.34	3.65 d (17.5)				
	<i>N</i> -Me	–	4.74 d (17.5) 2.68 s				

Chromospheres: δ_{H} 2.20 (s, 3H, 12- H_3), 2.53 (s, 3H, 11- H_3), 7.36 (d, $J = 7.5$ Hz, 1H, 7-H), 7.61 (d, $J = 7.5$ Hz, 1H, 8-H) δ_{C} 7.73 (CH_3 , C12), 15.04 (CH_3 , C11), 101.70 (C, C-1), 113.38 (C, C-4), 125.6 (CH, C-8), 127.59 (C, C-6), 129.9 (C, C-9a), 130.24 (CH, C-7), 132.61 (C, C-9), 140.40 (C, C-5a), 144.96 (C, C-4a), 166.40 (C, C-13), 166.24 (C, C-14), 179.01 (C, C-3)

that of RSP 01. This suggested that the carbonyl C=O group present on proline moiety (at the 5th position) in RSP 01 was replaced with C-OH on the 4th carbon of proline moiety in RSP 02. Further confirmation for the presence of carbonyl C=O group on proline ring (at the 5th position) in RSP 01 (Fig. S6) was confirmed by hetero-nuclear spectral data (^1H - ^1H -COSY, NOESY, TOSEY, HSQC, and HMBC) while RSP 02 structure was confirmed from the data ^1H NMR (Fig. S7), ^{13}C NMR (Fig. S8), and 2D NMR (Fig. S9 depicting ^1H - ^1H -COSY, NOESY, and TOSEY). Based on the above information, the chemical structure of both compounds RSP 01 and RSP 02 were deduced and reported in Fig. 3 and Fig. 4, respectively. Literature search revealed that a similar structure of RSP 02 was observed and reported as actinomycin XO beta (National center for biotechnology information. pubchem compound database; CID=197972, <https://pubchem.ncbi.nlm.nih.gov/compound/197972>). Hence, the authors concluded that this isolate RAB12 also produces actinomycin XO beta along with RSP 01.

Bioactivity of isolated compounds

The antioxidant activity

Antioxidant activity data revealed that both the compounds depicted concentration-dependent inhibition of DPPH activity. This can be evidenced from Fig. 5 that the percent inhibition of DPPH activity increased from 40 to 70% with the increase of compound concentration from 50 to 250 $\mu\text{g}/\text{ml}$, respectively, for both compounds. The rate of increase of antioxidant activity with respect to compound concentration differed from selected one concentration to another. When the concentration of the compound increased from 50 to 100 $\mu\text{g}/\text{ml}$, the antioxidant activity was increased from 40 to 53%; while the concentration of compound increased from 200 to 250 $\mu\text{g}/\text{ml}$, the antioxidant activity was increased from 70 to 74% in the case of RSP 01 and 68 to 71% in the case of compound RSP 02, respectively (Fig. 5). There was a limited increase in antioxidant activity with respect to ascorbic acid

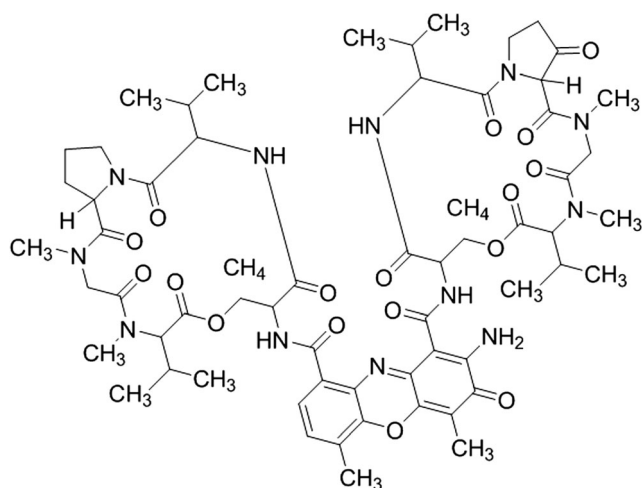


Fig. 3 Chemical structure of RSP 01 compound

over different concentrations and this standard compound showed the highest activity, i.e., 80%. This data suggested that though the isolated RSP 01 and RSP 02 compounds revealed potent antioxidant activity, these cannot replace the ascorbic acid. Similar trend was noticed with standard actinomycin D (Fig. 5). Saravana Kumar group working with ethyl extract of *Streptomyces lavendulae* strain SCA5 reported antioxidant activity; however, these authors did not isolate bioactive principle and also reported only 50% activity at a concentration of 507 $\mu\text{g/ml}$. This further confirms that the compounds reported in the present study have better antioxidant potential than reported by Saravana Kumar group (Saravana et al. 2014).

The two isolated molecules (RSP 01 and RSP 02) were further evaluated for in vitro antimicrobial activity against three Gram positive (*S. aureus*, *B. subtilis*, and *M. luteus*) and two Gram negative (*P. aeruginosa* and *S. typhi*) as well as a fungal strain, *C. albicans*, along with actinomycin D as standard using 100 μg each. It is clear from Table 2 that both isolated molecules were shown activity against all tested microorganisms indicating these molecules have potency to use them as antimicrobial agents (Table 2). It is also clear that the two actinomycin molecules of the present study have better bioactivity profile compared to standard actinomycin D (Table 2) except in the case of *S. aureus*. In fact, RSP 02 compound is through reported in the literature as actinomycin XOB however, this is the first report on antimicrobial property of the actinomycin XOB. Another compound, RSP 01 isolation, characterization, and bioactivity property, is reported for the first time. Bitzer isolated actinomycins with altered threonine and characterized its antimicrobial activity profile, in which the authors reported that one of the actinomycins containing chlorine revealed higher bioactivity against *E. coli*, *S. aureus*, and *B. subtilis*; however, the reported activity was less than standard actinomycin D

(Bitzer et al. 2009). Further analysis of the antimicrobial activity revealed that RSP 01 compound is better than RSP 02 as this can be evidenced from the fact that RSP 01 compound showed approximately 8 to 10% higher antimicrobial potency against all tested microbes.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

In view of the better antimicrobial profile of isolated compounds (RSP 01 and RSP 02) compared to actinomycin D, further evaluation with respect to cidal quantity requirements was detected by conducting the experiments of MIC and MBC. For determining the MICs for RSP 01 and RSP 02, compounds were tested against *P. aeruginosa*, *M. luteus*, *S. aureus*, *S. typhi*, *B. subtilis*, and *C. albicans*. The data suggested that all selected strains were susceptible to RSP 01 and RSP 02 and are higher potent than actinomycin D; however, the most potent activity was found against *C. albicans*. This can be evidenced from the fact that the MIC value for both compounds (RSP 01 and RSP 02) was observed to be 0.0039 $\mu\text{g/ml}$, whereas for actinomycin D, it was observed to be 0.062 $\mu\text{g/ml}$ against *C. albicans* (Table 2). Calculated MBC values against *P. aeruginosa* and *C. albicans* were found to be 0.0156 and 0.0078 $\mu\text{g/ml}$, respectively, for RSP 01 compound while it differed in the case of RSP 02 (Table 2). It is interesting to note that both isolated compounds were highly effective against *C. albicans* where the observed MIC and MBC were 0.0039 and 0.0078 $\mu\text{g/ml}$ and these values were approximately tenfold lower compared to actinomycin D (0.062 and 0.124 $\mu\text{g/ml}$) indicating their potential nature (Table 2) against *C. albicans*.

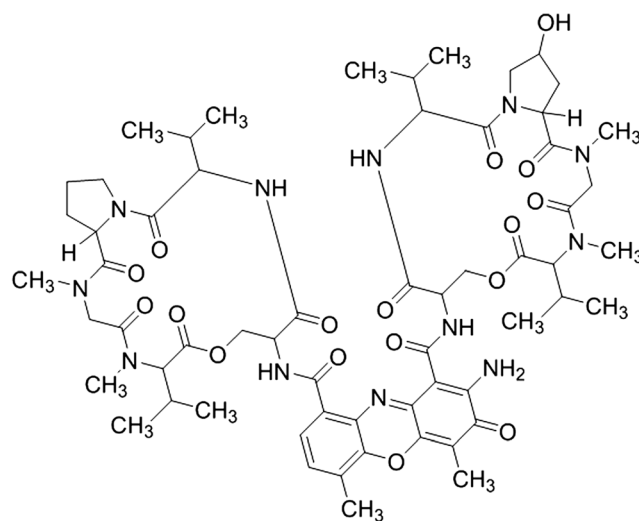


Fig. 4 Chemical structure of RSP 02 compound

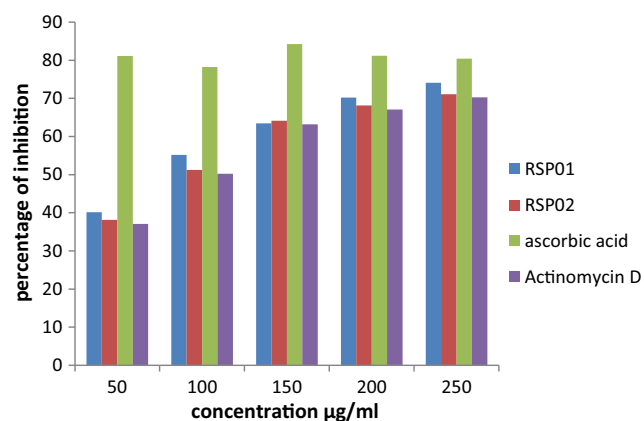


Fig. 5 Antioxidant activity of RSP 01 and RSP 02 compounds

Discussion

Antimicrobial resistance report of WHO 2016 (<http://www.who.int/mediacentre/factsheets/fs194/en/>) clearly indicated that newer resistance mechanisms are emerging as well as spreading globally resulting in superbug's development due to over/misuse of antibiotics. These will threaten our potential to treat common infectious diseases leading to prolonged illness, disability, and death in addition to developing a high risk in prevention and treatment of infectious medical procedures like organ transplantation, cancer chemotherapy, and diabetes management. Although continuous efforts have been made globally by researchers for the discovery of new antibiotics or development of a newer generation of antimicrobials by structural modification, yet success is very slim. Thus, there is an indispensable need for newer and novel antibiotics. One of the options could be exploring the novel, effective, and potential antimicrobial compound-producing microbial strains. In the current

study, authors made an effort to isolate an antibiotic-producing microbial strain, characterized at the molecular level, isolated the active principle and identified their structure. Authors report that the isolated strain belongs to *Streptomyces* sp. whose 16S rRNA gene sequence was deposited in NCBI under the accession number KY 203650. This strain showed 99% similarity with reported *Streptomyces* sp. 13,647 M (accession number: EU741143.1) in terms of 16S rRNA sequence; however, there is no report on antibiotic production by this strain, while the present isolate produced more than one potent antimicrobial compounds.

Comparative evaluation of active principle in terms of their antibiotic profile and molecular mass suggested that the produced antibiotic compounds belong to the class of actinomycin however slightly differ in molecular structure of actinomycin D. This could be exemplified from the fact that (a) the present isolated antimicrobial compounds (RSP 01 and RSP 02) molecular masses were observed to be 1269.61 (Fig. 3) and 1271.49 (Fig. S3) while actinomycin D has a molecular mass of 1255 (Praveen and Tripathi 2009); (b) RSP 01 compound revealed a ketocarbonyl group at the fourth carbon of proline moiety while RSP 02 showed a hydroxyl group at the third carbon of proline moiety (Figs. 3 and 4) whereas actinomycin D does not have any group at the above positions (Rebecca and Peter 2014); otherwise, the rest of the structure of RSP 01 and RSP 02 was similar to actinomycin D; (c) the bioactive profile (antimicrobial activity, MIC and MBC) of RSP 01, RSP 02 and actinomycin D differ (Table 2). Considering the structural similarity among RSP 01, RSP 02 and actinomycin D with slight modifications as mentioned above, newly isolated compound inhibitory mechanism may be similar to that of actinomycin D (DNA intercalation property); while noticed higher activity of RSP 01 may be attributed to presence of

Table 2 Antimicrobial activity of isolated compounds

Organisms	Antimicrobial profile of RSP 01 and RSP 02											
	RSP 01			RSP 02			Actinomycin D			Miconazole		
	ZI†	MIC*	MBC*	ZI†	MIC*	MBC*	ZI†	MIC*	MBC*	ZI†	MIC*	MBC*
<i>P. aeruginosa</i>	35	0.007	0.015	32	0.031	0.06	25	0.062	0.124	–	–	–
<i>M. luteus</i>	38	0.007	0.015	38	0.062	0.062	28	0.031	0.062	–	–	–
<i>S. aureus</i>	15	0.06	0.124	14	0.125	0.250	20	0.125	0.250	–	–	–
<i>S. typhi</i>	37	0.007	0.015	38	0.015	0.030	30	0.031	0.062	–	–	–
<i>B. subtilis</i>	38	0.03	0.062	35	0.015	0.030	30	0.031	0.062	–	–	–
<i>C. albicans</i>	40	0.003	0.007	38	0.003	0.007	32	0.062	0.124	–	7.8	23.4

Abbreviations: millimeter (mm), Minimum inhibitory concentration (MIC), Minimum bactericidal concentration (MBC) and Zone of inhibition (Z I)

*MIC and MIC are expressed in µg/ml

†Zone of inhibition in millimeter (mm)

ketocarbonyl group present on proline of beta chain of actinomycin D. Furthermore, the compound RSP 01 is reported first time while the RSP 02 compound was reported in literature (CID = 197,972, <https://pubchem.ncbi.nlm.nih.gov/compound/197972>). The structural and bioactive nature of isolated antimicrobial compound, RSP 01, further suggested that this compound is novel, first time reported, and has potential in human health care sector.

Critical bioactivity evaluation further suggested that the isolated RSP 01 compound has higher antimicrobial potential in comparison with actinomycin D and miconazole, standard antifungal; used as control available in the market (Table 2). For example, *C. albicans* growth was inhibited effectively by RSP 01 compound which can be evidenced from MIC values 0.0078 and 0.062 µg for RSP 01 and actinomycin D, respectively (Table 2), while the miconazole did not reveal any activity. This observation of the variation of MIC values further confirms that RSP 01 is more effective (approximately 10 times) to that of actinomycin D in terms of antimicrobial properties against tested bacterial and *C. albicans* strains (Table 2).

The productivity yield of the present isolate, *Streptomyces* sp. RAB12, towards RSP 01 and RSP 02 is observed to be higher compared to literature reported similar antimicrobial compounds from other *Streptomyces* strains. The fermentation yield of novel actinomycin RSP 01 from the present strain *Streptomyces* sp. RAB12 was observed to be 200 mg/10 L whereas RSP 02 showing a similar structural nature of actinomycin XOB was 127 mg/10 L while literature reported strain, *Streptomyces padanus* JAU4234, produced only 800 mg/200 L (Xiong et al. 2012). This indicated the potential nature of the present isolate in terms of novel antibiotic and productivity yield in addition to its antimicrobial potential towards *C. albicans* and other bacterial strains. Thus, the authors conclude that the present isolate could be a promising candidate for industrial-scale production of novel actinomycin RSP 01 which prevents bacterial and fungal infections effectively compared to presently market available actinomycin D.

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Compliance with ethical standards

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

Human and animal right and informed consent This article does not contain any studies with human participants or animals performed by any of the authors.

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