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Exploitation of potential bioactive compounds from two soil derived actinomycetes, *Streptomyces* sp. strain 196 and RI.24

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ABSTRACT

Due to emergence of drug resistant pathogens, nearly all available medicines are becoming ineffective against these life threatening pathogens so there is dire need for the discovery of compounds having unique modes of action. During our previous studies, actinomycetes designated as 196 and RI.24 were isolated, screened for bioactive compounds production and characterized using 16S rRNA gene sequencing. Colony 196 was identified as strain of *Streptomyces albolongus* (100% sequence similarity) and RI.24 as strain of *Streptomyces enissocaesilis* (100% sequence similarity). In current study, potential bioactive compounds produced by these strains were characterized. Cold extraction method was applied for taking out of bioactive compounds from actinomycetes. Minimum inhibitory concentration (MIC) determination of compounds from these strains showed activity nearly in the range of commercial antibiotics (strain 196 0.0075 mg/ml, RI.24 0.25 mg/ml and chloramphenicol 0.0075 mg/ml, ampicillin 0.025 mg/ml). Structural elucidation of these compounds was carried out using spectroscopic techniques of LC–MS/MS and ^{1}H NMR. Compounds K-252-*C*-Aglycone, indolocarbazole alkaloid, decoyinine, cycloheximide were detected from strain 196 whereas daunorubicin, hygromycin B, agecorynin F, indinavir-*N*-glucuronide and minocycline were identified from strain RI.24.Current study reports these compounds for the first time from strains of *Streptomyces albolongus* and *Streptomyces enissocaesilis*. Present investigation also suggests that strains 196 and RI.24 contain polyketide synthase-I (PKS-I) and non-ribosomal peptide synthetase (NRPS) gene clusters which are responsible for the production of bioactive compounds. The results of this study can be used by the scientific world or pharmaceutical industries for the development of new drugs/formulations by applying more advanced techniques.

1. Introduction

During mid-twentieth century discovery of penicillin and many other bioactive agents for treating contagious diseases has revolutionized the field of drug development. These inventions have led to the development of improved and effective antibiotics (Sharma et al., 2016). However, due to over the counter availability and overuse of antibiotics a large number of pathogens have developed resistance to the existing antibiotics. These pathogens were found to contain genes in their genome and plasmids which encode large number of resistant elements, greatly limiting therapeutic options against the bacteria (Wright, 2012). So, there is a dire need for invention and development of antibiotics that efficiently acts against deadly and life threatening pathogens. Microbes have long been considered as important reserves for isolation of useful molecules in the drug discovery paradigms (Hussain et al., 2017). Actinomycetes have vigorous ability to biosynthesize the potent bioactive secondary metabolites with broad range of biological activities (Berdy, 2012; Hussain et al., 2017). A small portion of the existing ecosystems have methodically been explored for isolation of microbial diversity (Hussain et al., 2017). Although, myriad molecules have been extracted from actinomycetes but only few have been analyzed for diverse biological activities. There are only 1% actinomycetes that have been cultured till date, thus bio-prospection of actinomycetes from diverse habitats for production of biologically important metabolites becomes an essential aspect (Subramani and Aalbersberg, 2013).

Present investigation is focused on extraction, identification and characterization of potent bioactive compounds from two actinomycete strains, *Streptomyces* sp. strain 196 and RI.24 isolated from two distinct habitats. The study is also aimed at PCR detection of PKS (Polyketide

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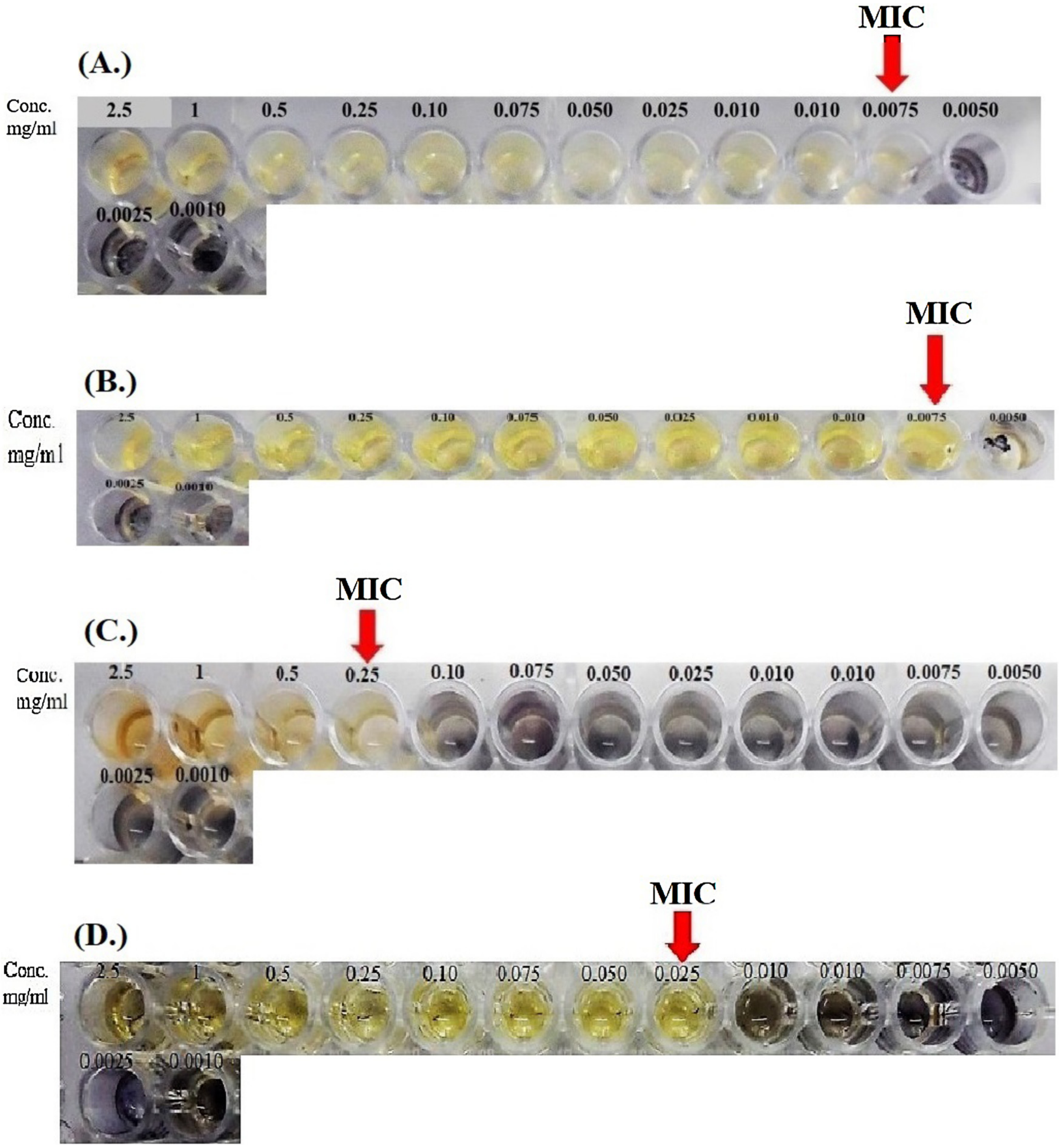


Fig. 1. Minimum inhibitory concentration (MIC) determination of *Streptomyces* sp. strain 196 and RI.24 extracts along with commercial antibiotics chloramphenicol and ampicillin as standards **(A.)** MIC of *Streptomyces* sp. strain 196 extract, **(B.)** MIC of chloramphenicol, **(C.)** MIC of *Streptomyces* sp. strain RI.24 extract, **(D.)** MIC of ampicillin.

Table 1
Minimum inhibitory concentrations of *Streptomyces* sp. strain 196 and *Streptomyces* sp. strain RI.24 extracts against pathogenic microorganisms.

S. No.	Pathogenic bacteria	MIC	
		Streptomyces sp. strain 196 extract (mg/ml)	Control-Chloramphenicol (mg/ml)
1.	*Bacillus cereus* MTCC 430	0.0075	0.0075
2.	*Staphylococcus aureus* MTCC 740	***Streptomyces* sp. strain RI.24 extract (mg/ml)** 0.25	**Control-Ampicillin (mg/ml)** 0.025

synthase) and NRPS (Non-ribosomal peptide synthetase) genes responsible for regulation of bioactive molecules production in the above mentioned strains.

2. Materials and methods

2.1. Actinomycete strains

Actinomycetes used in present study were isolated from soil samples of two distinct habitats, agricultural soil (Yamuna River, New Delhi, India) and radiation exposed soil (INMAAS, New Delhi, India) during our previous studies (Solanki, 2013; Kapur et al., 2018a, 2018b). 16S

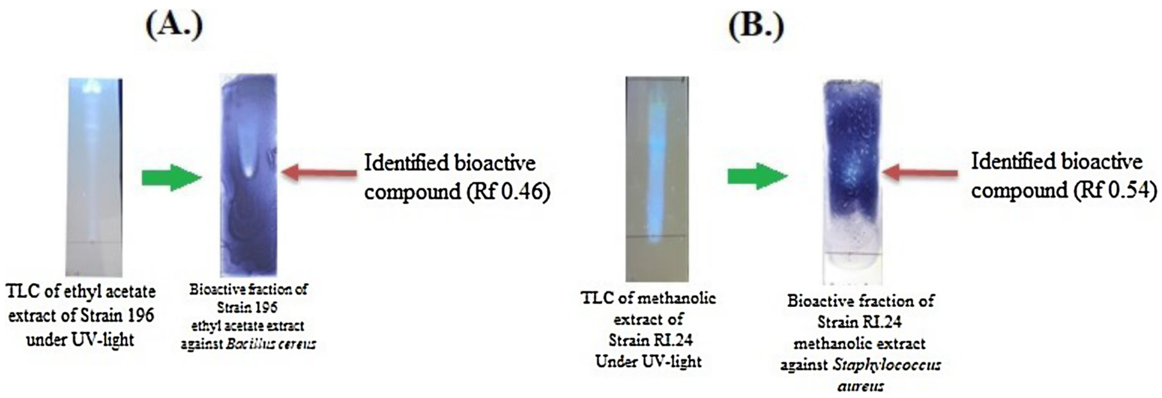


Fig. 2. Identification of actual bioactive fraction from crude extracts using immersion bioautography **(A.)** Bioactive fraction from strain 196 extract; **(B.)** Bioactive fraction from strain RI.24 extract.

Table 2
Chemical composition of bioactive compounds detected in LC–MS/MS analysis of extracts from *Streptomyces* sp. strain 196 and *Streptomyces* sp. strain RI.24.

Name of strain	RT(min.)	Compound name	Molecular weight
Streptomyces sp. strain 196	10.316	K-252-*C*-Aglycone, indolocarbazole alkaloid	311.4
	13.484	Decoyinine	279.24
	14.671	Cycloheximide	281.25
Streptomyces sp. strain RI.24	1.22	Daunorubicin	527
	1.22	Hygromycin B	527
	7.67	Agecorynin F (Flavonoid)	419
	10.33	Indinavir-*N*-glucuronide	789
	11.16	Minocycline (Tetracycline)	458

rRNA gene sequence homology revealed that strain 196 and RI.24 were 100% similar to *Streptomyces albolongus* and *Streptomyces enissocaesilis* respectively (Solanki, 2013; Kapur et al., 2018b). Present course of our study is focused on structural elucidation of bioactive compounds from these strains and identification of biosynthetic genes (PKS-I and NRPS) responsible for production of these molecules.

2.2. Extraction

Extraction of antimicrobial compounds from culture plates was performed using organic cold extraction method (Schimana et al., 2000; Solanki et al., 2015). *Streptomyces* sp. 196 and RI.24 were grown on Yeast extract malt extract (YM) medium plates for 10–15 days at 28 °C for production and diffusion of bioactive compounds. After incubation the well grown culture plates were chopped into small pieces and these pieces were added into the separate flasks. Ethyl acetate (Fisher Scientific) was added in strain 196 flask whereas methanol (Fisher Scientific) was added in strain RI.24 flask. These flasks were incubated at incubator shaker for 4–5 h at 28 °C and 200 rpm. The resulted solution was passed through filter paper (Whatman). Evaporation of solvents was done under reduced pressure in a rotary evaporator (BUCHI R-200, Switzerland).

2.3. Determination of Minimum inhibitory concentration (MIC)

MIC is defined as the lowest concentration of the bioactive agent that inhibits growth of microbes. During previous studies from our lab it was found that strain 196 showed maximum zone of inhibition against *Bacillus cereus* MTCC 430 whereas strain RI.24 against *Staphylococcus aureus* MTCC 740 (Solanki, 2013; Kapur et al., 2018a). In present study MIC was determined using microdilution method (Andrews, 2001; Solanki, 2013) for extracts from strains 196 and RI.24 along with positive controls chloramphenicol (Abbott Healthcare Pvt. Ltd., India) and ampicillin (Cadila Pharmaceuticals, India) respectively. Cultures of pathogenic strains *Bacillus cereus* and *Staphylococcus aureus* in log phase were diluted in their respective sterile growth medium so as to have a final concentration of 10^5 cfu/ml. Stock solutions of dried ethyl acetate extract of strain 196 was prepared in fraction amount of DMSO (Hi-Media) and maximum amount of sterile MQ water whereas for dried methanolic extract of strain RI.24 only sterile MQ water was used. Stocks from commercial antibiotics chloramphenicol having activity against *Bacillus cereus*; and Ampicillin having activity against *Staphylococcus aureus* were also prepared in autoclaved MQ water. Dilutions of these stocks were added to the wells of micro-titer plate and 100 μl from log phase culture of pathogenic bacteria having 10^5 cfu/ml concentration was also added to each well in the micro-titer plate and incubated at optimum temperature (Andrews, 2001). After 48 h of incubation, the plates were examined for inhibition of growth of pathogenic strain in each well. For better visualization of results 10 μl of MTT stain (Loba Chemie) having concentration 1 mg/ml was added in each well.

2.4. Bioautography and purification of bioactive compounds

Components of crude extracts were separated by silica gel TLC (60 F_{254}, 0.2 mm Merck, Germany). Solvent system dichloromethane: methanol (9:1) was used as mobile phase for ethyl acetate extract whereas *n*-butanol : acetic acid : water (3:1:1) was used for methanolic extract. The plates were air dried and visualized under UV light and different fractions were marked. A layer of soft agar medium containing log phase (10^5 cfu/ml) *Bacillus cereus* MTCC 430 was overlayed on strain 196 TLC plate whereas soft agar medium containing log phase (10^5 cfu/ml) *Staphylococcus aureus* MTCC 740 was overlayed on strain RI.24 TLC plate. These plates were incubated overnight at 30 °C (for 196) and 37 °C (for RI.24). Next day, MTT tetrazolium (Loba Chemie) (1 mg/ml) was used for staining of pathogenic layered TLC plates and incubated at 30 °C and 37 °C overnight (Taddei et al., 2006; Selvameenal et al., 2009; Solanki et al., 2015). White clear zones represents the presence of bioactive fractions whereas the rest of plate had purple or blue background which shows the live pathogenic cells stained with MTT tetrazolium. Rf values were calculated for each bioactive fraction. The actual bioactive fractions identified using bioautography were scraped along with silica gel from identical TLC plates of strain 196 and RI.24. Bioactive compounds were separated from silica gel by addition of ethyl acetate in 196 and methanol in case of RI.24 followed by centrifugation at 8000 rpm. Supernatants containing purified bioactive compounds were dried and subjected to ^{1}H NMR analysis.

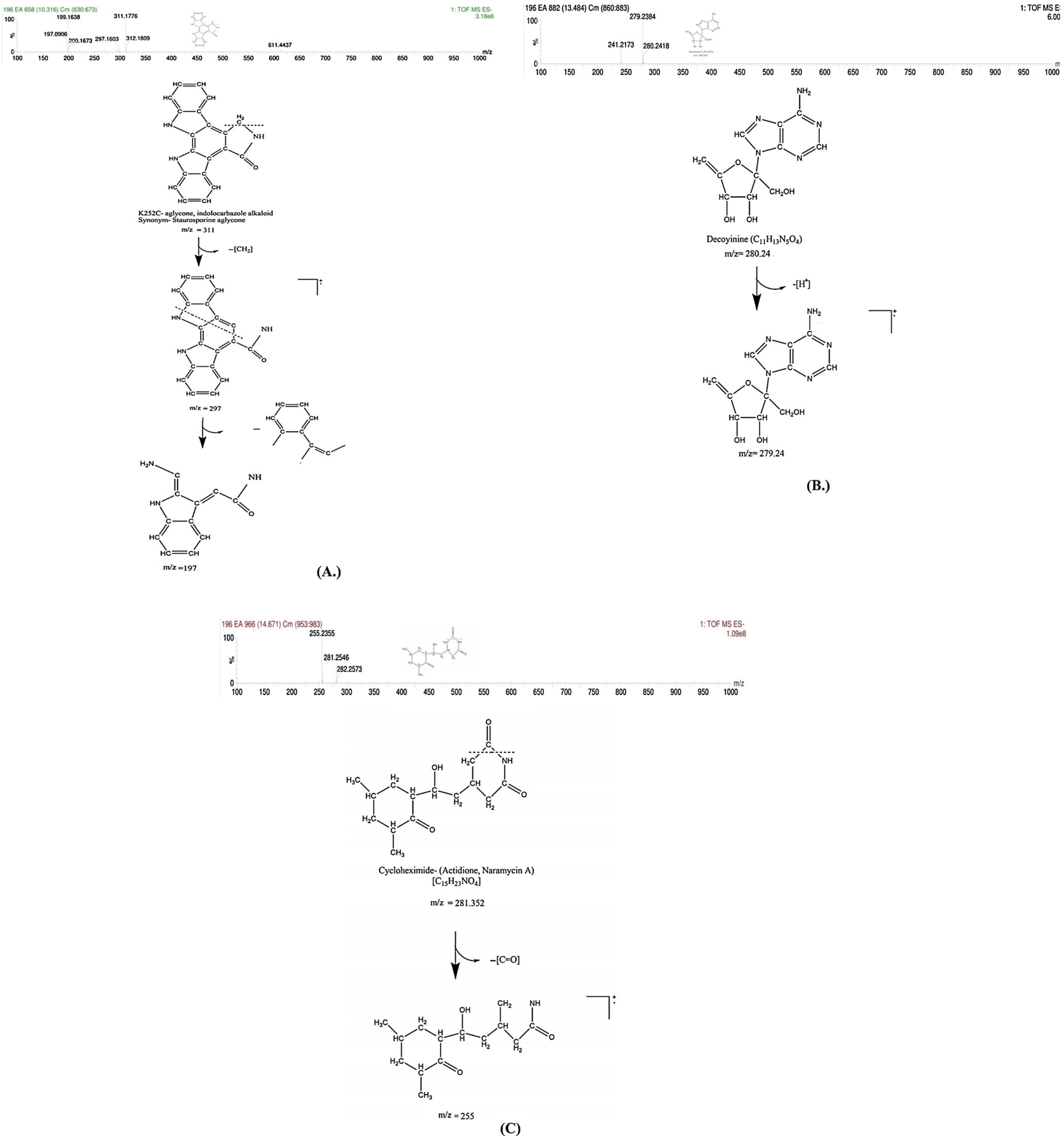


Fig. 3. ESI-MS and fragmentation patterns of compounds separated in LC–MS/MS analyses of *Streptomyces* sp. strain 196 crude extract/mixture **(A.)** K-252-aglycone, indolocarbazole alkaloid **(B.)** decoyinine **(C.)** cycloheximide.

2.5. Structural identification of bioactive compounds

2.5.1. LC–MS/MS analysis

Ethyl acetate extract of strain 196 and methanolic extract of strain RI.24 were subjected to LC–MS/MS analysis using Waters SYNAPT G2 with 2D nano ACQUITY System (Igarashi et al., 2005; Maskey et al., 2006; Solanki et al., 2015).

2.6. Proton Nuclear Magnetic Resonance (^{1}H NMR) of purified bioactive compounds

Bioactive fractions present in crude extracts of strains 196 and RI.24 were detected during bioautography. These fractions were purified and subjected to ^{1}H NMR for their structure elucidation (Igarashi et al., 2005; Maskey et al., 2006; Solanki et al., 2015). ^{1}H NMR was performed using Bruker Ultrashield 500 Plus ™ spectrometer fitted with a 5 mm PABBO probe. $CDCl_3$ (Deuterated chloroform) was taken as a solvent and tetramethylsilane was used as internal standard. Chemical shifts

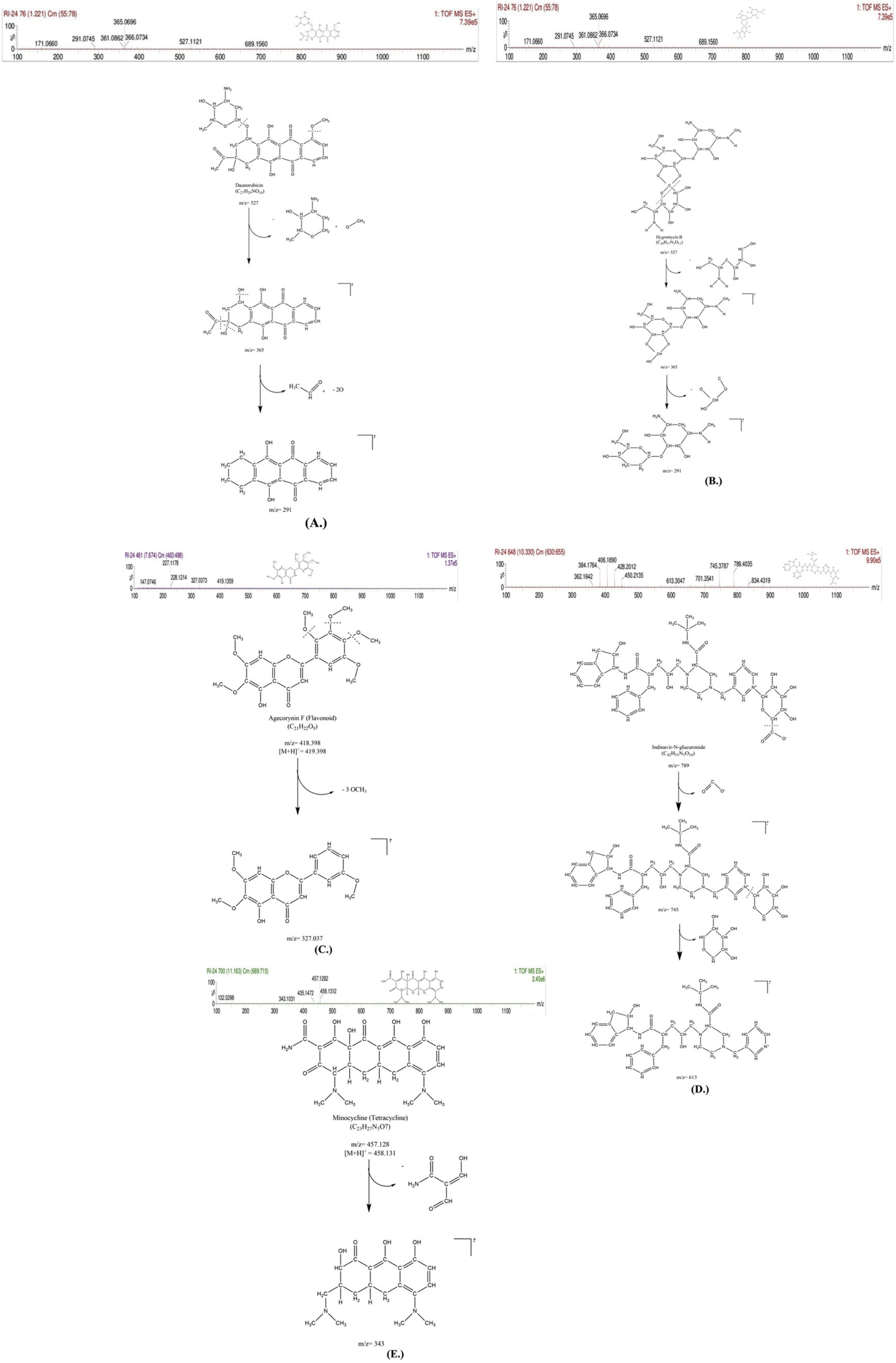


Fig. 4. ESI-MS and fragmentation patterns of compounds separated in LC–MS/MS analysis of *Streptomyces* sp. strain RI.24 crude extract/mixture **(A.)** daunorubicin **(B.)** hygromycin B **(C.)** agecorynin F (flavonoid) **(D.)** indinavir **(E.)** minocycline.

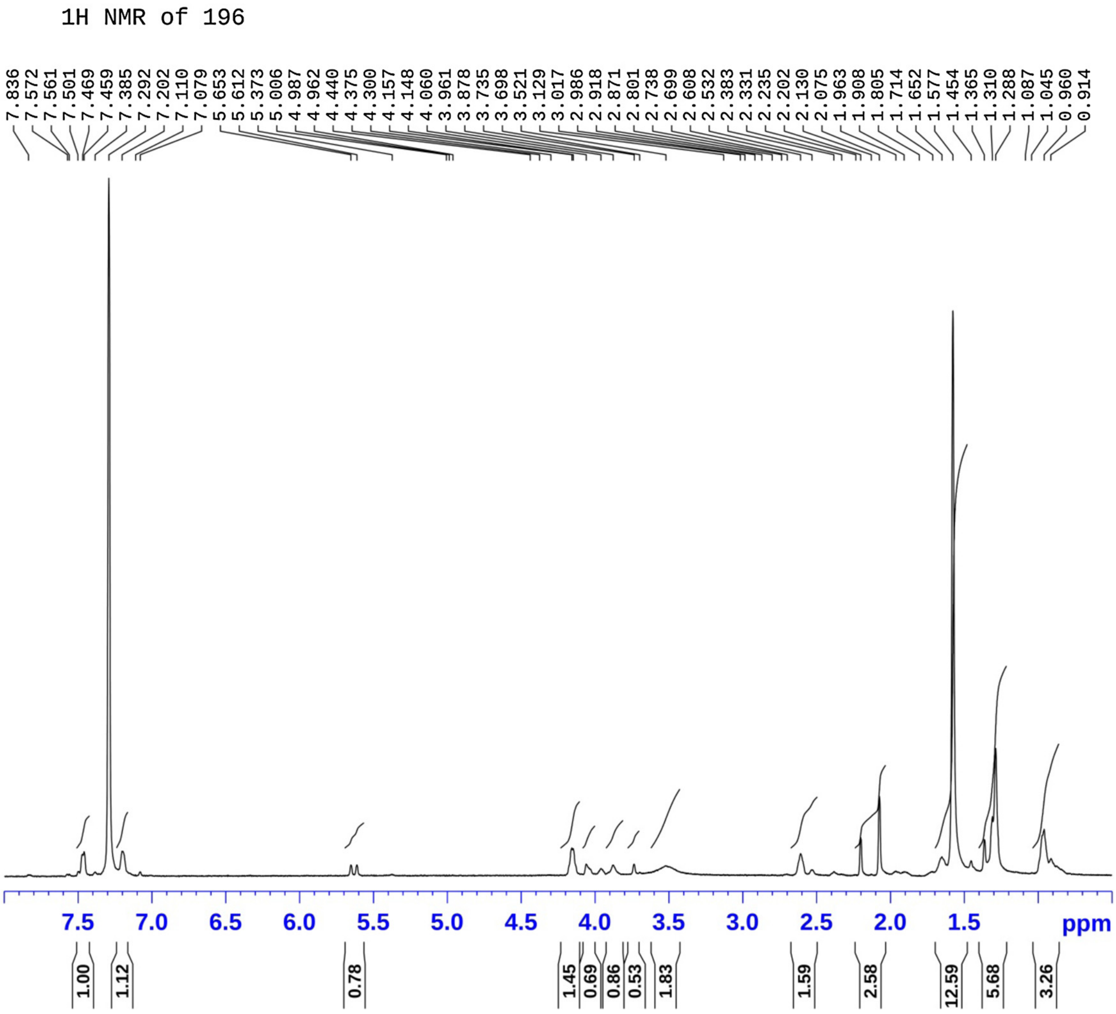


Fig. 5. ^{1}H NMR spectra of purified bioactive compound-1 from *Streptomyces* sp. strain 196.

were recorded in parts per million (ppm, δ) and coupling constants at 500 MHz.

2.7. Polymerase chain reaction (PCR) amplification and sequencing of bioactive compounds encoding biosynthetic genes in Streptomyces sp. 196 and RI.24

2.7.1. Genomic DNA extraction

Genomic DNA was isolated using the small scale method standardized for actinomycetes (Hopwood et al., 1985; Khanna and Solanki, 2012).

2.7.2. PCR amplification

Degenerate PCR primer pair K1F (5′-TSAAGTCSAACATCGGBCA-3′) and M6R (5′- CGCAGGTTSCSGTACCAGTA-3′) was used to amplify ketosynthase and methyl-malonyltransferase domain sequences of PKS-I whereas primers A3F (5′- GCSTACSYSATSTACACSTCSGG-3′) and A7R (5′- SASGTCVCCSGTSCGGTAS -3′) were used for amplification of adenylation domain sequences of NRPS gene (Ayusho-Sacido and Genilloud, 2004). PCR was performed in eppendorf *Mastercycler* gradient. Genomic DNA was used as template for Taq polymerase. Reactions mixture was prepared in a final volume of 100 µl containing: 5 µl of genomic DNA, 1 µl of each primer (Sigma) having 100 mM concentration, 2 µl of dNTP mix (Qiagen) having concentration 10 mM each, 1.3 µl of *Taq* polymerase (Qiagen) having concentration 5U/µl, 10 µl of recommended 10X *Taq* polymerase buffer containing 15 mM $MgCl_2$, 2 µl of DMSO (Himedia) and final volume was make up by 77.7 µl of autoclaved MQ water. Reaction conditions used for amplification are as follows: 5 min at 95 °C and 35 cycles of 30 s at 95 °C, 2 min at 58 °C, 4 min at 72 °C and 10 min at 72 °C. Amplicons were analyzed by 1.2% agarose gel stained with EtBr (ethidium bromide). The amplified bands of desired sizes were purified using Gel Extraction Kit (Qiagen). The purified products were sequenced using high throughput DNA sequencer (Applied Biosystems 3730) with specific set of PKS-I and NRPS primers. Sequences of genes were deposited at NCBI-GenBank database under the accession numbers MK343465, MK343466 and MK355721.

3. Results and discussion

3.1. Extraction

Bioactive compounds were extracted from well grown culture plates of actinomycetes using cold extraction technique. Bioassay guided extraction directed to use ethyl acetate as extracting solvent for the extraction of bioactive compounds from *Streptomyces* sp. strain 196 whereas methanol was used for RI.24. Evaporation of solvents under reduced pressure in a rotary evaporator yields a light yellow colored semi-solid residue of strain 196 and a brown colored residue for strain RI.24. The extraction protocol adopted in the present study was previously followed by many researchers for extraction of secondary metabolites from actinomycetes (Schimana et al., 2000; Solanki et al., 2015; Kapur et al., 2018a).

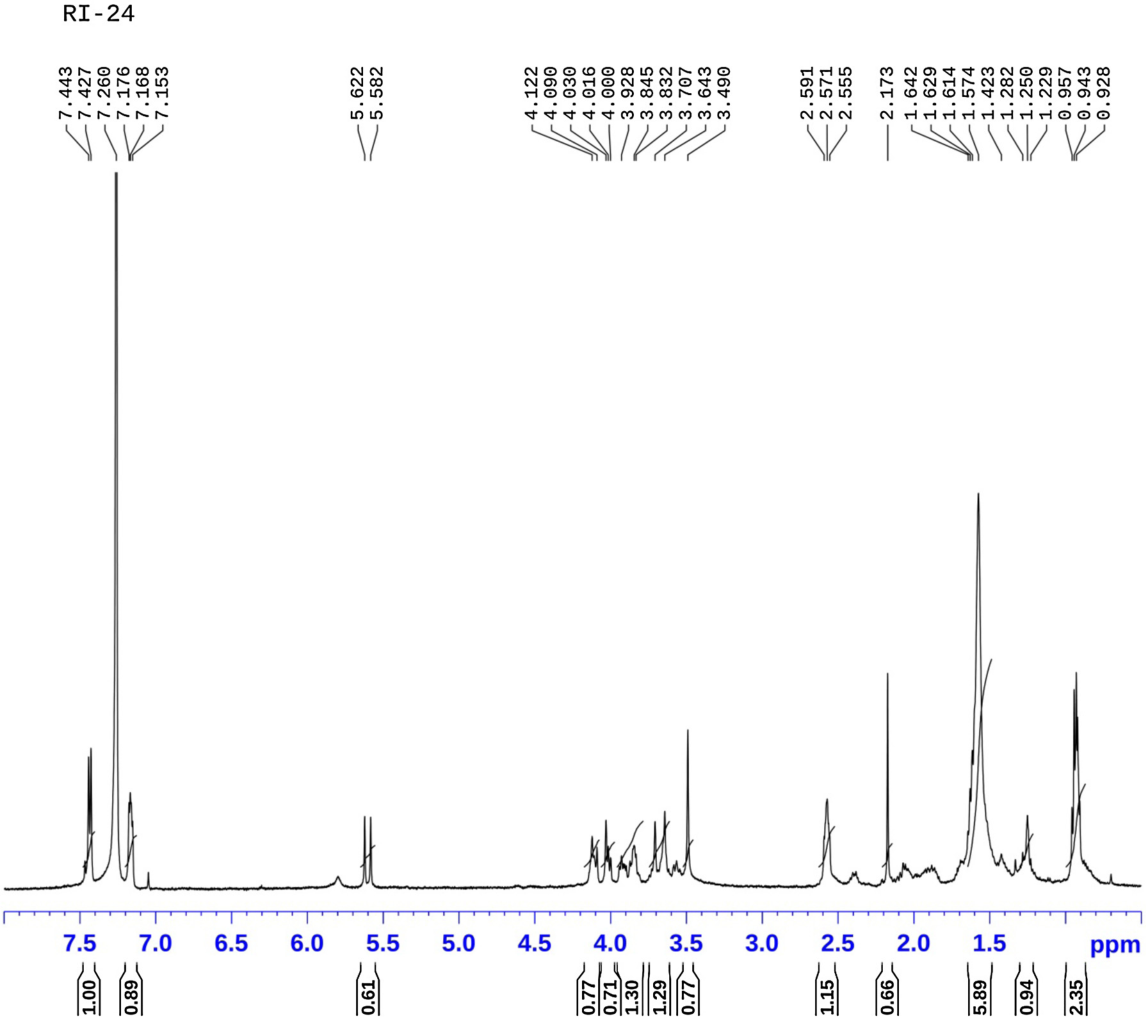
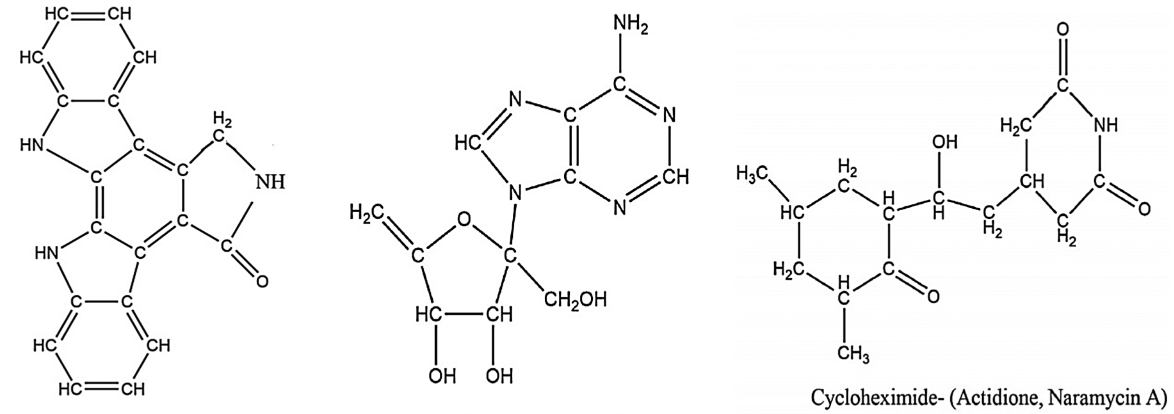


Fig. 6. ^{1}H NMR spectra of purified bioactive compound-2 from *Streptomyces* sp. strain RI.24.



K252C- aglycone, indolocarbazole alkaloid
Synonym- Staurosporine aglycone
m/z = 311

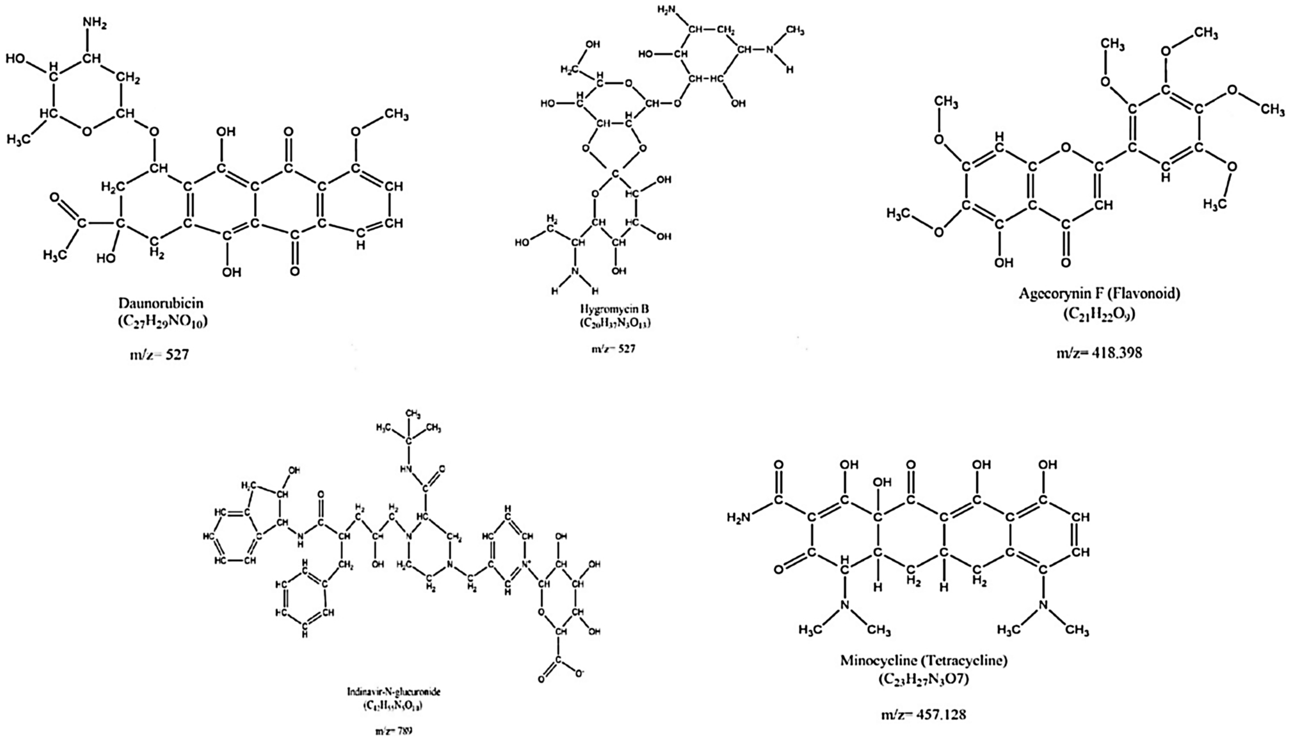
Decoyinine ($C_{11}H_{13}N_5O_4$)
m/z= 280.2425

Cycloheximide- (Actidione, Naramycin A)
[$C_{15}H_{23}NO_4$]
m/z = 281.352

Fig. 7. Chemical structures of compounds identified from LC–MS/MS and ^{1}H NMR analysis of bioactive natural products from *Streptomyces* sp. strain 196.

3.2. Determination of Minimum inhibitory concentration (MIC)

During our previous studies, strains 196 and RI.24 were screened for their activity against *Bacillus cereus* MTCC 430, *Fusarium oxysporum* MTCC 284, *Candida albicans* MTCC227, *Escherichia coli* MTCC 443 and *Staphylococcus aureus* MTCC 740. Strain 196 showed maximum activity against *Bacillus cereus* MTCC 430 whereas RI.24 against *Staphylococcus aureus* MTCC 740 (Solanki, 2013; Kapur et al., 2018a). In present course of our study, MIC determination of strain 196 and RI.24 extracts along with positive controls chloramphenicol and ampicillin was employed by microdilution method to evaluate their bactericidal/bateriostatic properties. The concentration effect of extracts from 196 and RI.24 was



Daunorubicin ($C_{27}H_{29}NO_{10}$) m/z= 527

Hygromycin B ($C_{20}H_{37}N_3O_{13}$) m/z= 527

Agecorynin F (Flavonoid) ($C_{21}H_{22}O_9$) m/z= 418.398

Indinavir-N-glucuronide ($C_{42}H_{55}N_5O_{10}$) m/z= 789

Minocycline (Tetracycline) ($C_{23}H_{27}N_3O7$) m/z= 457.128

Fig. 8. Chemical structures of compounds identified from LC–MS/MS and ^{1}H NMR analysis of bioactive natural products from *Streptomyces* sp. strain RI.24.

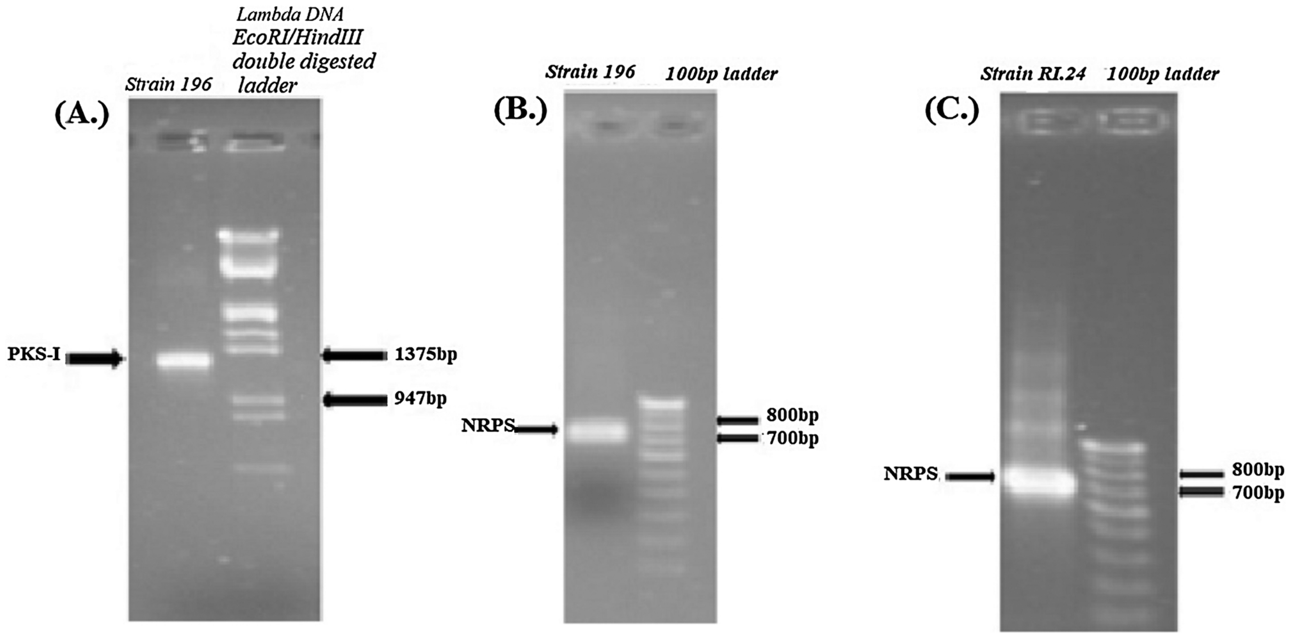


Fig. 9. Agarose gel electrophoresis of PCR amplified products of *Streptomyces* sp. strain 196 and strain RI.24 **(A.)** Amplification of strain 196 PKS-I using K1F/M6R specific primers **(B.)** amplification of strain 196 NRPS using A3F/A7R specific primers **(C.)** amplification of strain RI.24 NRPS using A3F/A7R specific primer.

examined against *Bacillus cereus* and *Staphylococcus aureus* respectively (Fig. 1). Results of our present investigation showed that inhibitory effect of strain 196 extract started at 0.0075 mg/ml against *Bacillus cereus* while extract of strain RI.24 suppressed growth of *Staphylococcus aureus* at 0.25 mg/ml (Fig. 1(**A.**) (**C.**), Table 1). The results were comparable to the positive controls; chloramphenicol started inhibitory effect at 0.0075 mg/ml against *Bacillus cereus* whereas ampicillin abolished *Staphylococcus aureus* growth at 0.025 mg/ml (Fig. 1(**B.**) (**D.**), Table 1). On the basis of MIC results it was concluded that extracts from the strains under study showed activity nearly in the range of commercial antibiotics.

3.3. Bioautography and purification of bioactive compounds

Myriad of compounds are produced by actinomycetes but the ones that are biologically active needs to be identified and such identification can be done by performing bioautography. In current study immersion bioautography was performed for both strain 196 and RI.24 extracts. In the result, strain 196 has contained a single bioactive compound (Compound-1) with Rf value 0.46 (Fig. 2(**A.**)) whereas strain

RI.24 also showed single bioactive compound (Compound-2) with Rf value 0.54 (Fig. 2(**B.**)). Furthermore, the identified bioactive compounds were isolated and purified using chromatographic methods. The purified compounds were dried and obtained in the form of powder which was further used for ^{1}H NMR studies. Bioactive compound identification and purification protocols adopted in present investigation were successfully used by many researchers during their studies (Selvameenal et al., 2009; Solanki et al., 2015).

3.4. Structural identification of bioactive compounds

3.4.1. LC–MS/MS analysis

Crude extracts/mixtures of natural products from *Streptomyces* sp. strains 196 and RI.24 were subjected to LC–MS/MS analysis for the identification and characterization of total bioactive compounds produced by these strains. LC–MS/MS analysis at positive mode of ethyl acetate extract of *Streptomyces* sp. strain 196 has been characterized on the basis of their molecular ion *(m/z)* and fragment ion peaks (Table 2). The total ion chromatogram (TIC) of ethyl acetate extract exhibits total six major peaks excluding several minor peaks.

The LC peak observed at Rt 10.32 min interval corresponded to the molecular ion peak at *m/z 311* (Fig. 3(**A.**)). The molecular structure was further confirmed from their characteristic fragment ion peak at *m/z*297 which originated due to loss of hydrocarbon ($-CH_2$) of 14amu. Further fragmentation of molecular ion peak resulted prominent daughter ion peak at *m/z*197 due to loss of disubstituted phenyl ring of 100amu. The molecular ion peak at *m/z*311 and characteristic fragmentation pattern suggested structural similarities with K-252-C-aglycone, indolcarbazole alkaloid also known as staurosporine aglycone (Fig. 3(**A.**)) (Nakano and Omura, 2009; Rajeshwaran and Mohanakrishnan, 2011; Fox et al., 2016). Similarly another LC peak observed at 13.48 min interval corresponded *m/z 280.24 (*Fig. 3(**B.**)). The molecular structure was elucidated by its major fragment ion peak at *m/z*279.24 which originated due to loss of hydrogen ion (-H$^+$) (Fig. 3(**B.**)). **Thus the LC**–MS/MS spectrum and fragmentation pattern suggested the structure as decoyinine or angustmycin A (Yuntsen, 1958; Guarino, 1967). The molecular ion peak observed at Rt 14.57 min interval in LC corresponded *m/z 281.35 (*Fig. 3(**C.**)). The molecule parent structure was elucidated by their fragment ion peak at *m/z*255 which originated due to loss of carbonyl moiety ($-CO$) of 28amu. The total spectrum of fragmentation pattern in LC–MS/MS suggested the structure of the peak with *m/z*281.35 as cycloheximide- (Actidione, Naramycin A) (Fig. 3(**C.**)) (Poetsch et al., 2010).

LC–MS/MS analysis of methanolic extract of *Streptomyces* sp. strain RI.24 has also been characterized on the basis of their molecular ion *(m/z)* and fragment ion peaks (Table 2). The total ion chromatogram (TIC) of methanolic extract in positive ionization mode exhibits total fourteen major peaks excluding several minor peaks.

The structure of the compound was elucidated as daunorubicin or daunomycin which showed characteristic molecular ion peak at *m/z*527.11. The structure was also confirmed by its fragmentation pattern and literature reports (Di Marco et al., 1981; Beijnen et al., 1987). Further fragmentation of the molecular ion peak at 527.112 gave daughter ion peaks at *m/z 365.06 and m/z*291.07 due to loss of substituted pyran ring, methoxy group, acetyl and hydroxyl moieties (Fig. 4(**A.**)). Hygromycin B is also identified in the methanolic extract upon LC—MS/MS analysis, which showed characteristic molecular ion peak at *m/z*527.11. The structure was further confirmed by its fragmentation pattern and literature reports (Neuss et al., 1970). With the exposure of energy, the molecular ion peak (527.11) fragmented to daughter ions. Removal of cyclohexyl ring along with side amino chain resulted fragment ion peak at *m/z*365.06amu as a base peak. Further fragmentation results formation of daughter ion peak at *m/z*291.07 due to loss of cyclic ether moieties (Fig. 4(**B.**)). Interestingly four metabolites belongs to class flavonoid namely, agecorynin F, agecorynin G, gardenin A and agehoustin E have been identified based on their mass spectrometric analysis. Highly advanced LC—MS/MS analysis help us to differentiate the actual metabolite based on their accurate molecular mass and fragmentation pattern. LC—MS analysis showed a LC peak at 7.67 min with the MS distinct molecular ion peak at *m/z*418.13, corresponding to agecorynin F (Fig. 4(**C.**)). The molecular formula deduced as $C_{21}H_{22}O_9$. The accurate molecular ion mass was matching with the literature molecular mass value below the acceptable error mass value of 3 ppm. Structural similarities were exactly found to flavonoid groups of compounds (Harborne and Wiliams, 1995; Taechowisan et al., 2014). The molecular ion peak was observed at *m/z*419.13. Further fragmentation of the molecular ion peak at 419.13, resulted daughter ion peak at *m/z*327.03, originated due to loss of three methoxy group ($-OCH_3$). A sharp LC peak at 10.33 min interval and characterized as indinavir-*N*-glucuronide from its fragmentation pattern, molecular ion and daughter ion peaks (Fig. 4(**D.**)). The molecular formula was calculated as $C_{42}H_{55}N_5O_{10}$. Molecular ion peak was showed *m/z*789.39 which was further fragmented to form daughter ion peak at *m/z*745 after removal of acid moiety ($-COO$) of 43amu. Another daughter ion was observed as *m/z*613, originated after sequential removal of trihydroxy pyran ring. LC—MS/MS characterization and comparison with the reported literature survey helped to identify the compound as indinavir-*N*-glucuronide (Balani et al., 1996; Gangl et al., 2002). Another compound was identified as minocycline, which is tetracycline type of antibiotic with having molecular formula $C_{23}H_{27}N_3O_7$. It exhibited sharp molecular ion peak at *m/z*457.13. It was eluted from the C-18 column at Rt 10.83 min. (Fig. 4(**E.**)). The molecular ion peak (M$^+$) at *m/z*457.13 was further fragmented to form daughter ion peaks at *m/z*343 after sequential removal of carbonyl ($-CO$), hydroxyl ($-OH$) and amide ($-CONH$) group (Fig. 4(**E.**)). Comparison with the literature reports and LC—MS/MS analysis help us to identify the compound as minocycline (Rodrigues et al., 2014; Tariq et al., 2018).

3.5. Proton nuclear magnetic resonance (^{1}H NMR) of purified bioactive compounds

During bioautography, one compound (compound-1) present in crude extract of strain 196 was found active against *Bacillus cereus* whereas another compound (compound-2) present in crude extract of strain RI.24 was found active against *Staphylococcus aureus*. These compounds were purified by chromatographic methods and subjected to ^{1}H NMR analysis for their structural elucidation. The structure of compound-1, isolated from bioactive fraction of *Streptomyces* sp. strain 196 was elucidated from its ^{1}H NMR spectra (Fig. 5), which showed characteristic hydroxyl proton at δ 5.00–5.61 ppm. The ^{1}H NMR spectra also suggested the presence of heterocyclic protons as multiplate at δ 7.07-7.57 ppm, attributing furan and pyrimidine ring protons. Another sharp peak was observed at δ 7.83 ppm allocated to amino proton. Olifinic protons were observed at δ 5.65 ppm due to de-shielding effects (Fig. 5). So the ^{1}H NMR data further confirmed the structure of the purified bioactive compound-1 as decoyinine (Fig. 7) (Yuntsen, 1958; Guarino et al., 1967) which was also characterized from its LC-MS/MS analysis.

Structure of compound-2, isolated and purified from *Streptomyces* sp. strain RI.24 was partially confirmed as minocycline or tetracycline from its ^{1}H NMR spectra (Figs. 6 and 8) and literature reports (Rodrigues et al., 2014; Tariq et al., 2018). Aromatic proton was assigned to δ 7.26-7.44 ppm as triplate. Dimethyl amino proton was assigned to δ 2.55 and δ 2.59 ppm as singlet with six protons. Sharp peak was observed at δ 2.57 ppm, attributed to N-CH proton. Two hydroxy protons were observed at δ 5.58 to 5.62 ppm as singlet. Besides, other two hydroxyl protons can be assigned to δ 4.0 and 4.12 ppm due to shielding effect. Hydrocarbons aliphatic protons were observed at δ 1.22-1.64 ppm. Though the peak regarding amino proton could not be detected (Fig. 6).

3.6. Polymerase chain reaction (PCR) amplification and sequencing of bioactive compounds encoding biosynthetic genes in Streptomyces sp. 196 and RI.24

3.6.1. PCR amplification

Polyketide synthase (PKS) and Non-ribosomal peptide synthetase (NRPS) are genes which encode multimodular enzyme complexes responsible for production of diverse bioactive compounds in actinobacteria (Hodges et al., 2012; Sharma et al., 2016). Diverse bioactive products obtained from this bacterial group have a broad range of applications in the fields like drug development, veterinary science and agriculture (Cane and Walsh, 1999). During detection of these gene clusters in strain 196 and RI.24 using specific degenerate primers, it was observed that strain 196 contains both PKS-I and NRPS genes whereas strain RI.24 was found to possess only NRPS gene (Fig. 9(**A.)–(C.)**). PCR of PKS-I gene of strain 196 yield a band of size ~1200 bp using K1F/M6R primer set which target PKS-I methyl-malonyl-CoA transferase modules and ketosynthase (KS) (Fig. 9(**A.)**). Resulted gene sequence was submitted at NCBI-GenBank database under accession number MK343465. The NRPS amplicon of strain 196 was found to be ~700bp size using A3F/A7R primer set which target NRPS adenylation domain sequence (Fig. 9(**B.)**). Obtained sequence was submitted at NCBI-GenBank database under accession number MK343466. In strain RI.24, no amplification was observed during PCR using PKS-I specific set of primer whereas PCR amplification of NRPS gene resulted in a band of size ~700bp using A3F/A7R primer set which target NRPS adenylation domain sequence (Fig. 9(**C.)**). This gene sequence was submitted at NCBI-GenBank database under accession number MK355721.

On the basis of PCR detection of PKS-I and NRPS genes in strain 196 and RI.24, it may be suggested that these genes are responsible for bioactive compound production. The results are comparable with the studies of researchers Ding et al. (2013), Passari et al. (2015), Samak et al. (2018) and Sharma et al. (2016), where it was observed that actinobacteria showing antimicrobial activity were found positive for the presence of one or both of these two gene clusters. Sharma et al. (2016) also reported in their study that antimicrobial activity of *Nocardia* sp. PB-52 is due to the engagement of PKS and NRPS genes.

4. Conclusion

In conclusion, present investigation demonstrate that *Streptomyces* sp. strain 196 and *Streptomyces* sp. strain RI.24 isolated from soil samples of two distinct habitats are novel sources of bioactive molecules. The study revealed that actinomycetes from diverse ecological habitats possess huge potential of bioactive compounds production. The potential bioactive compounds detected in present study are reported in the literature for having broad-spectrum biological activities such as antibacterial, antifungal, antitumor, antihypertensive etc. Thus, the results of this study have set the background for further exploitation of these highly potent strains for the purpose of drug development/formulation by pharmaceutical industries or scientific community.

Declaration of Competing Interest

The authors declare no competing interests.

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