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CHEMICAL PROFILING OF ANTIMICROBIAL COMPOUNDS FROM *STREPTOMYCES* SP.

Renu Solanki[a], Aditi Kundu[b], Payal Das[a] and Monisha Khanna[c*]

[a]Deen Dayal Upadhyaya College, University of Delhi, Shivaji Marg, Karampura, New Delhi 110 015.
[b]Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi 110 012.
[c]Acharya Narendra Dev College, University of Delhi, Govindpuri, Kalkaji, New Delhi 110 019.

***Correspondence for Author: Dr. Monisha Khanna**
Acharya Narendra Dev College, University of Delhi, Govindpuri, Kalkaji, New Delhi 110 019.

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ABSTRACT

Natural products from micro-organisms are an important source of antibiotics against drug resistant pathogenic microbes. Actinomycete genera especially *Streptomyces* are well known for production of antimicrobial compounds. In the present study, *Streptomyces sp.* isolated from HCH dump site, Lucknow was subjected to antimicrobial analyses. Structure elucidation of purified bioactive compounds was done by mass spectrometry (MS) and nuclear magnetic resonance (NMR) after their identification by bioautography. It was revealed that compound structures showed partial resemblance to known antibiotic groups indicating tentative presence of novel chemical moieties. One of the compounds from isolate L3.46 extracted in ethyl acetate had molecular weight of 681g/mol similar to mycinamicin III, a macrolide antibiotic. Mass fragmentation pattern of L3.46 compound showed partial resemblance to mycinamicin III. A second bioactive compound isolated from L3.46 extracted in methanol was found to have molecular weight of 771 g/mol. Its core structure showed resemblance to xantholipin, belonging to xanthone group of antibiotics. On the basis of structural studies, compounds were identified as Mycinamicin III and Xantholipin derivatives respectively.

KEYWORDS: Actinomycetes, Antimicrobial compounds, Structure elucidation, Mass spectrometry (MS), Nuclear magnetic resonance (NMR)

INTRODUCTION

Pathogenic microbes belonging to genera *Clostridium*, *Enterococcus*, *Mycobacterium, Neisseria, Pseudomonas*, *Salmonella, Staphylococcus* and *Streptococcus* are becoming resistant to existing antibiotics. These pathogenic microbes produce enzymes which can degrade or inactivate the target drug. Alternatively, reduced drug permeability, target site alteration and decreased intracellular accumulation due to antibiotic efflux.[1-7] Due to emergence of multidrug resistant (MDR) microbes, there is an urgent need to discover new antibiotics with unique modes of action. Effective treatments of the infection caused by MDR organisms require new chemical structures which can escape the developed resistance mechanisms.[8-10] Consequently, the demand for discovery and development of new and effective antimicrobial compounds is increasing to combat resistant pathogens.

Natural products from micro-organisms are an important source of antibiotics. Among microbes, actinomycetes are well known for antibiotic production.[11] Actinomycetes are Gram positive bacteria characterized by branching filaments. Their DNA has high GC content and they are widespread in nature. They are well known for production of antimicrobial compounds belonging to different chemical classes including aminoglycoside, ansamycin, anthracycline, β-lactam, glycopeptide, macrolide, naphthoquinone, peptide, xanthone to name a few. They possess a range of biological activities like antitumor, antibacterial, antifungal, antiviral, antidiabetic, antituberculosis, insecticidal and nematodal.[12-14] Actinomycetes genera are responsible for production of majority of the current antimicrobial compounds[.15-19]

Continuous screening of secondary microbial products from bacteria is essential for discovery of novel chemicals which can be developed as new therapeutic agents. Researchers are exploring diverse untapped habitats in an attempt to discover microbes with potential for production of novel chemical moieties.[20-23]

In the present study, *Streptomyces sp.* L3.46 was isolated from HCH dump site, Lucknow and subjected to antimicrobial analyses. Bioactive compounds were extracted using organic solvents of different polarity. Actual bioactive fractions were identified by bioautography. Structure elucidation of purified compounds was done using mass spectrometry and nuclear magnetic resonance (NMR) studies.

MATERIALS AND METHODS

Extraction of antimicrobial compounds

Extraction from culture broth

Actinomycete culture was inoculated in Nutrient medium.[24] Cultures were incubated on shaker for 7 days. Bioactive compounds from cell free culture filtrate were extracted using equal volume of organic solvent. Organic solvent phase was separated from aqueous phase and evaporated in a rotavapor at 40ºC-45ºC and get powdered form of extract.[25-26]

Extraction from culture plates

Actinomycete culture inoculated on YM medium plates were incubated at 28°C for 10 days. Bioactive compounds were extracted directly from culture plates. Agar medium was cut into small pieces and organic solvent was added. After 4-5 hours of shaking, organic solvent was separated from agar media and bacterial culture. Solvent was evaporated to obtain crude extract.[27]

Bioautography

Bioactive compounds were fractionated by thin layer chromatography.[25] Dichloromethane:Methanol (9:1) was used as the mobile solvent system for fractionation of ethyl acetate extracts. Another solvent system i.e. Butanol:Acetic acid:Water (3:1:1) was used for methanolic extracts. Developed chromatograms of culture extracts were dried and observed under UV at 365 nm for identification and marking of fractions. TLC plates were over layered with soft agar medium containing pathogenic culture in log phase (10^5 cfu's/ml) and incubated at 37°C overnight. Next day, thiazolyl blue tetrazolium bromide (MTT tetrazolium) (1mg/ml in distilled water) was spread over pathogen layered TLC plates and incubated at 30°C for 4 hours. Plates were observed for appearance of white inhibition zones (around marked fractions) against purple background to identify bioactive fractions by comparing with another TLC plate on which extracts had been run but not subjected to bioautography and their R_f values were also calculated.[28-29]

Purification of antimicrobial compounds

Crude extracts were fractionated as mentioned above by TLC. Bioactive fractions were identified with the help of bioautography. Bioactive fractions were scraped from TLC plates. Ethyl acetate was added to TLC scrapings from ethyl acetate extract and methanol was added to scrapings from methanolic extract, left for some time so that compound got dissolved in solvent. Silica was removed by centrifugation. Partially purified fractions were again separated by TLC and entire process was repeated till one clear band was observed. Purified compounds were further subjected to MS and NMR studies for structure elucidation.[29]

Structural Analyses of Compounds

Mass spectrophometric analyses

ESI-MS data was obtained using SYNAPT G2 High Definition Mass Spectrometry (HDMS) connected to a capillary (Waters Zspray^{TM} LackSpray) with electrospray ionization (capillary voltage 3.0 kV, cone voltages 69V, source temperature 80°C, desolvation temperature 300°C, desolvation gas flow 475 l/h). All samples were run in positive ion mode with a mass range of 50-2000.[30-31]

Nuclear Magnetic Resonance

NMR spectra were recorded with a Bruker Ultrashield 500 Plus TM spectrometer fitted with a 5mm PABBO probe using deuterated chloroform or methanol as solvent and tetramethylsilane was taken as the internal standard. The chemical shifts were recorded in parts per million (ppm, δ) and the coupling constants at 500 mHertz (MHz). Details of the experimental conditions were as follows: Operating temperature 28° C; Proton spectra recorded at 64K (F2), AQ 3.17 seconds, SW 20.66 ppm, D1 1 second, NS 128.[30-31]

RESULTS

During fermentation process, large numbers of secondary metabolites are produced by bacteria but actual fraction responsible for antimicrobial activity needs to be recognized. This can be achieved by bioautography. Fractionated metabolites on TLC plates are subjected to immersion bioautography which is considered as an efficient method for detection of fractions with actual antimicrobial activity and to determine their R_f values (Table 1).

In L3.46 ethyl acetate extract, three fractions were found bioactive against *Bacillus cereus*. Among these one fraction was also active against *Fusarium oxysporum*. In L3.46 methanolic extract, one fraction was found bioactive against *Fusarium oxysporum* and *Candida albicans*.

Bioactive fractions in each extract were purified by 2-3 rounds of TLC followed by confirmation of activity by bioautography.

In L3.46 ethyl acetate culture extract three bands having R_f values of 0.60, 0.56 and 0.45 showed activity but purple colored fraction with R_f 0.45 was purified for structural studies. On the other hand, methanolic extract of L3.46 culture showed one single bioactive fraction having R_f value of 0.64 was active against both *Fusarium oxysporum* and *Candida albicans*, that fraction was purified from rest of the fractions from TLC plates for further analyses.

Table 1: R_f values of bioactive fractions

S.No	Culture extracts	Solvent system	Active against pathogen	R_f value
1.	L3.46 (ethyl acetate extract, 20 mg/ml)	Dichloromethane:methanol (9:1)	*Bacillus cereus*	0.60 0.56 0.45
2.	L3.46 (ethyl acetate extract, 40 mg/ml)	Dichloromethane:methanol (9:1)	*Fusarium oxysporum*	0.60
3.	L3.46 (methanol extract, 20 mg/ml)	Butanol:acetic acid:water (3:1:1)	*Candida albicans*	0.64
4.	L3.46 (methanol extract, 20 mg/ml)	Butanol:acetic acid:water (3:1:1)	*Fusarium oxysporum*	0.64

Compound L3.46 (1)

An oily compound (R_f: 0.45, purple) was isolated from ethyl acetate extract of actinomycete L3.46 after purification by repeated rounds of TLC using dichloromethane: methanol (9:1) solvent system. ^{1}H NMR ($CDCl_3$, 500MHz) spectra of compound L3.46 (1) showed the presence of three hydroxy, methoxy and N-dimethyl groups. NMR spectrum of these compounds exhibited characteristic alkene proton peaks. Several broad peaks were observed at upfield indicating proton corresponding to alkane side chains and cyclohexane moieties. Proton NMR data also indicated the presence of aromatic moiety. Mass spectrum of the compound L3.46 (1) exhibited molecular ion peak at m/z 681 (Figure 1), indicating molecular formula $C_{36}H_{59}NO_{11}$ which was similar to that of mycinamicin III, belonging to macrolide class of antibiotics. ESI-MS/MS of the molecular ion peak (m/z 681) resulted in its fragmentation at m/z 316 due to loss of both the pyran moieties including side chains (Figure 2).

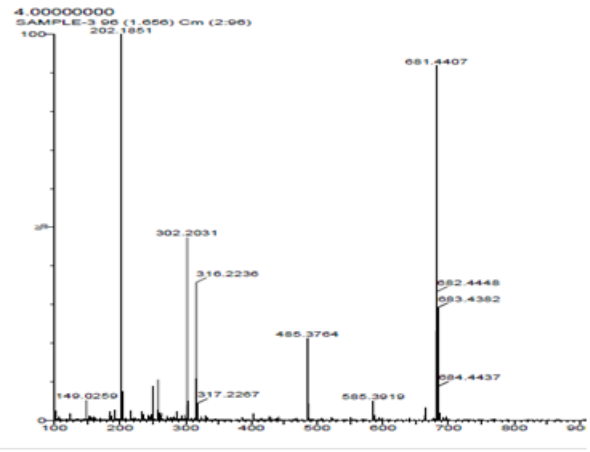

Figure 1: ESI-MS spectra of compound L3.46 (1)

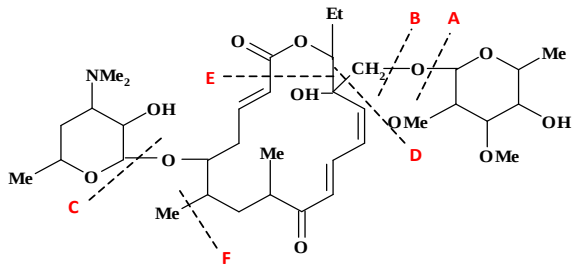

Figure 2: L3.46 (1) compound mass spectrum generation

Further two fragment ion peaks at m/z 302 and 202 were observed due to loss of methyl group and furan moiety, respectively. The structure of compound L3.46 (1) was tentatively confirmed as a mycinamicin isomer on the basis of ESI-MS spectra. Consequently, L3.46 (1) compound may be a mycinamicin III derivative.

Compound L3.46 (2)

An oily L3.46 (2) compound (R_f: 0.64, yellow) was isolated from methanol extract of actinomycete L3.46 after purification by TLC using butanol:acetic acid:water (3:1:1) solvent system. ^{1}H NMR (CD_3OD, 500MHz) spectrum of compound L3.46 (2) showed the presence of long hydrocarbon side chains and cyclohexane ring protons at δ 1.7-2.90. Protons at δ 5.6-5.8 were attributed to presence of alkene moiety. Presence of hydroxyl moiety was confirmed from splitting pattern and chemical shift value δ 3.49. Methoxy and aromatic protons were attributed to δ 3.4-3.8 and 6.45-7.9 respectively. Mass spectrum of the compound L3.46 (2) exhibited molecular ion peak at m/z 771 (Figure 3).

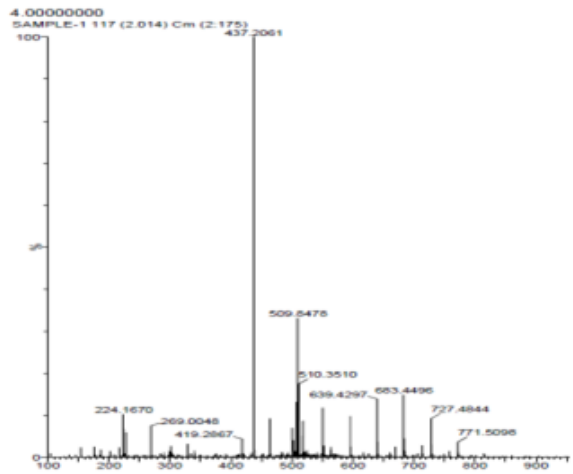

Figure 3: ESI-MS spectra of compound L3.46 (2)

Core structure was similar to that of xantholipin which belongs to xanthone group of antibiotics. On the basis of NMR data, it showed similarity to xantholipin in its core structure but differs from it in possessing extra side chains identified from mass fragmentation pattern. ESI-MS of the molecular ion peak (m/z 771) resulted in fragment ion peaks at m/z 727, 683, 639, 510 and 437 due to loss of $-COCH_3$, $-CH_2CH_2CH_3$, $-COCH_3$, $-(CH_2)_8-CH_3$ and –OH, $-COCH_3$ - CH_3, respectively (Figure 4).

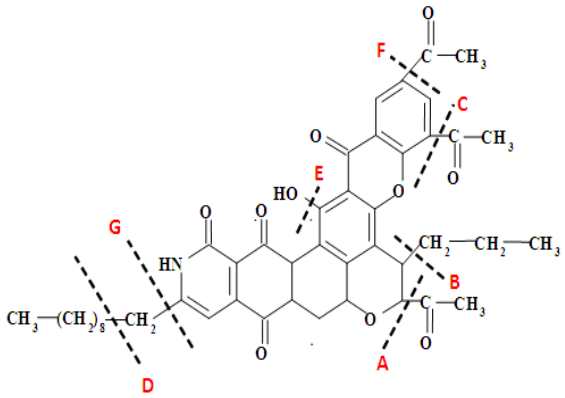

Figure 4: L3.46 (2) compound mass spectrum generation

DISCUSSION

In the present work, possible structures in the culture extracts of actinomycetes have been identified. Bioautography was used to identify the actual bioactive fraction(s). Immersion bioautography was used for localization and identification of bioactive zones after staining with 3-{4,5-dimethylthiazol-2-yl}-2,5-diphenyltetrazolium bromide (MTT). This salt was used because dehydrogenases of living microorganism convert tetrazolium salts to intensely colored formazan. Therefore, the point at which growth of microorganism had been inhibited due to presence of antimicrobials could be marked as cream-white spot against purple background.

It has been found that ethyl acetate extract of L3.46 possessed more than one bioactive fraction active against single sensitive strain whereas methanolic extract had a single bioactive fraction responsible for activity against more than one sensitive strain. Bioactive constituents from ethyl acetate and methanolic extracts of L3.46 were isolated, purified and subjected to ^{1}H NMR and mass spectrometry for structure elucidation.

Compound L3.46 (1) isolated from L3.46 ethyl acetate had a molecular weight of 681 g/mol which is similar to mycinamicin III, belonging to the macrolide antibiotic class. Mass fragmentation pattern indicated structural similarity with mycinamicin III peaks. Consequently, L3.46 (1) compound may be a mycinamicin derivative.

Structure activity relationship revealed that the aglycone functional group (Figure 5) of L3.46 (1) compound imparted antibacterial activity against *Bacillus cereus.*[32]

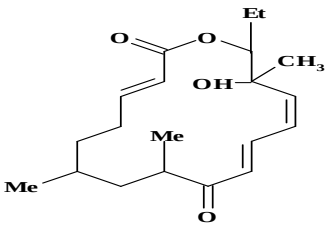

Figure 5: Bioactive moiety of compound L3.46 (1) (Aglycone)

Compound L3.46 (2) isolated from methanolic extract of L3.46 possessed a molecular weight of 771 g/mol. On the basis of NMR data, it showed similarity to xantholipin in its core structure but differs from it in possessing extra side chains and the structure could be interpreted on the basis of mass fragmentation pattern. Addition of these extra hydrocarbon and methoxy side chains attributed to difference in molecular weight, physical properties as well as biological activities of compound L3.46 (2) compared to xantholipin. Xantholipin belongs to xanthone group of compounds and isolated from a *Streptomyces* sp.[33] Actinoplanones A and B, Sch 56036, Sch 42137, albofungin, cervinomycins, simaomicins are other xanthone antibiotics that have been reported from *Streptomyces*, *Actinoplanes* and *Actinomadura.*[34-40] Structure activity relationship of the compound was studied and suggested that presence of 1,4 dioxygenated xanthone moiety (Figure 6) was responsible for bioactivity against *Candida albicans* and *Fusarium oxysporum.*[41] In addition, presence of ketonic functional group attached with ring also imparted antifungal activity to the L3.46 (2) compound (Figure 6).

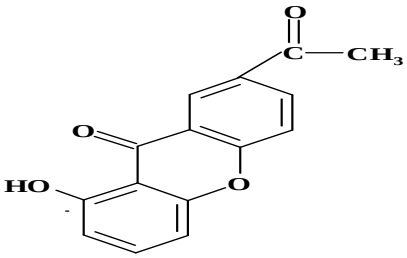

Figure 6: Bioactive moiety of compound L3.46 (2) (Xanthone group and ketonic ring moiety)

CONCLUSIONS

In the present study, structure elucidation of culture extracts from actinomycetes belonging to genus *Streptomyces* revealed moeities which showed partial resemblance to known antibiotics indicating the presence of novel chemical moieties in these extracts. The antimicrobial compounds have been identified as Mycinamicin III and Xantholipin derivatives respectively. Furthermore, these compounds belonged to diverse chemical groups including macrolides and xanthones.

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