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PRODUCTION OF ANTIMICROBIAL METABOLITES BY *STREPTOMYCES PUNICEUS* LC13

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ABSTRACT

Objective: Actinomycetes are a source of wide variety of secondary metabolite producers in drug discovery programs, accounting for approximately 30% of the total microorganisms in the soil rhizosphere. *Streptomycetes puniceus* LC13 strain was isolated from rhizosphere soil of *Lantana camara*, Jabalpur (M.P.), India and it was produce number of bioactive metabolite which was capable to discover novel drug formulation. **Methods:** The compounds were purified by solvent extraction, silica gel column chromatography (60-120 mesh) and Thin layer chromatography (TLC). The structures of the compounds were elucidated by Gas Chromatography and Mass Spectroscopy (GC-MS). **Results:** The results indicated that the *Streptomyces puniceus* LC13 produced seven peaks which have antimicrobial activity with R_f valve 0.49. **Conclusion:** It was concluded that rhizosphere soil is a promising source of secondary metabolites with highest antimicrobial activity.

KEYWORDS: *Streptomycetes puniceus, Lantana camara,* GC-MS, antimicrobial activity.

INTRODUCTION

Soil was a diverse habitat for growth of microorganisms and its produce a spacious range of "secondary metabolites" during later stages of growth under laboratory conditions.[1] Antimicrobial compounds are widely considered to play significant roles in microbial ecology, for instance through chemical warfare among bacteria or as weapons in a prey-predator interactions.[2] The resistance of numerous pathogenic bacteria and fungi to commonly used bioactive secondary metabolites is presently an urgent focus of research and producing new antibiotics that have a novel mechanism of action or are structurally distinct

from those currently in usage is one strategy to battle the spread of existing resistance mechanisms.[3-4] Actinomycetes have high G+C content in their DNA (69-73%), which is phylogenetically related from the evidence of 16S ribosomal cataloguing and DNA: rRNA pairing studies. They stand out as a unique group of prokaryotic organisms in two respects; the diversity of their morphology and their metabolic products.[5] In the present study, *Streptomycetes puniceus* LC13 was isolated from rhizosphere soil of *Lantana camara*, Jabalpur (M.P.), India and subjected to antimicrobial analyses against five pathogenic bacteria, five yeast and three fungi. Bioactive compounds were extracted using organic solvents of different polarity. These compounds were purified by column chromatography and Thin layer chromatography (TLC). The TLC products were tested against antimicrobial activity by agar well diffusion technique. Recognition of partially purified compounds was performed by Gas Chromatography and Mass Spectroscopy (GC-MS). For knowing the specific compound responsible for antimicrobial property which was applied in novel drug formulation.

MATERIALSANDMETHOD

Testorganisms

The antimicrobial activity of isolates were assayed using bacterial cultures of *Staphylococcus aureus* (MTCC 7443), *Escherichia coli* (MTCC 77), *Pseudomonas aeruginosa* (MTCC 741), *Salmonella enteric* (MTCC 3218) and *Micrococcus luteus* (MTCC 4300), also the fungal cultures of *Aspergillus niger* (MTCC 404), *Curvularia lunata* (MTCC 2030) and *Fusarium solani* (MTCC 3004) and yeast *i.e. Candida albicans* (MTCC 1637). All these clinical isolates were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The yeast cultures of *Candida parapsilosis*, *C. krusei*, *C. glabrata*, and *Cryptococcus neoformans* were procured from Medical Mycology Laboratory, Rani Durgavati Vishwavidalaya (R.D.V.V.), Jabalpur, M. P. The bacterial cultures were maintained at 37±2^{o}C on Nutrient agar media (Hi-media) slants while the fungal and yeast cultures were maintained at 28±2^{o}C on Saboraud's dextrose agar (Hi-media) slants. For routine transfer, about one loop of inoculum was streaked on the respective slant and store in refrigerator at 4^{o}C for further use.

Extraction of cell free supernatant from *Streptomycetes puniceus* LC13

A loopful culture of *Streptomycetes puniceus* LC13 was inoculated into 500 ml of Starch casein broth (Hi-media)[6] and incubated at 28±2^{o}C for 3 d. Bioactive compounds from cell

free supernatant were extracted twice by using equal volume of organic solvents from non-polar to polar for recovery of best antimicrobial product from solvent extraction method[7] Chawala *et al.*, 2011. The mixture was shaken vigorously for 15 min and was kept in undisturbed condition for 15 min to separate the solvent phase from aqueous phase and evaporated at room temperature and the residue (crude antibiotic extract) was stored till further use. Separate aliquots of organic and aqueous extract of isolate were assessed for their inhibitory potential against the test pathogens by Agar well diffusion technique.[8] The bacterial and fungal lawn of each test pathogen was prepared by swabbing the surface of Nutrient Agar Media (NAM) (Hi-media) and Sabouraud Dextrose agar (Hi-media) plates by spore/ culture suspension of each test pathogen and 6 mm wide wells were punched on each bacterial and fungal lawn with the help of sterilized cork borer. Each well on each plate was loaded with 50 µl each of organic and aqueous extract of isolate separately. Then, the plates were kept at 4°C for 30 min for diffusion of the antibiotic, and then they were incubated at 37±2°C for 24 h in case of bacteria and for yeast 28±2°C for 24 h while for mold 28±2ºC for 3-5 days. Thereafter, zone of inhibition around each well was measured and antimicrobial activity index was calculated as (DI – DW) / DW, where DI was the diameter of the inhibition zone including the well in mm, and DW was diameter of the well in mm. Therefore, the extract of isolate, which recorded remarkable antimicrobial activity, was further analyzed for purification of compounds.

Purification of bioactive compound

The crude antibiotic compound was dissolved in methanol (1 mg/ml) and further purified by column chromatography (35 ml x 10 mm) using mobile phase (*i.e*. Ethyl acetate: Methanol: Distilled water in the ratio of 60:35:5 used as a solvent system) and stationary phase material *i.e*. silica gel (60-120 mesh) (Hi-media). All the eluted fractions were assessed for their antimicrobial activity against test organisms by agar-well diffusion technique and the fractions with antimicrobial activity were stored as active fractions. They were further purified by thin layer chromatography (TLC). The dried TLC plates were sprayed with ninhydrin and the activity of each ninhydrin positive band was again testedby agar well diffusion technique.

Gas Chromatography and Mass Spetroscopy (GC – MS) analysis

The bioactive fraction was identified by GC-MS [SHIMADZU QP2010] from Advanced Instrumentation Research Facility (AIRF) Laboratory, Jawaharlal Nehru University, New

Delhi, India. The identification of components were doneby comparison of retention time and fragmentation pattern, as well as with mass spectra in the NIST11and Wiley08 spectral library stored in the computer software (version 1.10 beta, Shimadzu) of the GC-MS. The spectrum of the unknown components was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were studied. The relative percentage of each extract constituent was expressed as percentage with peak area normalization.

Statistical analysis

The data for antimicrobial activity was presented as mean ± standard deviation from three replicates and the analysis was carried out using Statistical Package for Social Sciences software, version 16.0 (SPSS Inc., Chicago, USA). One way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test was performed to compare treatments of antimicrobial activity.

RESULTS

In the present investigation, the number of organic solvents i.e., hexane, n-butanol, chloroform, ethyl acetate and methanol were used according to its increasing polarity and the highest antimicrobial activity was shown only against ethyl acetate (Fig. 1). After complete evaporation oily golden brown residue was obtained. The crude antibiotic compound was partially purified by column chromatography. From this, 10 fractions were collected and it was tested against all selected test organisms, among them three fractions were active against all tested organisms and the highest antimicrobial activity index of the test pathogen was showed by fraction-III (Fig. 2). Therefore for further purification fraction-III was selected.

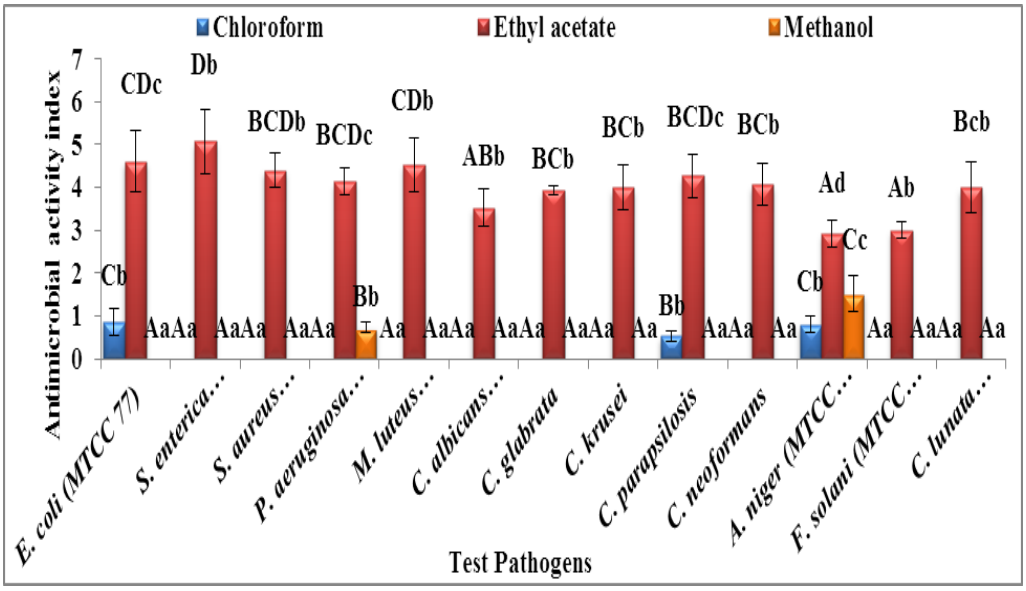
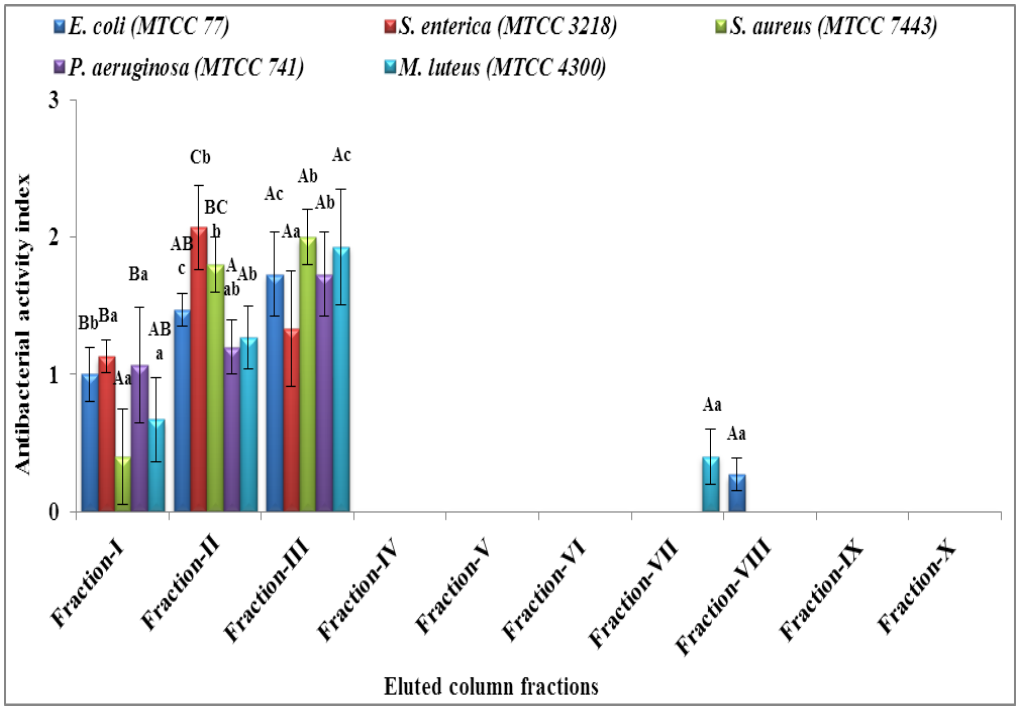
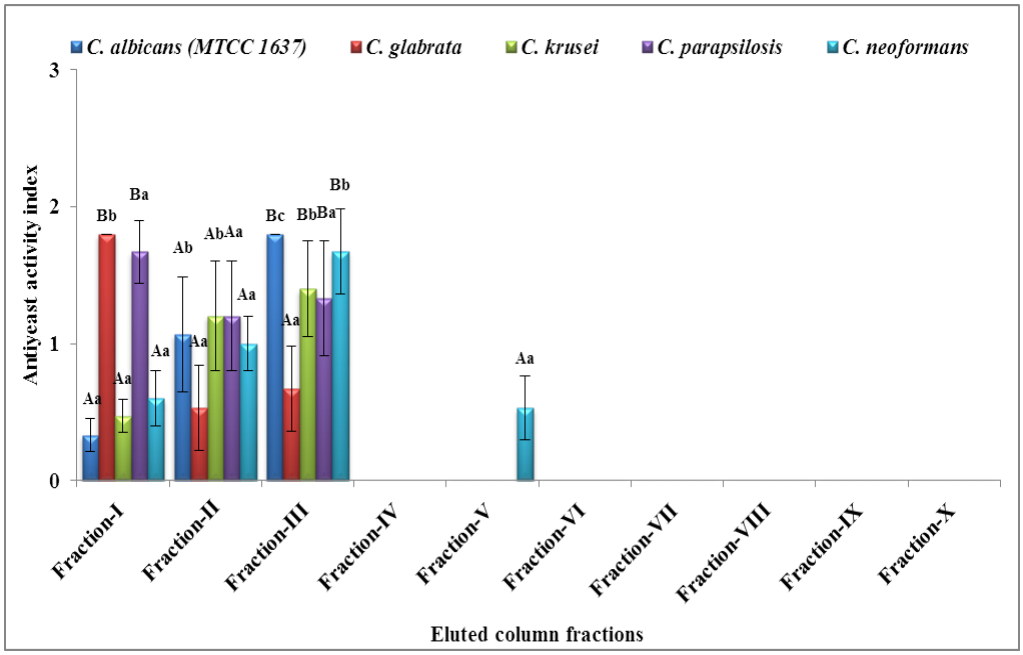


Fig. 1: Antimicrobial activity index of organic extract of the *Streptomycetes puniceus* LC13 against test pathogens.

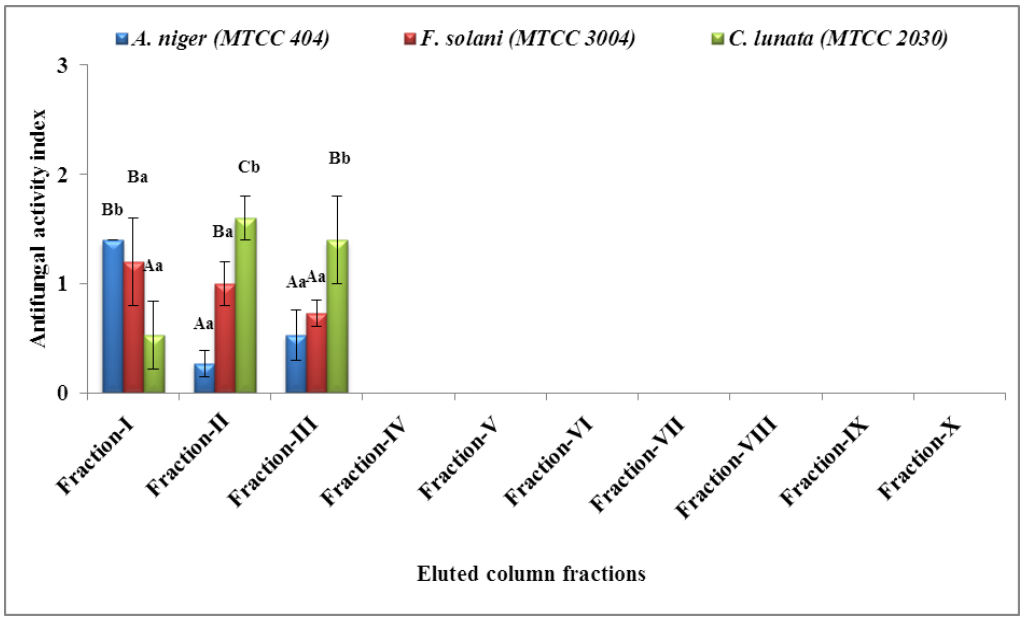
[data are presented as mean ± standard deviation (n=3); The significantly different means (at $p<0.05$) are indicated by different capital alphabets within test pathogens and small alphabets within organic solvents].



(A)



(B)



(C)

Fig. 2: Antimicrobial activity index of the *Streptomycetes puniceus* LC13 using eluted column fractions against test pathogens (A) Antibacterial (B) Antiyeast (C) Antifungal

[data are presented as mean ± standard deviation (n=3); The significantly different means (at $p<0.05$) are indicated by different capital alphabets within test pathogens and small alphabets within eluted column fractions]*0 is not taken in statistical analysis.

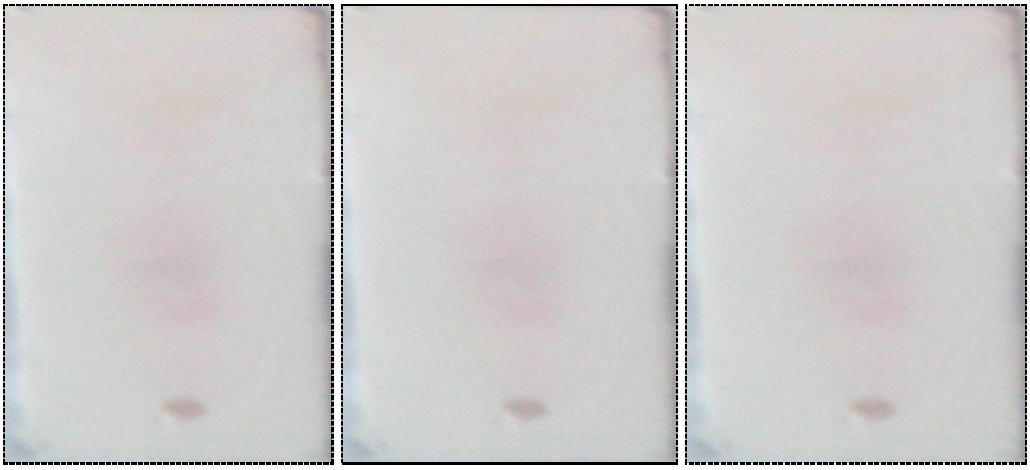


Fig. 3: Thin layer chromatography (TLC) chromatogram of *Streptomycetes puniceus* LC13 in ninhydrin spray using eluted column Fraction-III.

It was again purified on TLC and showed one band of pink colour with R_f value of 0.49 was active against all tested organisms (Fig. 3). The bioactive fraction-III was further analysed by GC-MS.GC-MS chromatogram of fraction-III clearly showed presences of seven peaks at retention time of 25.861, 29.943, 33.474, 37.234, 43.280, 44.826 and 51.470 with area of 2.66, 5.64, 31.43, 10.83, 33.62, 8.22 and 7.61 min, respectively (Fig. 4). 1,2-benzenedicarboxylic acid (retention time- 43.280 min; area%- 33.62) was present in highest concentration. The other compounds recorded were 2,4,6-tri-t-butyl phenol; 2-propenenitrile, 2-(methoxymethyl); phthalic acid, butyl 8-methylnonyl ester; 9- octadecenoic acid (Z); octocrylene and stigmasterol acetate (Table 1).

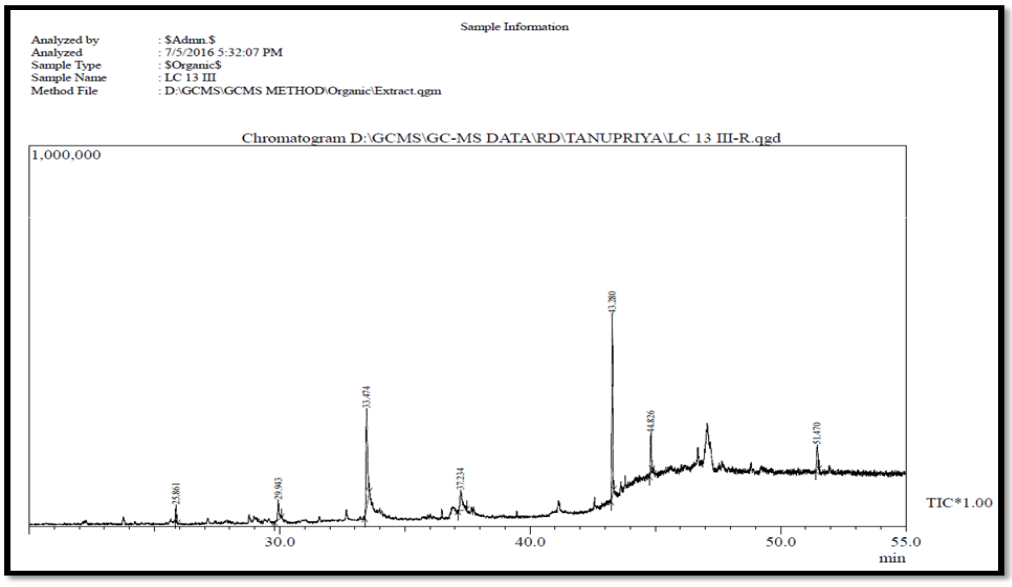
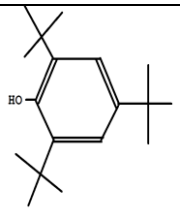
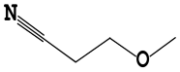
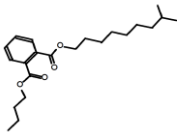
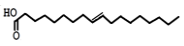
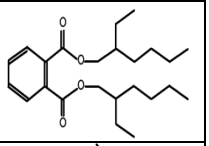
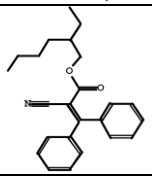
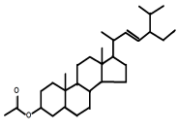


Fig. 4: Gas Chromatography and Mass Spectroscopy (GC-MS) chromatograms of the ethyl acetate extract of *Streptomycetes puniceus* LC13 (fraction-III).

Table 1: Bioactive compounds identified in ethyl acetate extract of *Streptomycetes puniceus* LC13 (fraction-III) (Compounds are listed in ascending order of retention time).

Peak No.	Retentiontime (min)	Area%	Compound name	Molecular weight	Molecular formula	Molecular structure
1	25.861	2.66	2,4,6-TRI-T-Butyl Phenol	262	$C_{18}H_{30}O$	
2	29.943	5.64	2-Propenenitrile, 2-(Methoxymethyl)	97	C_5H_7NO	
3	33.474	31.43	Phthalic acid, Butyl 8-Methylnonyl Ester	362	$C_{22}H_{34}O_4$	
4	37.234	10.83	9- Octadecenoic Acid (Z)	282	$C_{18}H_{34}O_2$	
5	43.280	33.62	1,2-Benzenedicarboxylic Acid	390	$C_{24}H_{38}O_4$	
6	44.826	8.22	Octocrylene	361	$C_{24}H_{27}NO_2$	
7	51.470	7.61	Stigmasterol acetate	454	$C_{31}H_{50}O_2$	
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DISCUSSION

Actinomycetes produce large numbers of secondary metabolites with antimicrobial potential. The moderate degree of polarity is a characteristic of most antibiotics therefore most of the crude antibiotic was soluble in ethyl acetate.[9] Fraction-III showed highest activity against most of the tested pathogens. The GC-MS result of fraction-III revealed that presences of seven peaks with different volatile compounds. 1,2-Benzenedicarboxylic Acid (retention time- 43.280 min; area%- 33.62) was present in highest concentration. Similarly, Fraction 10 showed good activity against *Staphylococcus epidermidis* (31.25 µg/mL) and *Malassezia pachydermatis* (500 µg/mL) and the active principle (fraction 10) was identified as 2,4-bis (1,1-dimethylethyl) phenol.[10] Fatty acid esters namely phthalic acid, dibutyl ester, bis(2-

ethyl hexyl) maleate and 1,2-benzenedicarboxylic acid were reported to possess anti inflammatory[11] and antibacterial activity.[12] The findings of Awla *et al*.[13] demonstrates that the presence of 22 different volatile compounds in the ethyl acetate crude extract by GC-MS. Recent studies have illustrated that a number of these compounds can lead to direct inhibition of fungal and bacterial pathogens.

CONCLUSIONS

Streptomycetes puniceus LC13 produce 1,2-benzenedicarboxylic acid, an antimicrobial compound, and promises to be a suitable candidate for industrial production of antimicrobial compounds. Phthalates and other fatty acid compounds of the present study appear to be the effective antimicrobial agents. Therefore, these volatile compounds may be usedin novel drug formulation after detailed study.

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