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Original Article

Antimicrobial efficacy of *Nocardiopsis* sp. MK_MSt033 against selected multidrug resistant clinical microbial pathogens

P. Muthukrishnan[a], D. Chithra Devi[a], Ashraf A. Mostafa[b], Khawla Ibrahim Alsamhary[c], Neveen Abdel-Raouf[c,d], Essam Nageh Sholkamy[b,*]

[a] *Department of Microbiology, P.S.G. College of Arts and Science, Coimbatore 641 014, Tamil Nadu, India*
[b] *Department of Botany and Microbiology, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia*
[c] *Department of Biology, College of Science and Humanities in Al-Kharj, Prince Sattam Bin Abdulaziz University, Al-Kharj 11942, Saudi Arabia*
[d] *Botany and Microbiology Department, Faculty of Science, Beni-Seuf University, Beni-Seuf 65211, Egypt*

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ABSTRACT

Background: Actinomycetes show an active role in microbial disease control and antimicrobial metabolism production and were shown the greatest potentialities as sources of antimicrobial agents. The past few decades, an extensive literature has accumulated on the production of bioactive compounds from actinomycetes, particularly genus *Streptomyces.*
Methods: The actinomycetes were isolated with starch casein nitrate (SCN) agar medium. The prospective isolate was subjected to antimicrobial metabolites production. Further, the bio-extract was evaluated their biological properites by agar well diffusion assay and finally the extract was anaylized through GC–MS.
Results: In the present study, isolated 9 actinomycetes and the isolates were examined for their antifungal activities. Of these nine isolates, the isolate MK_MSt033 picked out from the rest, hences, it showed significant control towards the selected microbial pathogens. The prospective strain MK_MSt033 was determined as *Nocardiopsis* sp. The strain displayed effective antimicrobial activities against both bacterial and fungal pathgoens such as *Escherichia coli*, *Staphylococcus aureus*, *Pencillium* sp., and *Aspergillus flavus* respectively. Subsequently the chemical nature of the compounds produced by the potential isolate MK_MSt033 and it was successfully determined by GC–MS and it were 18 compounds with different retention time.
Conclusion: The identified isolate *Nocardiopsis* sp. MK_MSt033 exhibited potential antimicrobial activities against selected microbial pathogens. Thus, the soil inhabiting *Nocardiopsis* sp. has explored for pharmaceutically active compounds with promising medical applications.

Introduction

Generally, natural products have played an essential role in controlling and prevention of human diseases during thousands of years [1]. The pharmaceutical agents derived from natural sources are playing a vital role for our therapeutic approaches since starting due to their chemical diversity and various biological activities against diseases and its causitve agents. The cost effective and local accessibility of the natural products have made them an important source of drugs especially in underdeveloped countries. Approximately more than ten thousands various antibiotics have been isolated from bacteria, actinomcycetes and of filamentous fungi [2].

However, the Actinomycetes predominantly *Streptomyces* species are accountable for the biosynthesis of approximately 60% of the known antimicrobial products and further 15% are produced by other actinomycetes species i.e., *Micromonospora*, *Actinomadura*, *Actinoplanes*, *Nocardia*, *Streptosporangium*, *Streptoverticillium* and *Thermoactinomyces* [3]. Most of the antimicrobial products purified so far are invented from actinomycetes *viz.*, genus of *Streptomyces* and *Micromonospora*. The actinomycetes produce an extensive variety of secondary metabolites and more than 70% of the naturally obtained antibiotics that are presently in therapeutical uses [4].

Actinomycetes show an active role in microbial and its disease control and antimicrobial metabolics production and were

* Corrsponding author.
E-mail addresses: elisi@ksu.edu.sa, Essam_92003@yahoo.com (E. Nageh Sholkamy).

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shown the greatest potentialities as sources of antimicrobial agents. The past few decades, an extensive literature has accumulated on the production of bioactive compounds from actinomycetes. Particularily polyene macrolide antibiotics consists a large group and predominalty originated from soil actinomycetes, the common genus belongs to the *Streptomyces* [5]. Since 1950 over two hundred polyene macrolides drugs were recuperated and documented. They elicit primarily with antifungal [6], antibacterial [7], antiprotozoal [8], antiviral [9], and antitumour [10] activities.

A prospective antibiotic innovative approach in the earlier peroids were dpends on whole cell screening of secondary metabolites from microorganism, after a while it was sustained in the middle due to rediscovery of known compounds along with the increasing incidence of antibiotic resistance by the microbial pathogens and limited production of antifungal antibiotics to treat the mycotic diseases [11]. Accordingly, there is a need for the production of new, safe and more efficacious antimicrobial drugs is a foremost confront to the pharmaceutical industry as on today [12].

Globally researchers are constantly working to discover novel efficient drugs to fight aganist multi drug resistant microbes and new antibiotics are still being found [13]. However, the possibility of discovering new antibiotics only by random screening of microorganisms is reduced now-a-days. Based on these facts, a research work has been proposed to discover new antibiotics from soil associated actinomycetes in this study. Therewith, to explore novel new antibiotics, our approach is to conduct intensive screening of different soil samples from agricultural farming area which is an unexamined space and it is expected to provide persistent results near isolation of new species neither actinomycetes nor newer antimicrobial compounds.

Materials and methods

Sample collection

Agricultural soil samples were procured from different groundnut cultivated agriculture lands. The soil samples were collected at depths of 6–10 cm and collected soil samples were dried for one week to reduce other bacterial and fungal populations. The samples were collected in sterile polythene bags, sealed compactly and stored at 4 °C for further studies.

Isolation of actinomycetes

Isolation of actinomycetes was accomplished by spread plate method with diluted samples. One g of soil sample was serially diluted upto 10^{-6}. The 0.1 mL of soil suspension was taken from 10^{-4}, 10^{-5} and 10^{-6} dilutions then it was spread on sterile Strach Casein Nitrate (SCN) agar plates accompanied with 50 μg/mL of streptomycin and 10 μg/mL of cyclohexamide to control bacterial and fungal growth respectively and plates were incubated at 30 °C for 5 days [14]. After incubation, actinomycetes colonies were purified and subcultured on SCNA plates and stored at 4 °C for further studies.

Characterization of actinomycetes

Cultural and morphological characteristics

Morphological and cultural characteristics of the isolated actinomycete isolates were examined by inoculating the strains onto SCN agar plates. After incubation of 5 days at 30 °C, the cultural and morphological characteristic features of the isolates were observed. The morphological characterizations of actinomycete isolates were carried out with JEOL JSAL (SEM) under different magnifications such as 2,500×, 5000× and 25,000×.

Biochemical characterization of actinomycete isolates

A biochemical characteristic of the selected isolates was performed followed by the Bergey's Manual of Determinative Bacteriology [15], to identify and confirm the isolated actinomycete into genus level. Biochemical characteristics were determined by various biochemical tests such as indole, methyl red and Voges Proskauer tests, triple sugar iron test, gelatin hydrolysis, starch hydrolysis and enzyme reactions such as urease, catalase and oxidase tests [16]. Carbohydarate utilization tests were completed to identify the utilization of different carbohydarate as a carbon source such as glucose, lactose, mannitol, xylose and sucrose.

Physiological characterization

Effect of temperature and pH. To determine the optimum temperature for the growth, the isolates were grown in the International Streptomycetes Project (ISP) 2 broth and incubated at the temperature ranging from 15 °C to 37 °C for 72 h. Similarly, pH obligatory for maximum productivity of seconday metabolites and growth of acinomycte was recognized by adjusting the pH of the fermentation medium with different pH ranges from 3 to 9. After sterilization, the respective strain was inoculated and incubated at 30 °C for 72 h in the rotary shaker at 100 rpm and the optical density was determined at 600 nm using UV–vis spectrophotometer [17].

Screening of actinomycetes for antimicrobial activities

Microbial pathogens

Bacterial pathogens like as *Staphylococcus aureus* ATCC 43300, *Streptococcus pyogenes* ATCC 12344, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27584 and *Proteus vulgaris* ATCC 9765 were used for this investigation. The test fungal pathogens used for this study were *Aspergillus niger, Aspergillus flavus, Candida albicans, Malassezia furfur* and *Penicillium* sp., All the microbial pathogens were obtained from Microbiology Lab., King Khalid University Hospital, Riyadh, Saudi Arabia and PSG Institute of Medical Research and Science, Coimbatore, Tamil Nadu, India. The microbial pathogens were subcultued and maintiained bacteria with nutrient agar and fungal pathogens in potato dextrose agar for routine experiment use and stored at −4 °C for the long term.

Screening by cross streak method

Antimicrobial activity of actinomycetes was determined by spectra-plak method (perpendicular streak method) [18]. Sabouraud dextrose agar (SDA) plates were prepared and inoculated the strains with single streak in the centre of the plate and incubated at 30 °C for 4 days. After 4 days of incubation, mycelial discs by picking the fungal spore of 6 mm diameter were cut from fungal plates then it was positioned at 2.5 cm away from the pregrown actinomycetes colony without touching and incubates the plates at room temperature for 3 days. If, the fungal pathogen is sensitive to the antibiotic producing actinomycetes, then it will not grow near the actinomycetes colonies. Antagonistic activity was evaluated by inhibition zone around the actinomycete isolates.

Biosynthesis of metabolites by potential isolate

Fermentation process

Shake flask fermentation method was performed for the biosynthesis of seconday metabolites with potential isolate MK_MSt033. The 250 mL of International *Streptomyces* Project (ISP) 2, Modified nutreient glucose (MNG), ISP 4 medium was prepared and sterilized in 500 mL of Erlenmayer flask then it was inoculated with isolate MK_MSt033. Then the flasks were incubated at 30 °C for 15

days under agitation at 150 rpm [19]. Finally, ISP 2 medium was selected for mass production of bioactive compounds.

Extraction of antimicrobial compounds

After completion of fermentation process, fermented broth was centrifuged at 3000 rpm for half an hour to remove biomass and cell debris. After centrifugation, supernatant was separated and the antifungal compound was recovered by solvent extraction method with ethyl acetate [20]. Brefily, filtrate and ethyl acetate was mixed in the ratio of 1:1, and then transferred into separating funnel. After vigorous shaking it was kept for 4 h to complete separation process. The organic (solvent) phase containing antimicrobial compound was removed and it was concentrated with rotary evaporator 80 °C and the dried active compound was collected and stored at 4 °C for further studies.

Antimicrobial activity

The compounds which have been isolated from fermentation medium were tested for their antimicrobial activity by well diffusion method [21]. The selected bacterial and fungal pathogens were swabbed on the NA and SDA (supplemented with 1% olive oil) plates respectively and then it was kept for 30 min under undisturbed condition. After 30 min wells were made by using sterile cork borer and then the partially purified extracts was loaded in the wells at 3 different concentrations includes, 50 100 and 200 μL. After incubation, the inhibition zone was determined and results recorded.

Molecular identification of isolate MK_MSt033

DNA isolation and PCR amplification

The DNA was isolated from the actinomycete isolates using Chromous Genomic Isolation Kit (RKT09). Isolated DNA was amplified by direct PCR with the aid of 16s rRNA specific primers (16SF 5′ AGAGTRTGATCMTYGCTWAC 3′ and 16SR 5′ CGYTAMCTTWTTACGRCT 3′). The reaction mixture contained 1 μL of DNA, 400 ng of 16s forward primer, 400 ng of 16s reverse primer, 4 μL of deoxynucleoside triphosphate mixture (dNTPs), 10 μL of 10X taq DNA polymerase assay buffer and 1 μL of Taq DNA polymerase enzyme. The PCR was accomplished according to manufactures instructions and conditions

16s rRNA sequencing and phylogenetic analysis

The 16S rRNA gene was sequenced partial with the PCR products directly as sequencing template with above specified primers. The sequencing reactions performed out with an ABI 3500 XL automated DNA sequencer and Big Dye Terminator version 3.1 cycle sequencing kit as per company's instructions. The identification of isolate was attained by 16S rRNA sequences comparision obtained with the other 16S rRNA sequence data from the public databases GenBank by using NCBI Basic Local Alignment search tools BLAST n program (http://www.ncbi.nlm.nih.gov/BLAST). The distance matrix was created by the Jukes-Cantor corrected distance model. The phylogenetic trees generated with Weighted Neighbor joining with alphabet size 4 and length size 1000 with boot strap values [22].

Partial purification of antimicrobial active compound

Thin layer chromatography (TLC)

Thin layer chromatography was performed to separate the active compound in the ethyl acetate extract of isolate MK_MSt033. The crude compound was spotted at 3 cm from the bottom of silica gel coated plate by using micropipette. A chamber was poured with solvent (mobile phase) containing chloroform and methanol in 4:1 ratio and the plate was placed in to the chamber [23]. The chamber was covered with a lid and allowed the solvent to travel with sample and left for 60 min [24]. The dried plate was examined under the ultra violet light and then the plate was exposed to iodine vapor for 30 min. After 30 min, brown colour spot was appeared and the Rf values was measured and recored.

Minimum inhibitory concentration (MIC) determination

Minimum inhibitory concentration (MIC) of the partially purified bioactive compound (PPBC) was testified by broth micro dilution technique [25]. Briefly, the purified bioactive compound was dissolved in with dimethyl sulfoxide (DMSO). The MIC was performed in 96 well plate contains the capacity of 300 μL volume. The mixture included, 185 μL of nutrient broth (bacteria) and sabouraud dextrose broth (fungal spore), 10 μL of the PPBC and 5 μL of the mid log phase and fungal spores were used respectively. Later, the plate was incubated at 37 °C for 17 h (bacteria) and 30 °C for 3 days (fungi). After incubation, the plate was visualized for observing the growth of the microbial pathogens. Standard streptomycin and nystatin was used as the positive control aganit bacterial and fungal culture respectively. The experiment was repeated three times.

Identification of antimicrobial compound

Fourier transform infrared spectroscopy (FTIR)

The brown colored substance obtained from the TLC plate was collected and given for the analytical method FTIR spectroscopy [26]. The infrared spectra were recorded on Shimadzu IR-470 model and scanned in the range of 400–4000 cm^{-1}. The resulting FTIR spectral pattern is then analyzed and matched with known signatures of identified compounds in the FTIR library.

Gas chromatography–mass spectrometry (GC–MS)

The identification and characterization of antimicrobial compounds was done by GC–MS. Partially purified bioactive compound was dissolved with methanol and it was analyzed through the GC–MS system [27], equipped with Thermo GC-Trace Ultra Version: 5.0, and thermo MS DSQ II with DB 5 – MS capillary standard non polar column. Column condition was programmed with initial oven temperature at 70 °C raised to 260 °C at 6 °C/min. Helium gas was used as a carrier gas at the flow rate of 1.0 mL/min. The spectra were analyzed from the NIST library.

Results and discussion

The current study principally focused on isolation, screening and production of bioprospective *Streptomyces* sp. and it antimicrobial compounds against microbial pathogens.

Isolation of actinomycetes

Actinomycetes are widely disseminated in the nature and are also predominantly as a soil inhabitant hence agricultural soil sample was preferred for isolation of actinomycetes and evaluated their antimicrobial potentials.

The various types of actinomycetes were isolated from the soil samples and the colonies were observed with different color, size and shape. The colony morphology was diversed in nature such as mucous, rough, lucid, powdery and slimy appearances (Fig. 1). Then the unique colonies with different morphology were selected and sub cultured on SCN agar. Finally, 9 isolates were selected with actinomycetes characteristics for further studies.

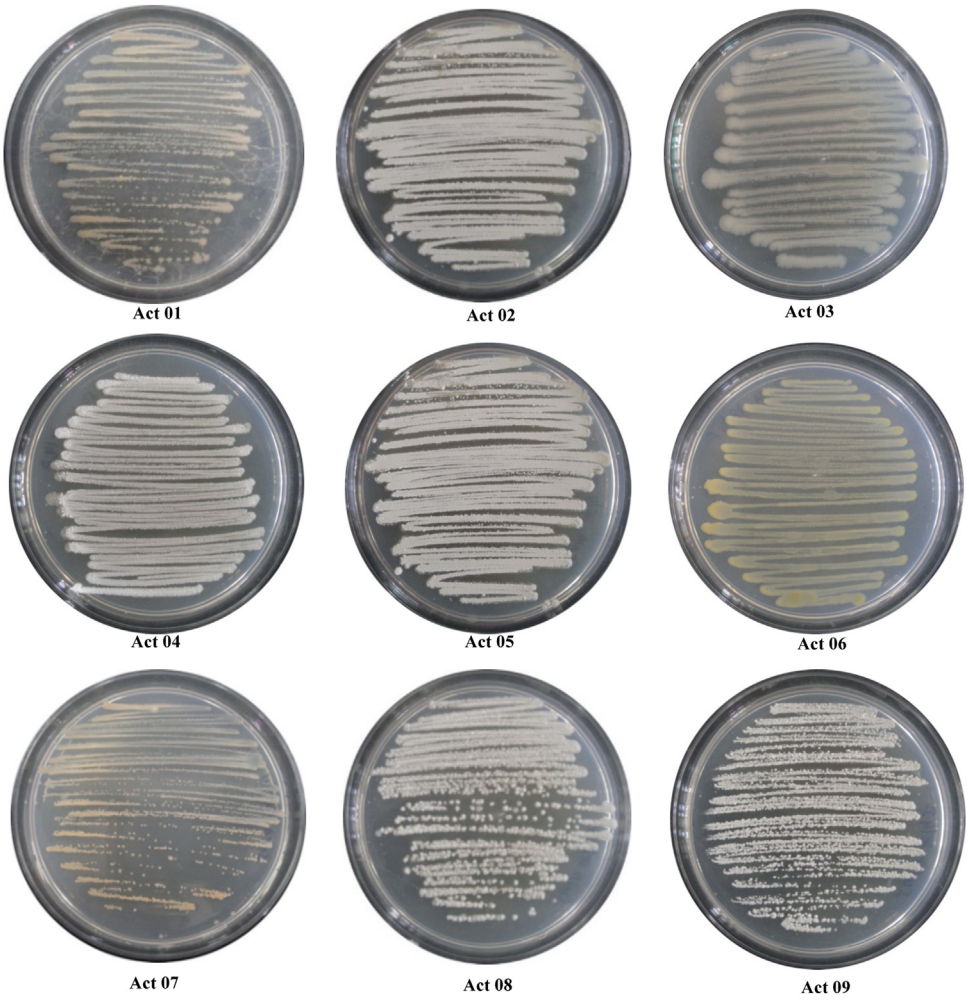

Fig. 1. Isolated actinomycete isolates from agricultural farming soils with different colonies morphology (Act 01–Act 09).

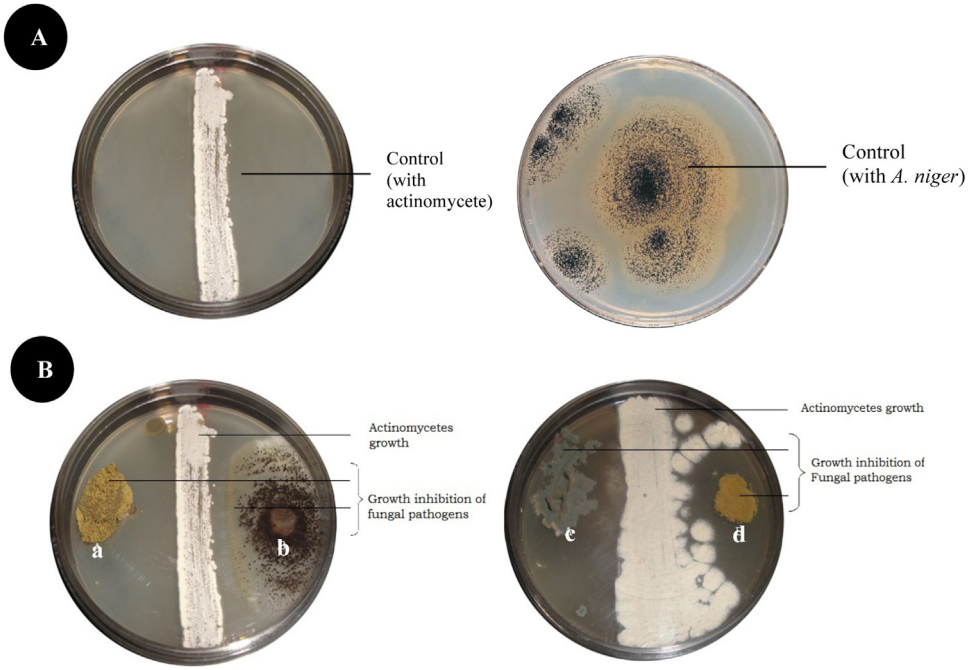

Fig. 2. Priliminary screening of antifungal activity of isolate MK_MST033.
(A) Control plates (with actinomycetes and with *A. niger*); (B) a) *A. flavus*; b) *A. niger*; c) *Penicillum* sp. d) *M. furfur*.

Table 1
Cultural, morphological, biochemical and physiological **c**haracteristic features of isolate MK_MSt033.

Characteristics	Observations			
	Mycelium type	Colony nature	Colony colour	Pigmentation
Cultural	Aerial	Chalky and rough	Grey	Brown
	Gram straining	Mycelial nature		
Microscopic	Gram positive	Delicate coiled filaments with fragmented spores		

	Indole	MR	VP	Citrate	TSI
Biochemical	Negative	Positive	Negative	Positive	Alk/Alk
	Oxidase	Catalase	Urease	Starch	Gelatin
Physiological	Positive	Positive	Positive	Positive	Negative

	Glucose	Lactose	Mannitol	Sucrose	Sorbitol	Xylose
Sugar utilizations	Positive/acid	Positive/acid	Positive/acid	Positive/acid	Positive/acid	Positive/acid

Screening of actinomycete isolates for antimicrobial metabolities

The antimicrobial metabolities producing actinomycete isolates were identified through primary screening by Spectra- Plak method. The preliminary screening displayed most effective activity against tested drug resistant bacterial pathogens. The significant antimicrobial activities were observed against *E. coli*, *S. aureus* and *P. vulgaris*. Whereas, *A. niger* and *A. flavus* is more sensitive and their growth was inhibited by the compound produced around the central streak of actinomycetes (Fig. 2). Among the 9 isolates, the isolate MK_MSt033 was the effective one in inhibiting the growth of all the fungal pathogens with considerable values hence it was considered as a prospective strain for further studies.

Morphologically characteristics of isolate MK_MSt033

The characteristics of isolate MK_MSt033 were inoculated with different ISP medium. After incubation, the plates showed different characteristics such as shape, size, surface, margin, color, odor, pigmentation and different growth nature. Thus, the isolated strain MK_MSt033 was showed excellent growth in ISP 2 and colony features was medium sized, grey colour with powdery and rough nature (Fig. 3A). The isolated strain MK_MSt033 was gram positive and non-motile organism and short rods (Fig. 3B). The similar type characteristics features strain was isolated from previous study by Vijayakumar et al. [28]. The cultural and morphological characteristics of isolate MK_MSt033 were presented in the Table 1.

Biochemical and physiological characterization

The isolated strain 33 was subjected to various biochemical tests to characterize and identify the isolated strains and it helps identify the isolates in to genus level. Among 9 isolates, the isolate 33 showed IMViC as follows; indole positive with red colour ring formation, methyl red negative, voges Proskauer negative and citrate positive with formation of blue colour (Fig. 3C). The isolate MK05 exhibited alkaline slant and alkaline butt without H_2S and gas production in triple sugar iron test. The isolate 33 was produced amylase and catalase enzyems (Fig. 3C). The biochemical test result of isolate 33 was displayed in the Table 1. The sugar fermentation test results were showed in the Table 1 and Fig. 3D for the isolate 33.

Effect of temperature and pH

In this study, isolated strain 33 was seeded in to ISP 2 broth and incubated at four distinct temperatures such as 15, 20, 30 and 37 °C. After incubation, the growth rate was increased linearly in their temperature ranges from 15 °C to 30 °C. Although, it was showed decrease in their growth rate when it was crossed the 30 °C–37 °C. The isolate 33 was attained maximum growth at 30 °C with highest OD value at 600 nm (Fig. 4).

The isolate 33 was allowed to grow in ISP 2 broth consist of four different pH ranges (3, 5, 7 and 9) and incubate at 30 °C. After incubation, the isolate 33 was grown at acidic, neutral and alkaline pH. However, at pH 7 the isolate 33 exhibited maximum growth and higher OD value at 600 nm (Fig. 4).

Molecular identification of isolate MK_MSt033

The 16s rRNA sequence of the potential isolate 33 was sequenced and strain was identified as *Nocardiopsis* (Fig. 5a). The sequenced data of isolate 33 was analyzed with the NCBI blast search engine by NCBI accession number and it showed the similarities to *Nocardiopsis* sp. M048 (AY764033) with the score of 0.998. The evolutionay tree was assembled using weighted Neighbor joining method and the phylogenetic tree confirmed that the isolate 33 was *Nocardiopsis* sp. (Fig. 5b). Thus, based on the cultural, biochemical and molecular characteristic features the isolate 33 was belongs to the genus *Nocardiopsis* and strain MK_MSt033.

Production and extraction of antimicrobial metabolites

The antimicrobial metabolites production was accomplished by the shake flask fermentation process with ISP 2 medium. The ISP 2 fermentation medium was prepared and seeded with *Nocardiopsis* sp. strain MK_MSt033 and incubated under agitation. During the fermentation process, growth rate of strain MK_MSt033 was in high speed during first 3 days, after that the thick masses of mycelium were developed in the flasks and the medium color was changed from yellowish brown to brown. Thus, the production of secondary metabolites is often inclined by the nutritionl compostion, physicochemical conditions and organism to organism [29].

After getting the sufficient growth, the compound was extracted from the mass cultures by solvent extraction process. After completion of extraction process, the solvent was completely evaporated at 60 °C and final brown colored powder was obtained. The powder compound was soluble in water, dimethyl sulfoxide (DMSO), NaOH (2.5 M) and H_2SO_4; however the compounds are not dissolved in Na_2CO_3 and HCl (1.5 M). Correspondingly, the previous findings different solvents such as *n*-butanol [29], methanol [30], petroleum ether, chloroform and benzene [31], were exploited for compound extraction.

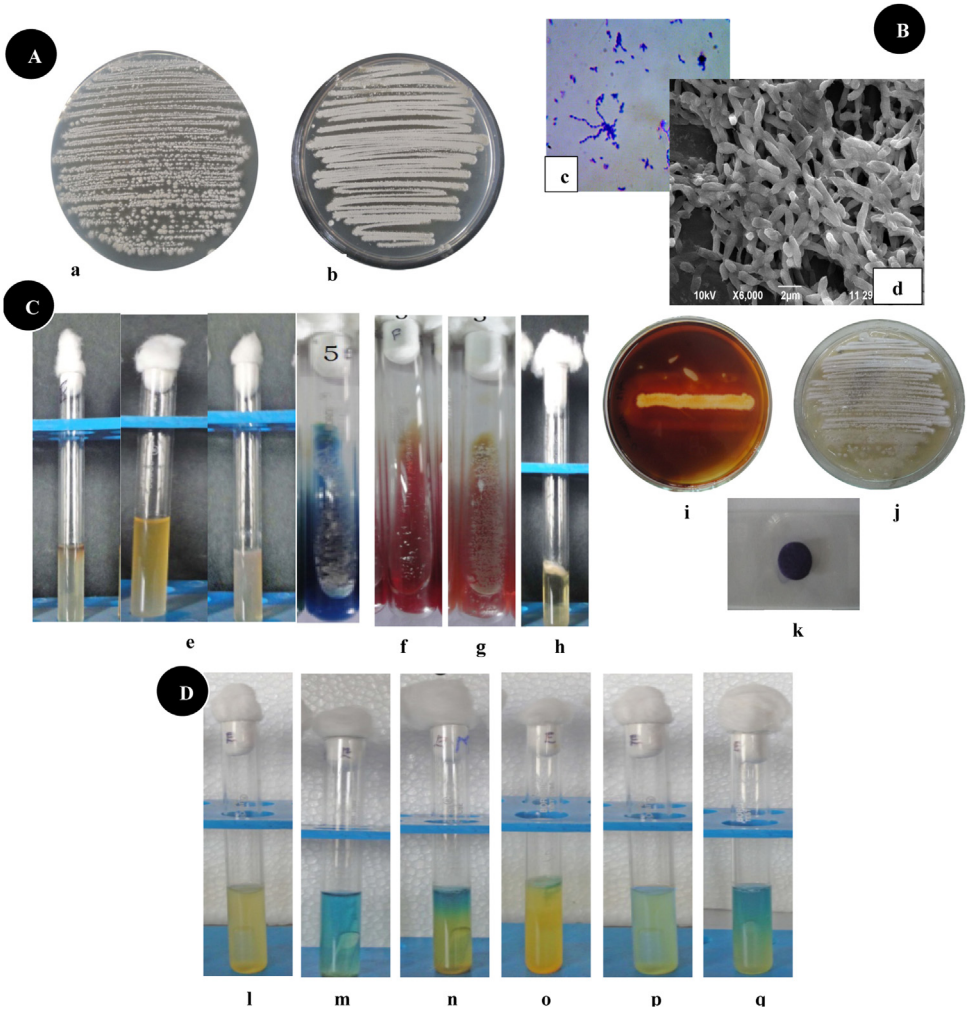

Fig. 3. (A) Growth, colony morphology, (B) Microscopic appearance, (C) Biochemical characteristics (D) Sugar utilization of actinomycete isolate MK_MSt033; a) colonies on Actinomycetes isolation agar; b) colonies on Starch Casein Nitrate Agar; c) light microscopic view (1000×); d) scanning electron microscopic view (6000×); e) IMViC: Indole – negative, methyl red – positive, Voges Proskauer – negative, citrate – positive; f) urease test – positive; g) TSI – Alk/Alk; h) gelatin liquefaction – negative; i) amylase – positive; j) oxidase – positive, k) catalase – positive; l) glucose; m) lactose; n) mannitol; o) sucrose; p) sorbitol; q) xylose.

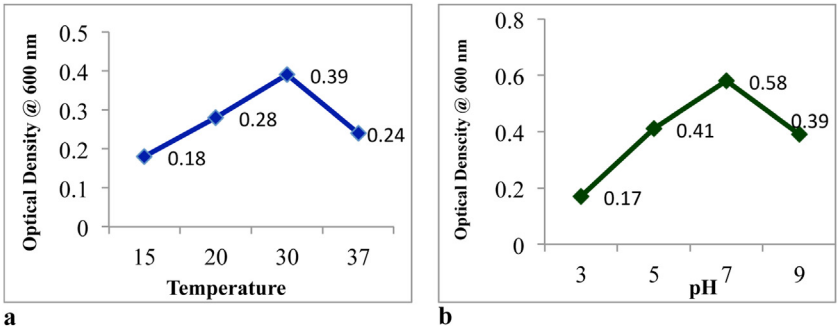

Fig. 4. The effects of temperature and pH on growth of isolate MK_MSt033. a) Effect of temperature (optimum is 30 °C); b) Effect of pH (optimum is 7).

Purification of antimicrobial compound

TLC was accomplished to separate the active compound in the ethyl acetate extract of strain MK_MSt033. The solvent system used to separate the compound was chloroform and methanol with the ratio of 4:1 and only one spot was observed after the exposure to iodine vapor. The Rf value for the compound present in the effective strain MK_MSt033 was 0.54.

Antimicrobial activity of partially purified compound

The partillay purified compound of ethyl acetate extract of strain MK_MSt033 was tested for antimicrobial activity against five bacterial such as *S. aureus*, *S. pyogenes*, *E. coli*, *P. aeruginosa* and *P. vulgaris* and fungal pathogens namely *A. niger*, *A. flavus*, *C. albicans*, *M. furfur* and *Penicillium* sp., and the inhibition zone was estimated and tabulated (Tables 2a,b). The *Nocardiopsis* sp. MK_MSt033 exhib-

>isolate MK_MSt033 (1376 bp)

GTCGAGCGGTAAGGCCCTTCGGGGTACACGAGCGGCGAACGGGTGAGTAACACGTGAGCAA
CCTGCCCCTGACTCTGGGATAAGCGGTGGAAACGCCGTCTAATACCGGATACGACCCGCCAC
CTCATGGTGGAGGGTGGAAAGTTTTTCGGTCAGGGATGGGCTCGCGGCCTATCAGCTTGTTGG
TGGGGTAACGGCCTACCAAGGCGATTACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACT
GGGACTGAGACACGGCCCAGACTCCTGCGGGAGGCAGCAGTGGGGAATATTGCGCAATGGG
CGAAAGCCTGACGCAGCGACGCCGCGTGGGGGATGACGGCCTTCGGGTTGTAAACCTCTTTT
ACCACCAACGCAGGCTTCCAGTTCTCTGGAGGTTGACGGTAGGTGGGGAATAAGGACCGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTCCGAGCGTTGTCCGGAATTATTGGGCG
TAAAGAGCTCGTAGGCGGCGTGTCGCGTCTGCTGTGAAAGACCGGGGCTTAACTCCGGTTCT
GCAGTGGATACGGGCATGCTAGAGGTAGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGA
AATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGCCTTACCTGACGC
TGAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAAC
GTTGGGCGCTAGGTGTGGGGACTTTCCACGGTTTCCGCGCCGTAGCTAACGCATTAAGCGCCC
CGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGC
GGCGGAGCATGTTGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGTTTGACATCACCC
GTGGACTCGCAGAGATGTGAGGTCATTTAGTTGGCGGGTGACAGGTGGTGCATGGCTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCCATGTTGC
CAGCACGTAATGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGA
TGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAAACATGCTACAATGGCCGGTACAA
TGGGCGTGCGATACCGTAAGGTGGAGCGAATCCCTAAAAGCCGGTCTCAGTTCGGATTGGGG
TCTGCAACTCGACCCCATGAAGGTGGAGTCGCTAGTAATCGCGGATCAGCAACGCCGCGGTG
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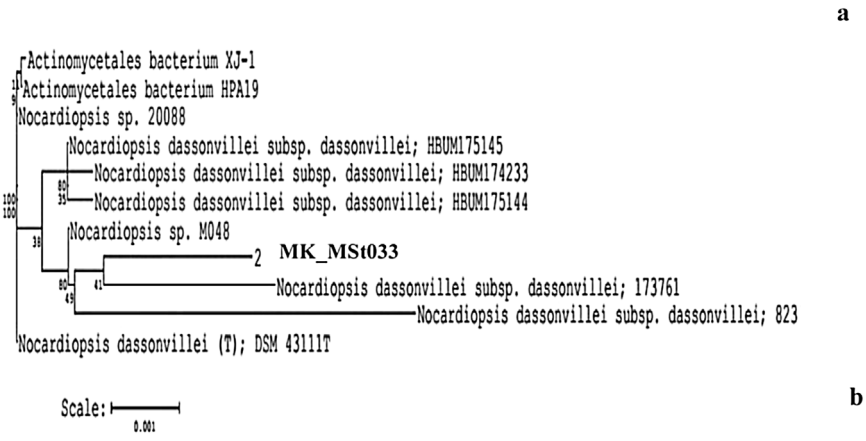

Fig. 5. Molecular idnetification of isolate MK_MSt033.
a) 16S rRNA sequnce of isoalte MK_MSt033.
b) Phylogenetic tree showing relationship of isoalte MK_MSt033 with other isolate from NCBI.

Table 2a
Antibacterial activities of partially purified bioactive compound from isolate MK_MSt033.

Bacterial pathogens	Zone of inhibition (mm)/concentration (μg)		
	25	50	75
E. coli ATCC 25922	7.00 ± 0.0	10.00 ± 0.5	16.00 ± 0.3
P. aeroginosa ATCC27584	ND*	8.00 ± 0.2	12.00 ± 0.4
P. vulgaris ATCC9765	10.00 ± 0.3	12.00 ± 0.2	16.00 ± 0.6
S. aureus ATCC43300	9.00 ± 0.1	13.00 ± 0.1	17.00 ± 0.5
S. pyogenes ATCC12344	11.00 ± 0.2	15.00 ± 0.4	15.00 ± 0.4

Note: ND* — Not detected.

Table 2b
Antifungal activities of partially purified bioactive compound from isolate MK_MSt033.

Fungal pathogens	Zone of inhibition (mm)/concentration (μg)		
	25	50	75
Aspergillus niger	9.00 ± 0.2	12.00 ± 0.1	17.00 ± 0.2
Aspergillus flavus	11.00 ± 0.4	17.00 ± 0.5	19.00 ± 0.6
Penicillium sp.	6.00 ± 0.1	14.00 ± 0.4	20.00 ± 0.4
Candida albicans	8.00 ± 0.2	14.00 ± 0.2	17.00 ± 0.1
Malassezia furfur	8.00 ± 0.3	14.00 ± 0.2	18.00 ± 0.3

ited significant activities towards the tested bacterial and fungal pathogens. The potential strain exhibited antimicrobial activity against bacteria *viz*., *E. coli* (16.00 ± 0.3), *P. aeroginosa* (12 ± 0.4), *P. vulgaris* (16.00 ± 0.6), *S. aureus* (17.00 ± 0.5) and *S. pyogenes* (15 ± 0.4) (Table 2a), and antifungal activity against such as *Penicillium* sp. (20.00 ± 0.4), *M. furfur* (18.00 ± 0.3), *A. niger* (17 ± 0.2), *C. albicans* (17 ± 0.1) and *A. flavus* (19.00 ± 0.6) (Table 2b and Fig. 6). Related findings of previous study were reported [32] from the ethyl acetate extract of *Nocardiopsis* sp., VITSVK5 (FJ973467) displayed antimicrobial activity against *A. fumigatus* (23 mm), *A. flavus* (15 mm) and *A. niger* (12 mm). Similarly, another finding of Vijayakumar et al. [33], revealed that the ethyl acetate extract of *Nocardiopsis* sp., isolated from the soils showed maximum activity against *C. neoformans* (26 mm) followed by *C. albicans* (21 mm), *E. coli* (19 mm), *P. miraiblis* (18 mm), *B. subtilis, S. typhi* and *P. aeruginosa* (16 mm), *E. faecalis* and *S. dysenteriae* (15 mm) and *S. aureus* (13 mm).

Minimum inhibotry concentration (MIC)

Table 3, displayed the MIC values of the bioactive compound along with streptomycin and nystatin as standard drugs. The bac-

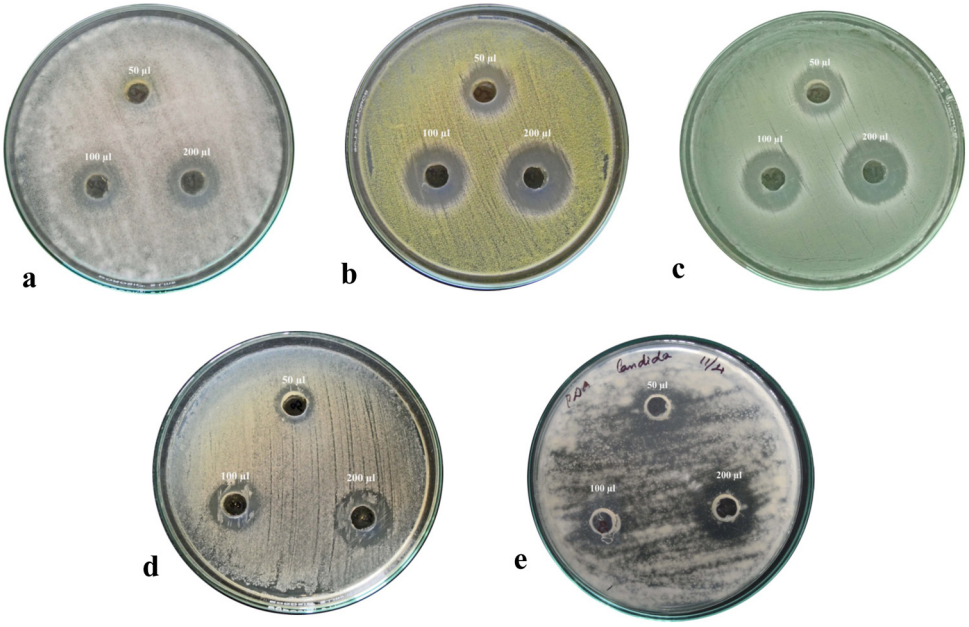

Fig. 6. Antifungal activity of partially purified antimicrobial compound.
a) *Aspergillus niger*; b) *Aspergillus flavus*; c) *Penicillum* sp.; d) *Malssezia furfur;* e) *Candida albicans*.

Table 3
Minimum inhibitory concentration of bioactive compound of strain MK_MSt033.

Microbial pathogens	MIC µg/mL	
	PPBC	Streptomycin
Bacterial pathogens		
E. coli ATCC 25922	15.60	7.80
P. aeroginosa ATCC27584	250.00	15.60
P. vulgaris ATCC9765	62.50	15.60
S. aureus ATCC43300	31.25	7.80
S. pyogenes ATCC12344	62.50	7.80
Fungal pathogens		Nystatin
Aspergillus niger	31.25	15.60
Aspergillus flavus	125.00	15.60
Penicillium sp.	15.60	7.80
Candida albicans	31.25	7.80
Malassezia furfur	15.60	15.60

terial pathognes, *E. coli* exhibited the MIC value of 15.60 µg/mL and *S. aureus* MIC value was noted 31.25 µg/mL when compared with other bacterial pathogens. Conversely, the fungal pathogens MIC values were range between 15.60 and 125.00 µg/mL. Overall, the minimum (15.60 µg/mL) and maximum (250.00 µg/mL) MIC value was observed against *E. coli, Pencillium* sp., and *P. aeruginosa* respectively. Collectively, the purified bioactive compound from *Nocardiopsis* sp. strain MK_MSt033 shown potent MIC values (15.60 µg/mL) against tested microbial pathogens. The MIC values of the bioactive compound of strain MK_MSt033 was comparatively equal and less for some microbial pathogens than the commercial antibiotic streptomycin (Table 3).

Identification of antimicrobial compound

Identification and characterization of antimicrobial compounds present in the effective isolate MK_MSt033 was done by FTIR and GC–MS analysis.

Fourier transform infrared spectroscopy (FTIR)

Through FTIR analysis, the functional groups present in the antimicrobial compounds were identified. The results of FTIR analysis showed broad absorption at the region of 3,700 to 3,200 cm^{-1}, and it is indicated the presence of O—H stretching of alcohol and phenol groups (Fig. 7). The another broad absorption showed at 1200–1000 cm^{-1} indicating the presence of C—O stretching of alcohols, ethers, acetates and fluorine containing compounds. The N—H stretching of amine groups were identified at 2400–2000 cm^{-1} region and some peaks were appeared in the region of 850–550 cm^{-1} thus indicates the presence of organic halogen compounds like chlorine and bromine containing compounds. Other groups like sulfur containing compounds were absorbed at the region of 700–400 cm^{-1} and it was confirmed by the presence of its C—S stretching. The FTIR analysis result was presented in Fig. 7.

Gas chromatography–mass spectrometry (GC–MS)

The analysis of bioactive compounds in the strain MK_MSt033 was further confirmed by GC–MS analysis. Through this analysis various compounds were identified with different retention time and relative abundance. The identified compounds contains various functional moieties includes acids, ketones, amides, amines, azoles, alkenes, quinones, coumarin and some aromatic hydrocarbons (Fig. 8 and Table 4).

The compound which was identified as 4-(Benzimidazol-1′-yl)-5-phenylisoxazole at the retention time 3.21 comprise azole compounds as their functional moiety. This azole compounds inhibit fungal cytochrome P 450 3A dependent C14-α-demethylase which is responsible for the synthesis of ergosterol [34], since ergosterol exists in cell membranes of fungi, yet not in animals, it is an expedient target for antifungal drugs, therefore this compound is considered as the biologically active compound thus have ability to inhibit the fungal pathogens.

The compound such as *N-n*-propyl-[2-(4-chlorophenyl) imidazo [1,2-a] pyridin-3-yl] acetamide was identified at the retention time of 11.08 and this compound has Imidazo [1,2-a] pyridin-3-yl] as their functional moiety. This imidazo [1,2-a] pyridine has significant importance in the pharmaceutical industry owing to the interesting biological activities displayed over a broad range of therapeutic classes, exhibiting anti-inflammatory antibacterial, antifungal properties [35].

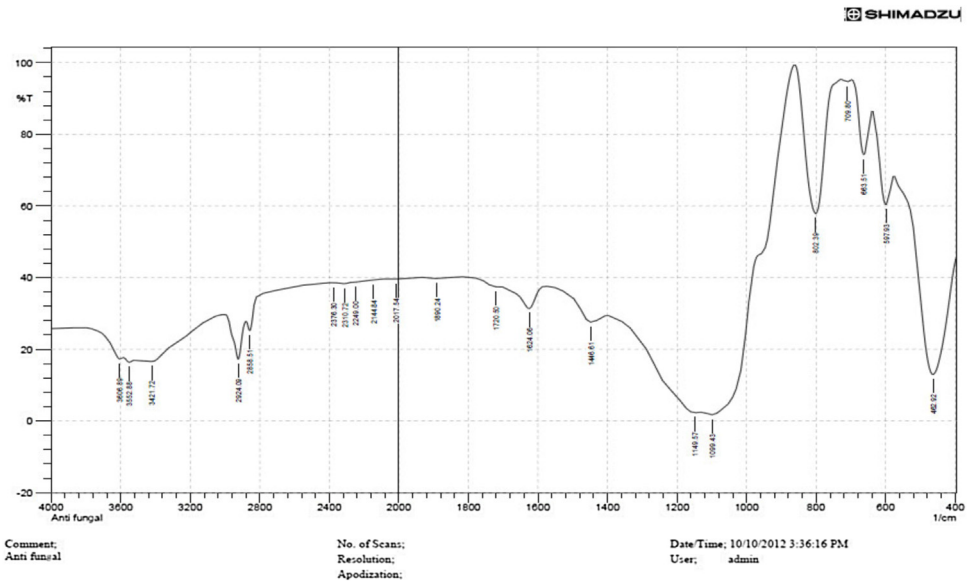

Fig. 7. FTIR spectrum of antimicrobial compound from isolates MK_MSt033.

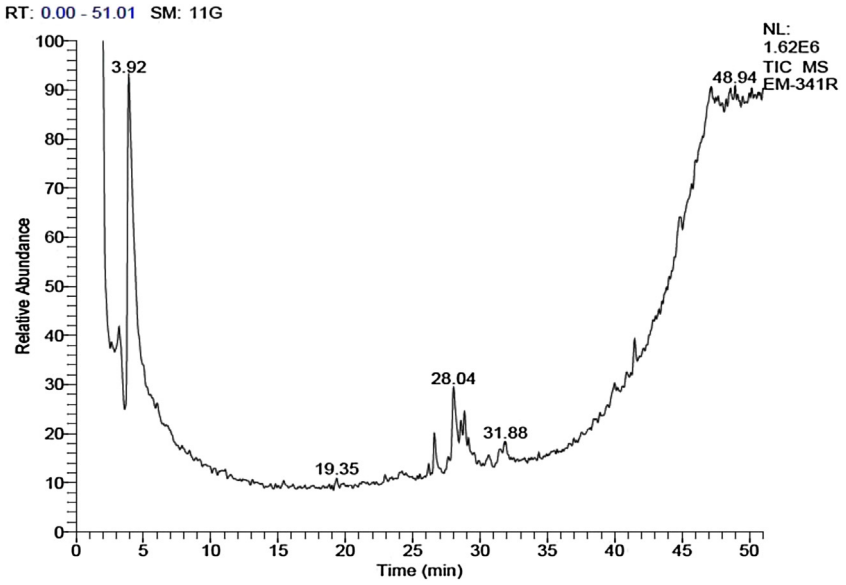

Fig. 8. GC–MS spectrum of ethyl acetate Extract of isolate MK_MSt033.

Table 4
Metabolic profiling for ethyl acetate extracts of isolate MK_MSt033.

Retentiontime	Compound name	Probability	Molecular formula	Molecular weight
3.21	4-(Benzimidazol-1′-yl)-5-phenylisoxazole	74.02	$C_{16}H_{11}N_3O$	261
5.04	[(4-Methyl-1-penten-4-yloxy) diphenylsilyl] trimethyl stannane	91.94	$C_{21}H_3OSiSn$	446
11.08	*N*-*n*-Propyl-[2-(4-chlorophenyl) imidazo[1,2-a] pyridin-3-yl] acetamide	98.73	$C_{18}H_{18}ClN_3O$	327
19.35	3-(*o*-Anisyl)-3-phenyl propenal	77.47	$C_{16}H_{14}O_2$	238
27.62	4(Phenylsulfonyl)-2 methyl-1-hydroxy undecan-3-one	75.23	$C_{18}H_{28}O_4S$	340
28.04	10,15-Dimethyl-11,12,13,14-tetrahydro-1,6-methano[18]-annulene	78.85	$C_{21}H_{18}$	270
29.16	N1-*tert*-Buoxycarbonyl-N2-(benzyloxycarbonyl)-3-methyl butan-1,2-diamine	88.01	$C_{18}H_{28}N_2O_4$	336
31.43	Butyl 2-amino-5-methyl benzoate	97.55	$C_{12}H_{17}NO_2$	207
31.88	4-Chloro-3-diethoxy methyl coumarin	78.33	$C_{14}H_{15}ClO_4$	282
39.99	2-Bromo-1,4,5-trimethoxy naphthalene	94.34	$C_{13}H_{13}BrO_3$	296
41.47	(3aS(*),9á)-(+-)-9-Acetoxy-5,6,8,9-tetrahydro-1-hydroxy-4H cyclopenta[a][1,3]dioxolo[4,5-h]pyrrolo[2,1-b][3] benzazepin-2(3H)-one	96.49	$C_{19}H_{19}NO_6$	357
42.83	3-Chloro-2,2,5,8-tetra hydroxy-3,6,7-trimethy l-2,3-dihydro -1,4-naphthoquinone	40.49	$C_{13}H_{13}ClO_6$	300
45.70	5-Methyl-bis(1′,3′-phenylene)-32-(crown-10)	94.51	$C_{29}H_{42}O_{10}$	550
48.94	5,6-Dihydro-4H-benzo[de]-7,9-dichloro-2,5-dimethyl [2,7]naphthyridin-1-one	51.99	$C_{13}H_{12}Cl_2N_2O$	282

The compound, 4-chloro-3-diethoxy methyl coumarin was identified at the retention time of 31.88 and it has the probability about 78.33. The identified compound has coumarin as their functional group and several studies revealed that the coumarin (2H-L benzopyran-2-one) and its derivatives are naturally found in plants and some of them were isolated from microorganisms, for example, novobiocin, coumermycin A1, and chlorobiocin [36].

Two carbazole containing compounds such as 5,6-dihydro-4H-pyrazino [3,2,1-j,k] carbazole and 4-ethyl-3-methyl-9H-carbazole-2-carboxylate were identified from the extract of isolate 33 at the retention time about 44.80 and 48.61 respectively. This carbazoles are aromatic heterocyclic organic compounds and many carbazole alkaloids have been isolated from various plants and microorganisms [37].

The important group of quinone rich compounds was identified through GC–MS analysis from the potential isolate 33. The compound identified from the sample was named as 3-chloro-2, 2, 5, 8-tetrahydroxy-3, 6, 7-trimethyl-2,3-dihydro-1,4-naphthoquinone along with the quinone as their one of the functional group. This quinone groups are widely distributed in nature and they are also synthesized by some actinomycetes [38]. The bicyclic aromatic quinones and 1,4 naphthoquinones are the most common type of naphthoquinone present in nature with potent activity in a variety of biological targets such as cytotoxic, antimicrobial, antifungal, antiviral, and antiparasitic activities.

Another one quinone containing compound was identified as 1,3-dimethoxy-8-methyl anthraquinone and it contains anthraquinone as their functional group. This Anthraquinone is another important group of compounds having diverse biological activities. When compared with the literature, the closely related compound was identified as 1,8-dihydroxy-2-ethyl-3-Methylanthraquinone and it is Polyketide group of compound produced by *Streptomyces* sp. FX-58 [39], and this compound exhibit cytotoxic activity against cancer cell lines through various pathways inducing apoptosis [40].

Conclusion

In the present study, totally 9 actinomycete isolates were isolated and all the isolates were tested for their antimicrobial activity. Of these 9 strains, one isolate number 33 picked out from the rest, because it exhibited significant activity towards tested bacterial and fungal pathogens. The identification of isolate 33 to the genus level resulted in the discovery of the potential strain, *Nocardiopsis* sp. and it was the family Thermonosporaceae is a non-streptomycete group of actinomycete. An attempt was made to recognize the chemical nature of the compounds produced by the effective isolate 33 and it was successfully determined by GC–MS. The GC–MS result of ethyl acetate extract showed 18 considerable compounds with different retention time intervals. Of these identified compounds, some compounds were previously determined and studied with their antifungal and cytotoxic properties and thus indicates the presence of antimicrobial compound in the isolate MK_MSt033. The identified compounds were used with their diverse derivatives in the pharmaceutical and agro industries and it showed significant biological activities such as antagonistic agents, pharmacological agents, neurological agents and enzyme inhibitors, agrobiologicals and regulatory activities. Thus, it is established that the soil inhabiting actinomycetes have a countless potential to biosynthesis of secondary metabolite against microbial pathogens.

Competing interests

None declared.

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