Asian Journal of Chemistry; *Vol. 36, No. 6 (2024), 1315-1320*

ASIAN JOURNAL OF CHEMISTRY

https://doi.org/10.14233/ajchem.2024.31441

Synthesis and Chemical Characterization of Secondary Metabolites of Weeds using FTIR and NMR Techniques

Syed Khalida Izhar[1], Shareen Fatima Rizvi[1], Aamina Shahab[1], Aliya Firdaus[1], Rohit Sharma[2], Shazia Qamar[1], Saba Siddiqui[3], Arshi Siddiqui[1] and Uzma Afaq[1,*]

[1]Department of Biosciences, Integral University, Lucknow-226026, India
[2]Department of Biotechnology Engineering and Food Technology, University Institute of Engineering, Chandigarh University, Chandigarh-160014, India
[3]Integral Institute of Agricultural Science & Technology, Integral University, Lucknow-226021, India

*Corresponding author: E-mail: uzmaafaq89@gmail.com; uzmaafaq@iul.ac.in

Received: 26 February 2024; *Accepted*: 5 April 2024; *Published online*: 31 May 2024; AJC-21643

Soil microorganisms have the ability to produce bioactive chemicals that can be utilized in the development of a bioherbicide for the purpose of biological weed control. A total of 34 bacterial strains were isolated and tested for their herbicidal activity against both grass and broadleaf weeds. From this group, six effective isolates were chosen for the purpose of characterization and identification. The actinomycete isolates were identified as *Microbacterium* sp and *Streptomyces* sp. based on an analysis of their morphological, biochemical and physiological characteristics. Out of the initially chosen 6 strains, the bacterial fermentation broths from one particular isolate strain exhibited herbicidal activity that resulted in a 95% reduction in the growth of weed when compared to the control. The *Streptomycetes* isolates underwent a germination inhibition assay to determine the presence of herbicidal properties. A total of 8 crop seeds were examined for herbicidal efficacy using *Streptomycetes* isolates and no growth inhibition was observed in the crop seeds. The herbicidal activity of *Streptomyces* isolates was tested on four weed seeds. *Streptomyces* inhibits the growth of nut grass. The current investigation establishes that *Streptomyces* isolates has the potential to function as a bioherbicide against nut grass. The strain SKI-[Z] was shown to have a 99% resemblance to *Actinobacterial* spp. through phylogenetic study of 16S rRNA gene sequencing. The FT-IR spectrum shows alkynes (-C≡C), alkenes (=C-H), amines (N-H), aliphatic amine (C-N) and alkanes (-C-H) and the NMR peak having ketone groups (C=O), di-ketone group (>C=O), ether (R-O-R) and aldehyde group (-CHO). Furthermore, upon ^{1}H NMR analysis of the secondary metabolites, the prominent peaks intensities at δ 2.4, 3.68 and 4.1 ppm correspond to the ketone groups (C=O), di-ketone group (>C=O), ether (R-O-R) and aldehyde group (-CHO) were found.

Keywords: Weeds, Actinomycetes, Bioherbicides, Biological control, Spectroscopic analysis.

INTRODUCTION

Weeds are highly adaptable species that quickly adjust their behaviour through phenological plasticity to better suit prevailing conditions [1]. Farmers and weed scientists are increasingly worried about recent advancements in weed control technology. The economic losses caused by weeds in crop production are among the highest compared to other pests. Weeds still cause major crop losses, even if weed control measures have gotten better. The use of herbicides is expected to rise because of the increased focus on decreased tillage and low-input agriculture. Growers typically employ herbicides once infestation becomes apparent, hence postemergence herbicides are expected to capture a larger portion of the market. Due to their exceptional capacity to generate useful secondary metabolites, including agro-active chemicals, *Actinobacteria* are highly suitable for screening studies, particularly in the field of biological weed control [2,3]. A literature survey indicates that report on bioherbicides from actinobacteria is minimal and reported Herbimycin, an antibiotic *Streptomyces* strains [4,5]. Dhanasekaran *et al.* [6] reported that bioherbicidal activity of *Streptomyces* isolates against *Ehinochilora crusgalli.* Keeping these points in view, the present study has been undertaken with the objectives of screening of actinobacteria for herbicidal

This is an open access journal, and articles are distributed under the terms of the Attribution 4.0 International (CC BY 4.0) License. This license lets others distribute, remix, tweak, and build upon your work, even commercially, as long as they credit the author for the original creation. You must give appropriate credit, provide a link to the license, and indicate if changes were made.

activity against the selected weeds and characterization and identification of bioherbicides producing actinobacteria.

Cyperus rotundus, also known as nutgrass or purple nutsedge, is a perennial weed which is notorious for its detrimental effects in several locations. *C. rotundus* is native to Africa, but it has spread to several places worldwide and is considered a problematic invasive species in many agricultural and horticultural settings [7]. Studying the herbicidal efficacy of actinomycetes extracted from crop soil against *C. rotundus* is the purpose of this study.

Attempts to explore the potential of actinobacteria as a source of bioherbicides and several studies have identified actinobacterial strains that produce herbicidal compounds. The development of bioherbicides involves screening and isolating actinobacterial strains, identifying the active compounds responsible for herbicidal activity and optimizing production methods. Nevertheless, it is essential to acknowledge that the utilization of bioherbicides is still in its early phase of advancement and their widespread use in the market may necessitate additional investigation, experimentation and regulatory approval. Additionally, the efficacy of bioherbicides can vary depending on environmental conditions, target weed species and application methods.

EXPERIMENTAL

The weed seeds were collected from ICAR-Directorate of Weed Research, Jabalpur, India. The rhizospheric soil samples were collected from different geographical locations as listed in Table-1 using a sterile spatula. The collected rhizospheric soil samples were placed in sterilized plastic bags and then stored under a low temperature (at 4 ºC) environment until processing for isolation [8].

Isolation: Each pre-treated soil sample (1 g) was vigorously agitated in a test tube containing 9 mL of distilled water at room 25 ± 2 ºC. For every soil sample, the serial dilution method was used to prepare dilutions up to 10^{-9}. A 20 µL from dilution 10^{-6} to 10^{-9} were spread on actinomycetes isolation agar (AIA) [9].

Screening of actinobacteria through morphological analysis: The actinobacteria colonies, characterized by their white and powdery appearance, were isolated and subsequently subjected to analysis of their morphological properties upon being introduced to a specific agar media. After isolation of the pure colonies, each colony was identified based on their colonial morphology, the colour of hyphae, the colour of aerial mycelium and microscopy. Subsequently, each of them was placed separately on a designated agar medium to ensure their short-term preservation. Only bacteria that are classified as Gram-positive were selected for additional screening [10].

Biochemical identification of actinobacteria: Following initial investigations, all isolates strain that exhibited positive results based on their morphology and Gram staining were chosen for further biochemical analysis. The biochemical tests used for identification were the indole production, methyl red, catalase, casein hydrolysis, starch hydrolysis, motility, Voges-Proskauer test and carbohydrate fermentation [8].

Initial assessment of antibacterial activity of the identified isolates: The actinobacterial isolates on Mueller Hinton agar for bacteria and potato dextrose agar for fungi using the Perpendicular streak method were streaked and then incubated the plates at 28 ºC [11]. After making the actinobacteria streaks perpendicular to the already streaked pathogenic strains were incubated at 30 ºC. In accordance with Bano *et al.* [12], the zone of inhibition was evaluated 72 h after incubation for fungus and 24 h after incubation for bacteria.

Secondary antimicrobial screenings of positive isolates: Further, the positive isolates were single-streaked on agar to check their antimicrobial activity through the good diffusion method. The well diffusion method was used to study the effects of pathogenic bacteria and listed in Table-2.

Molecular characterization using 16S rRNA amplification of isolated Actinobacterial strains: The molecular characterization was carried out at Bio-kart India Pvt. Ltd., Bangalore, India and involved PCR amplification, sequencing and restriction analysis with a 16S rRNA sequence amplification. The 16S ribosomal sequence amplification was conducted using the primers F243 (5' GGATGAGCCCGCGGCCTA

TABLE-1
MORPHOLOGICAL, BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF THE BACTERIAL STRAIN SKI-[Z]

Morphological tests	Bacterial strain SKI-[Z]	Biochemical test	Bacterial strain SKI-[Z]	Physiological tests	Bacterial strain SKI-[Z]
Gram staining	Positive	Carbohydrate fermentation	+	Optimum temperature for growth	27 °C
Pigmentation	Yellow	Casein	+	Growth at NaCl (%)	1
Surface	Smooth	Indole	+	Optimum pH for growth	6.5
Elevation	Flat	Methyl red	+	Optimum time	24 h
Cell shape	Log-like fragmented hyphae	Voges-Proskauer	+	Optimum rpm	150
		Catalase	+		
Growth	Well grown	Motility	–		
		Urease	+		

TABLE-2
ACTIVITY OF ACTINOBACTERIAL STRAINS SKI-[Z] FOR AGRICULTURAL CROP BY MOIST CHAMBER TECHNIQUE

Plants tested	*Triticum aestivum*	*Oryza sativa*	*Vigna radiata*	*Cicer arietinum*	*Len culinaris*	*Cicer arietinum*	*Pisum sativum*
SKI-[Z]	–	–	–	–	–	–	–

3') and 1378R (5' CGGTGTGTACAAGGCCCGG 3'). The next step was to use the neighbor-joining DNA distance technique in MEGA6 to build a phylogenetic tree [13].

The technique of 16S rRNA sequence amplification served as a means of molecular characterization for conducting secondary screening of the isolates were chosen following the initial screening process. The strains were classified further using the BLAST program in the GenBank database. Isolate SKI-[Z] has had its partial 16S rRNA gene sequence submitted to the NCBI GenBank (OR681911).

Secondary metabolite production and extraction: The process of submerged-state fermentation was used for the production of secondary metabolites by the actinobacteria, following the methodology outlined by Salim *et al.* [14] with certain modifications. An inoculum of 5 mL of SKI-[H], SKI-[Z] and SKI-[X] strain suspension was added into 1000 mL of ISP-2 broth (containing 5 g of yeast extract, 10 g of malt extract, 6 g of dextrose and a pH of 7.3). The combination was thereafter left to incubate for 25 days at 150 rpm [15]. The fermented broth was centrifuged for 10 min at 4 ºC and 10,000 revolutions per minute to extract secondary metabolites. The resultant supernatant was vigorously combined with ethyl acetate in a 1:1 proportion and agitated at 180 rpm for 10 h. The upper layer, which included bioactive chemicals, was collected using a separating funnel. Subsequently, the layer was subjected to rotary vacuum evaporation under the condition's temperature of 45 ºC and a rotation speed of 200 rpm. In this procedure, the solvent was removed to obtain the extract in its crude form. To prepare the stock solution, 1 mL of methanol was added to the crude extract. The mixture was then preserved for further analysis.

Plate assay of herbicidal activity of selected actinobacterial strain: The weed seeds (*Cyperus rotundus*, *Lantana camara*, *Chenopodium album*, *Aveana fatua* L., *Cuscuta* spp. and *Phalaris minor* Retz.) and crop seeds (*Triticum aestivum*, *Oryza sativa*, *Vigna radiata*, *Cicer arietinum*, *Len culinaris*, *Cicer arietinum* and *Pisum sativum*) were positioned on the petri plates covered with absorbent cotton, which was thoroughly saturated with the prepared secondary metabolite stock solution from SKI-[H], SKI-[Z] and SKI-[X] strains, respectively followed by incubation in a growth cabinet at 28 ºC. The herbicidal activity was detected after 5 days of incubation [7].

Paper towel assay of herbicidal activity of selected actinobacterial strain: The rolled paper towel assay was employed to assess the effectiveness of applying secondary metabolites from SKI-[H], SKI-[Z] and SKI-[X] strains onto crop and weed seeds to examine the suppression of seed growth. *Streptomyces* secondary metabolite stock solution was acquired by saturating a 10 days old culture of starch casein agar plates with sterile distilled water. The seeds of crops and weeds were sterilized on the surface and then immersed in *Streptomyces* spore suspensions for 30 min. Afterwards, they were dried in a laminar flow hood. The seeds were dispersed onto a dampened, sterilized 12 cm × 30 cm paper towel. The towel was rolled, enclosed in a plastic bag and subjected to incubation for 5 days at ambient temperature. Each treatment utilized three towels and the experiment was conducted on three separate occasions [6].

Media trails: *C. rotundus* seeds were washed with 5% sodium hypochlorite for disinfection of seeds. Murashige and Skoog (MS) media were autoclaved and supplemented with 100 mL/L cell-free filtrate of SKI-[Z], SKI-[H] and SKI-[X] cultures after complete cooling. The disinfected seeds were sowed in the resulting media once completely solidified. The flasks were incubated in the plant tissue culture (PTC) lab at 25 ºC for 72 h under dark conditions. The flasks were transferred to the light area of the PTC and left for 10 days at 30 ºC [16].

RESULTS AND DISCUSSION

The utilization of chemical pesticides has been employed to manage the growth of unwanted plants and insects. However, this practice has led to the development of resistance in weeds, making them more challenging to control [9]. Furthermore, several researches have been undertaken to ascertain the compounds generated by microbes as potential substitutes for man-made chemicals. *Streptomyces* is a prominent and extensively researched bacteria that resides in soil.

The pretreatment of soil samples with $CaCO_3$ ensured the termination of growth of the other undesirable microbes before further processing. The taxonomic characteristics of weeds including shoot, leaves, flower, stem, fruits and seed were documented and recognized as *Parthenium hysterophorous* L., *C. rotundus* L., *Aveana fatua* L., *Cuscuta* spp. and *Phalaris minor* Retz. The fully developed seeds and underground storage organs of the aforementioned undesirable plants were gathered, treated and subsequently utilized for their herbicidal properties.

SKI-[Z] demonstrated the maximum antimicrobial activity against almost all bacterial and fungal species compared to other isolates. The results of antimicrobial screening are summarized in Table-3.

Molecular characterization of the selected isolates using 16S rRNA Sequence amplification: The phylogenetic analysis verified that the isolates were classified under the *Streptomyces* genus SKI-[Z] as *Streptomyces griseus* with a similarity of 98%. The isolate SKI-[Z]'s partial 16S rRNA gene sequence has been recorded in the NCBI GenBank under the accession number OR681911. The strain's phylogenetic tree can be observed in Fig. 1 on the NCBI platform.

Characterization of secondary metabolite extract of SKI-[Z]: An investigation using GC-MS was conducted on

TABLE-3
RESULTS OF ANTIMICROBIAL SCREENING AGAINST PATHOGENIC BACTERIA AND FUNGI

Pathogenic strain	*E. coli*	*P. aeruginosa*	*K. pneumoniae*	*B. subtilis*	*S. aureus*	*A. niger*	*A. flavus*	*A. parasiticus*
SKI-[Z]	+++	++++	++	+++	+++	+++	+++	+++

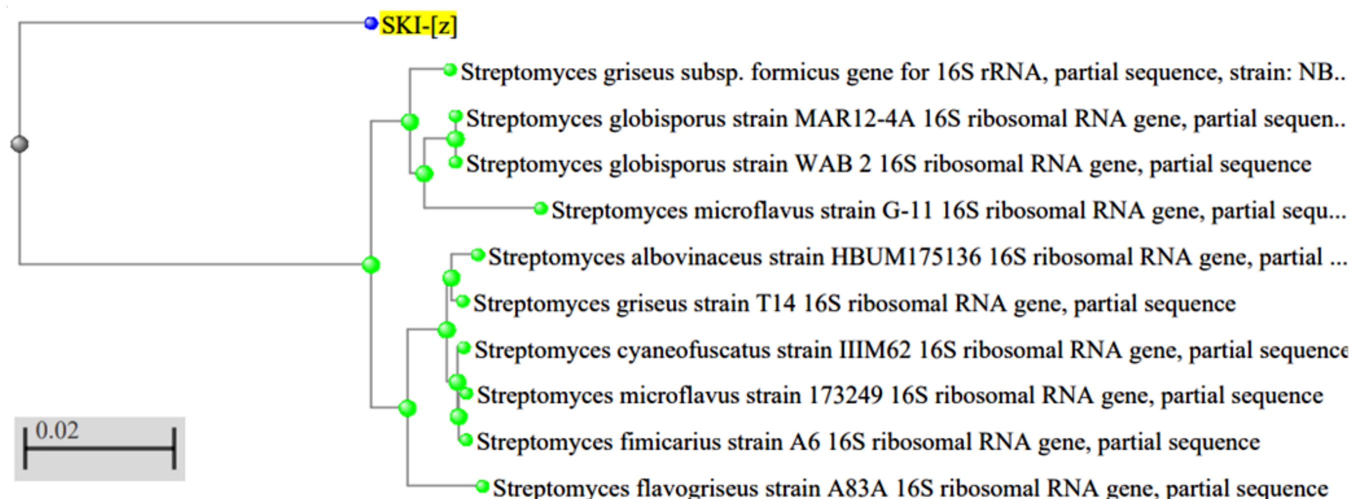

Fig. 1. Phylogenetic tree of *Streptomyces griseus* SKI-[Z] drawn using UPGMA tree (MEGA X software) with the evolutionary distances showing the relationship of Actinobacteria with the known sequences of related genera

the metabolite extracts of SKI-[Z]. Several parameters such as peak area, molecular weight, formula and similarity index, were examined to identify the compounds. Fig. 2 displays the GC-MS analysis of the ethyl acetate extract obtained from *Streptomyces griseus* SKI-[Z]. The compounds derived from the GC-MS analysis are presented in Table-4.

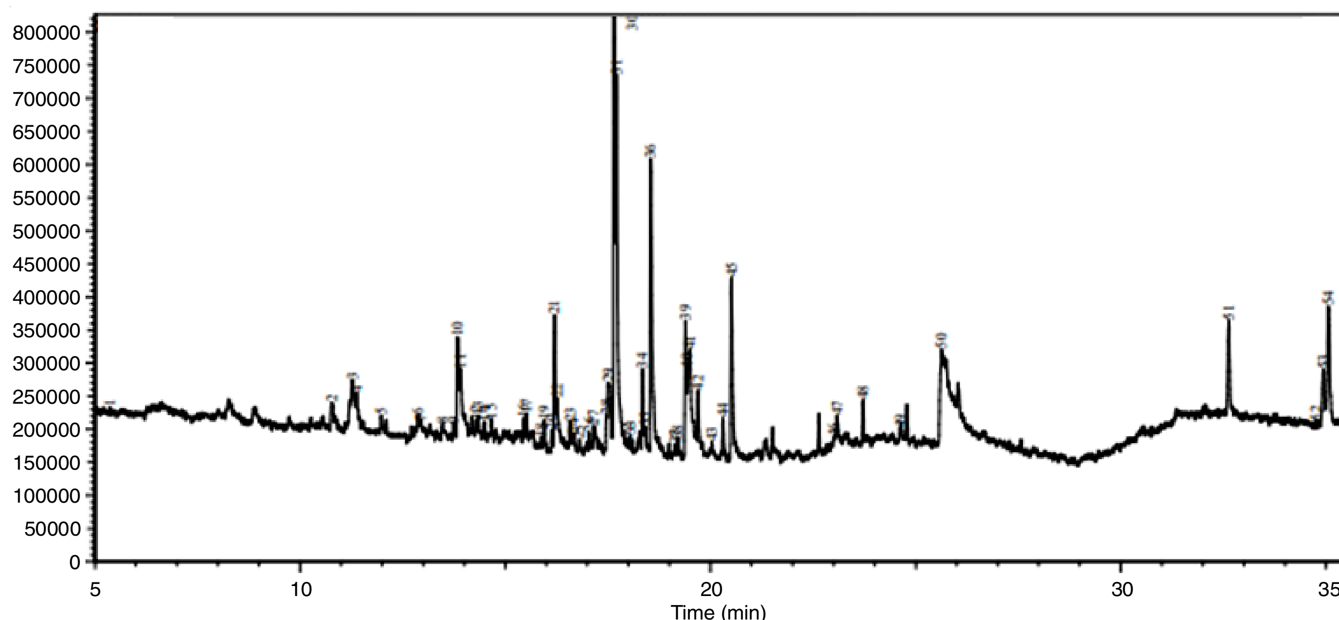

Fig. 2. GC-MS spectrum shows the secondary metabolite extract obtained from *Streptomyces* sp. *Streptomyces griseus* SKI-[Z]

TABLE-4
IDENTIFICATION OF THE CHEMICAL COMPOUNDS AND THEIR CORRESPONDING BIOLOGICAL ACTIVITY IN ACTINOBACTERIAL EXTRACT SKI-[Z] USING GC-MS ANALYSIS

Peak	Rotational time (min)	Area (%)	Name	m.w. (g/mol)	m.f.	Similarity index (%)
30	17.650	11.94	Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$	98
31	17.709	11.73	Benzenepropanoic acid	292	$C_{18}H_{28}O_3$	92
36	18.546	10.38	*cis*-10-Heptadecenoic acid, methyl ester	282	$C_{18}H_{34}O_2$	91
45	20.511	5.14	*cis*-10-Nonadecenoic acid, methyl ester	310	$C_{20}H_{38}O_2$	95
21	16.202	3.32	1-Nonadecene	266	$C_{19}H_{38}$	90
39	19.389	3.56	9,12-Octadecadienoic acid (Z, Z)-, methyl	294	$C_{19}H_{34}O_2$	94
50	25.635	3.75	13-Docosenamide, (Z)-	337	$C_{22}H_{43}NO$	88
51	32.628	2.75	Phenol, 2,4-bis (1,1-dimethyl ethyl)-, phosphite (3:1)	646	$C_{42}H_{63}O_3P$	89
54	35.059	5.58	*Tris*(2,4-di-*tert*-butyl phenyl) phosphate	662	$C_{42}H_{63}O_4P$	95
10	13.839	3.34	*n*-Pentadecanol	228	$C_{15}H_{32}O$	93

The characterization of the secondary metabolite obtained from SKI-[Z] exhibit a strong and broad absorption at 3304 cm^{-1} is attributed due to carboxylic acid (O-H) group in the FT-IR spectrum. Another broad peak at 2941.88 cm^{-1} implies the presence of alkanes (C-H) and 2830 (-CHO) aldehydic group. Moreover, the peaks at 2359, 1449, 1114, 1020 and 628.86 cm^{-1} indicate the presence of alkynes (-C≡C), alkanes (-C-H), aliphatic amine (C-N), alkenes (=C-H) and amines (N-H), respectively (Fig. 3).

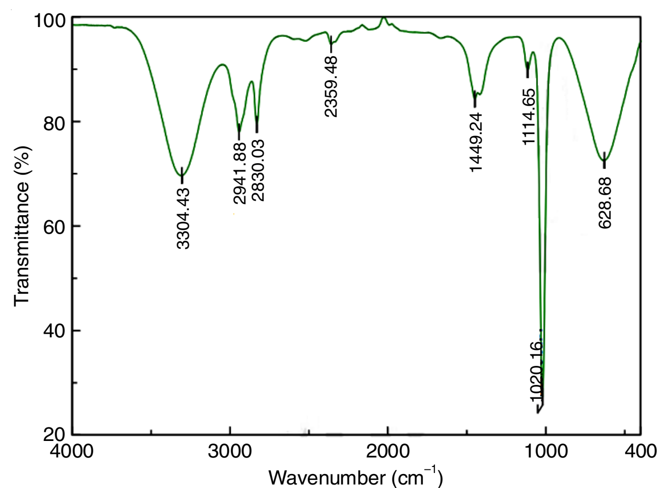

Fig. 3. FTIR of secondary metabolite of SKI-[Z]

^{1}H NMR spectral studies: Secondary metabolites obtained after each trial were characterized using ^{1}H NMR spectroscopy. Fig. 4 shows the prominent peaks of SKI-[Z] at δ 2.4, 3.68 and 4.1 ppm are due to ketone groups (C=O), di-ketone group (>C=O), ether (R-O-R) and aldehyde group (-CHO), respectively.

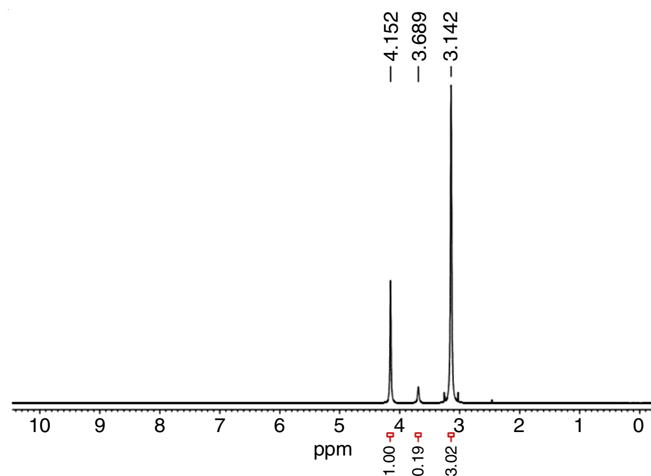

Fig. 4. NMR of secondary metabolite of SKI-[Z]

Present investigations found that 5-10% of actinomycetes synthesized secondary compounds having herbicidal capabilities, aligning with the findings. The taxonomic investigations revealed that SKI-[Z] shares a 98% identity with *Streptomyces griseus*. This strain synthesizes a distinctive antibiotic compound that contains sulphur. Additionally, it assists in the prevention of lactic acidosis in animals. Reports that one *Streptomyces* species is capable of producing over 25 distinct active chemical kinds [13]. Against *C. rotundus*, SKI-[Z] exhibited strong herbicidal activity in this investigation. The distinct modes of action exhibited by secondary metabolites synthesized by soil actinomycetes make them promising candidates for the development of herbicides. Preliminary results of this research indicate that the extract obtained from the actinobacterial isolate *Streptomyces* sp. can function as a bioherbicide, effectively managing the proliferation of weed *C. rotundus.* Nevertheless, this study could not establish definitive data about the mechanism through which the *Streptomyces* sp. extract demonstrates bioherbicidal activity.

Conclusion

A recent study focusing on the *Streptomyces* isolates sheds light on their potential in combating such notorious weed, *Cyperus rotundus*, commonly known as nutgrass. From the soil samples, 34 bacterial strains were isolated and evaluated for their herbicidal activity against grass and broadleaf weeds. As analyzed through morphological, biochemical and physiological features, the researchers identified six potent isolates belong to the genera *Microbacterium* spp. and *Streptomyces* spp. One particular strain demonstrated remarkable herbicidal activity, leading to a striking 95% reduction in the growth of *Cyperus rotundus* compared to untreated controls. This finding marked a significant breakthrough in the quest for effective bioherbicides targeting this resilient weed species. Further experiments were performed to investigate the herbicidal capabilities of the *Streptomyces* isolates, while the crop seeds showed no signs of growth inhibition in the presence of these isolates, but a significant suppression of weed seed germination was observed.

ACKNOWLEDGEMENTS

The authors acknowledge Department of Science and Technology (DST-FIST) project number [SR/FST/LS-1/2017/13(C)] for providing funds to establish the Central Instrumentation Facility at Integral University. A competent authority of the University has approved the manuscript and the assigned communication number is IU/R&D/2024 MCN0002485.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

REFERENCES

1. D.R. Clements and V.L. Jones, *Agronomy*, **11**, 284 (2021); https://doi.org/10.3390/agronomy11020284
2. C.-I. Mawang, A.-S. Azman, A.-S.M. Fuad and M. Ahamad, *Biotechnol. Rep.*, **32**, e00679 (2021); https://doi.org/10.1016/j.btre.2021.e00679
3. P. Sharma and D. Thakur, *Sci. Rep.*, **10**, 4104 (2020); https://doi.org/10.1038/s41598-020-60968-6
4. S. Omura, Y. Iwai, Y. Takahashi, N. Sadakane, A. Nakagawa, H. Oiwa, Y. Hasegawa and T. Ikai, *J. Antibiot. (Tokyo)*, **32**, 255 (1979); https://doi.org/10.7164/antibiotics.32.255

5. Y. Li, Z. Sun, X. Zhuang, L. Xu, S. Chen and M.Li, *Crop Protect*., **22**, 247 (2003); https://doi.org/10.1016/S0261-2194(02)00189-8
6. D. Dhanasekaran, K. Ambika, N. Thajuddin and A. Panneerselvam, *Arch. Phytopathol. Pflanzenschutz*, **45**, 505 (2012); https://doi.org/10.1080/03235408.2011.587988
7. D. Dhanasekaran, N. Thajuddin and A. Panneerselvam, *Nat. Prod. Res.*, **24**, 521 (2010); https://doi.org/10.1080/14786410802299281
8. Q. Li, X. Chen, Y. Jiang and C. Jiang, eds.: D. Dhanasekaran and Y. Jiang, Morphological Identification of Actinobacteria, In: Actinobacteria: Basics and Biotechnological Applications, Intech; Rijeka, Croatia, pp. 59–86 (2016).
9. G.H. Puttaswamygowda, S. Olakkaran, A. Antony and A.K. Purayil, Present Status and Future Perspectives of Marine Actinobacterial Metabolites, In: Recent Developments in Applied Microbiology and Biochemistry, Academic Press, Chap. 22, pp. 307–319 (2019).
10. A. Sapkota, A. Thapa, A. Budhathoki, M. Sainju, P. Shrestha and S. Aryal, *Int. J. Microbiol.*, **2020**, 2716584 (2020); https://doi.org/10.1155/2020/2716584
11. P. Ganesan, A.D. Reegan, R.H.A. David, M.R. Gandhi, M.G. Paulraj, N.A. Al-Dhabi and S. Ignacimuthu, *Alex. J. Med.*, **53**, 101 (2017); https://doi.org/10.1016/j.ajme.2016.03.004
12. S.A. Bano, S. Naz, B. Uzair, M. Hussain, M.M. Khan, H. Bibi, U. Habiba, S. Nisa and M. Israr, *Braz. J. Biol.*, **83**, e245585 (2023); https://doi.org/10.1590/1519-6984.245585
13. N. Bano, S. Siddiqui, M. Amir, Q. Zia, S. Banawas, D. Iqbal and Roohi, *Saudi J. Biol. Sci.*, **29**, 1858 (2022); https://doi.org/10.1016/j.sjbs.2021.10.038
14. F.M. Salim, S.A. Sharmili, J. Anbumalarmathi and K. Umamaheswari, *J. Appl. Pharm. Sci.*, **7**, 69 (2017); https://doi.org/10.7324/JAPS.2017.70909
15. X. Gong, W. Xiang, X. Cao, Y. Yu, Y. Hao, L. Li, Q. Wang, H. Zou and C. Qian, *Antonie van Leeuwenhoek*, **113**, 2053 (2020); https://doi.org/10.1007/s10482-020-01477-4
16. Q. Li, X. Chen, Y. Jiang and C. Jiang, eds.: D. Dhanasekaran and Y. Jiang, Cultural, Physiological and Biochemical Identification of Actinobacteria, In: Actinobacteria: Basics and Biotechnological Applications, Intech; Rijeka, Croatia: pp. 87–111 (2016).