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Original Article

Antimicrobial Activities of the Secondary Metabolite Extracted from *a Nocardia* Strain

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Abstract

Background & Objective: The emergence of antibiotic-resistant bacteria and limited treatment options for these infections is a challenging problem in the world. There is increasing interest in isolating bioactive molecules from actinobacteria and elucidating their antimicrobial mechanisms at the moment. The current study aimed to investigate the antimicrobial activities of a *Nocardia* strain (N4) that was previously isolated from the soil samples in Tehran, Iran.
Materials & Methods: The cultural, morphological, and physiological characteristics of the strain were determined and the identity of the strain was confirmed using 16SrRNA gene sequencing. The strain was screened for antimicrobial metabolite production. The in vitro anti-microbial activity of the extracted metabolite was assessed by agar well diffusion and minimum inhibitory concentrations methods. Finally, the structure of the extracted metabolite was confirmed by HPLC and mass spectrometry methods.
Results: The isolate shared 99% sequence similarity with *Nocardia soli*. The strain showed broad-spectrum antimicrobial activities against some pathogenic bacteria (*Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Klebsiella pneumoniae ATCC 700603, Salmonella Typhimurium ATCC 14028* and *Shigella sonnei RI366*) and fungi (*Aspergillus niger ATCC 1015* and *Aspergillus fumigatus ATCC 1022*) in the agar well diffusion method. The ethyl acetate extract of the strain exhibited the highest antimicrobial activity against *E. coli* (MIC= 4.67±3.055 µg/mL) and *K. pneumoniae* (MIC=3.33±1.155 µg/mL). The metabolite showed high similarity with $C_21H_{28}N_7O_{14}$.
Conclusions: The current study confirmed the presence of a bioactive metabolite in strain N4, suggesting that this may be a promising candidate for discovering bioactive components with broad antibacterial activities against a variety of pathogens.

Keywords: *Nocardia soli*; antimicrobial activity; HPLC; Bioactive metabolite

Introduction

The widespread incidence of microbial infections and persistent usage of antimicrobial agents have led to the emergence of multidrug-resistant (MDR) strains (1). Multidrug-resistance bacteria and fungi with high morbidity and mortality rates in humans have been reported to have spread widely in recent years (2, 3). Additionally, hospitalization time and associated costs are significantly longer in patients with MDR bacteria compared to those with susceptible strains (4). The widespread occurrence of nosocomial infections following the emergence of MDR pathogens such as *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Pseudomonas aeruginosa, Acinetobacter baumannii,*

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and *Enterobacter* species have been a major critical public health concern in recent decades (5).

Although antibiotics have saved many lives against infectious diseases, they can contribute to the increase in antibiotic resistance (6). There is a great need to develop potent, sustainable, and broad-spectrum antimicrobial compounds and metabolites with biological significance for potential use against MDR pathogenic bacteria (7, 8). A common strategy is to identify novel antimicrobial metabolites produced by various microorganisms isolated from different environments. Soil microorganisms are known as excellent resources for the isolation and identification of products with therapeutic importance. Among these, the actinomycetes represent a broad range of valuable and prominent source of pharmaceutically active metabolites (9). Actinobacteria are the most abundant source of such secondary metabolites with pharmaceutical properties, such as antibacterial, antiviral, anticancer, antiparasite, and immunosuppressive activity (10, 11). Approximately, 75% of commercially available antibacterial agents are produced by the dominant genus *Streptomyces* (12). However, rare actinomycetes, such as the genus *Nocardia*, have recently garnered attention as a potential source of bioactive metabolites and antibiotic molecules (13, 14). Numerous interesting biologically active compounds such as *nargenicin* (15), *transvalencin* (16), and *nocardithiocin* (17) have been previously reported from the genus *Nocardia*. They are Gram-positive, aerobic, and partially acid-fast bacillus that has been reported in the genus (18). *Nocardia* sp. isolated from Indian soils produced bioactive metabolites that showed potent antibacterial activity against *S. aureus* and *Klebsiella pneumoniae* (19). Based on this, the present study was conducted to investigate bioactive metabolites in *Nocardia* strain isolated from soil sampled in Tehran, Iran, to determine its antibacterial and antifungal properties.

Materials & Methods

Isolation of an Actinomycetes Strain and Phenotypic Assay

Soil samples were collected at depths of 3–5 cm below the surface from randomly selected districts in Tehran. The soil samples were placed in sterile zip-lock bags and stored at 4 °C. Five grams of soil sample were suspended in the physiological water (0.9% NaCl) to prepare 10-fold serial dilutions (from 10^{-3} to 10^{-5}). The dilutions were cultured separately on actinomycete isolation agar medium (AIA) and Potato Dextrose Agar (PDA) medium (Merck KGaA, Darmstadt, Germany). Then, the plates were incubated at 28° C for 1-4 weeks. Cream to white-colored colonies were cultured on nutrient agar, sabouraud dextrose agar, and blood agar media (Merck, Germany), then subjected to paraffin baiting technique in order to isolate *Nocardia* strains (20). During the screening of *Nocardia* spp. N4 strain was isolated and investigated for further analysis. The pure culture of the isolate was sub-cultured and maintained in agar casein starch with 10% glycerol.

Preliminary Screening of Antimicrobial Activity

Antibacterial activity of the N4 isolate against several Gram-positive and Gram-negative human bacterial and fungal pathogens (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, Methicillin-Resistant *Staphylococcus aureus* [MRSA] ATCC 33591, *Staphylococcus saprophyticus* ATCC 15805, *Shigella sonnei* ATCC 9290, *Klebsiella pneumoniae* ATCC 700603, *Shigella dysentery* RI 366, *Salmonella Typhimurium* ATCC 14028, *Enterococcus faecalis* ATCC 51299, and *Bacillus cereus* ATCC 11778, *Aspergillus niger* ATCC1015, *Candida. Albicans* ATCC10231, and *Aspergillus fumigatus* ATCC1022) was investigated using the cross-streak method based on the previously described method (21). The growth of bacterial and fungal pathogens was assessed. The aim was to identify strains that did not show any growth on the plate.

Biochemical Assays

The N4 strain was cultured on nutrient blood agar. Biochemical tests performed in this study include Gram, partially acid-fast, and acid-fast

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staining, growth in lysozyme broth (resistance to lysozyme), hydrolysis of casein, gelatin, tyrosine, urea, xanthine, and hypoxanthine, production of acid from carbohydrates, nitrate reduction test, and Simmons citrate test (22, 23).

Phylogenetic Analysis

DNA extraction of N4 isolate was performed using the extraction kit (AccuPrep Genomic DNA Extraction Kit Cat. No. K-3032, Bioneer Corporation, Korea). A set of primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Bioneer, South Korea) was used to amplify the fragment of the 16S rRNA gene (24, 25). CR amplification was carried out with an initial denaturation of 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and an extension at 72 °C for 10 min. The PCR product was checked in 1% agarose gels (Cinnagen Inc, Iran) and assessed in a LED transilluminator (ETS Vilber-Lourmat, France). After purification, 20 µl of the PCR products with 5 µl (0.1 concentration) of forward primer was sent to the sequencing service (Life BioScience, UK). The sequence quality was tested using Finch TV software version 1.4.0. The phylogenetic tree was constructed using MEGA version 7 to show the relationship between the isolate and other isolates using the Neighbor-Joining (NJ) method.

Extraction of Antimicrobial Metabolites

The N4 isolate was cultured in the 250 ml of brain heart infusion agar medium (BHIA, Merck, Germany) and kept on a shaker incubator (150 rpm) at 30º C for 5 days. Then, 10% of this medium was transferred to 1L flask containing 250 ml of Yeast Extract - Malt Extract (YEME) liquid medium and incubated at 30º C and 200 rpm on a rotary shaker for 7 days. After fermentation, the liquid medium was centrifuged at 12000 rpm for 4 minutes at 4º C. The supernatant was mixed with ethyl acetate (ratio of 1: 1) and maintained at room temperature for 1 hour. After forming two layers, the ethyl acetate phase was separated from the organic phase and removed. Finally, the organic phase containing the antibacterial metabolite was collected and concentrated in a rotary vacuum evaporator. Further purification was conducted using silica gel column chromatography (255 cm, Silica gel 60, Merck). Fractions showing antibacterial activity were combined and further purified using HPLC semi-preparative column as described previously (26).

Antibacterial Activity of Ethyl Acetate Extract Agar Well Diffusion Method

The antibacterial activity of the extract was assayed using the agar well diffusion method. The McFarland 0.5 turbidity standard of human pathogenic bacteria and fungi was swabbed on Mueller-Hinton agar plates. A well of 6 mm diameter was punched on agar plates and filled with 100 µl of the metabolites. The plates were incubated at 37º C overnight for bacteria and 30º C for 48 hours for fungi (27). The diameters of the inhibition zone were assessed and scored as follows: 0–5 mm as no inhibition (0); 6–10 mm as weak (1+); 11–15 mm as moderate (2+) and > 15 mm as strong antimicrobial activity (3+) (28, 29).

Minimum Inhibitory Concentrations (MICs) Method

Minimum inhibitory concentrations of antimicrobial metabolites were determined using the microdilution method in 96-well microtitre plates (30). 100 µl of sterile Mueller Hinton Broth was added to each well. Then, 100 µl of dilutions of the metabolite were added to the first well, and then serially diluted 1 10 down the rows of the plates. The inoculums of human pathogens and fungi were prepared (0.5 McFarland) and poured into each well. The plates were incubated at optimum temperature and time for each organism. Gentamicin and 1% DMSO were used as positive and negative controls respectively. The turbidity of each well was measured by the spectrophotometer (Jinoe, UK) at 620 nm.

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Inhibitory Effect of Metabolite on Fungal Pathogens

The inhibitory effect of metabolites against fungal strains was further investigated. The fungal suspension of *A. niger* and *A. fumigatus* which were treated with different concentrations of metabolite were incubated at 28º C for 94 hours. The fungal suspension was extracted using a reparatory funnel with previously weighted aluminum foil. The wet weight was determined for positive control and different concentrations of metabolite. Each sample was dried at 80º C for 3 hours and then weighed again to obtain the dry weight. The percentage of fungal growth (X) was obtained through the following formula:

The dry weight of the control sample * X = Dry weight of treatment group* 100

The percentage of fungal inhibition activity was obtained by subtracting X from100.

The Activity of Antimicrobial Metabolites in Rabbit Serum

MICs against *P. aeruginosa* were determined with some modifications. *P. aeruginosa* (10^6 cells/ ml) were inoculated into Luria–Bevrtani (LB) broth medium containing 20% rabbit serum, extracted metabolites (64 µg /ml), and Gentamicin (64 µg /ml) and incubated in a shaker incubator at 37º C for 5 hours. Then, the absorbance of the cells was determined at 600 nm. Then the growth curve of the bacteria was plotted (31). *Pseudomonas aeruginosa* was used as control.

Stability of the Antimicrobial Metabolites

The stability of the extract against temperature and Proteinase K enzyme was investigated based on the previously described method (32). Briefly, the extracted metabolite was exposed to 60 ° C and proteinase K (50 mg/ml) for 30 minutes. Then the antimicrobial activity was tested on nutrient agar plates.

Chemical Characterization of the Purified Compound

Crude extract of the N4 isolate was subjected to high-performance liquid chromatography (HPLC) on a C18, 3µm column with water: methanol (80:20) as mobile phase. The possible molecular formula and compound of the metabolite were determined under suitable conditions in Mass Spectra (26).

Results

Phenotypic and Phylogenetic Characteristics

The present investigation involved the isolation of a potential Nocardia strain from soil sample obtained in Tehran, Iran. After the incubation period, the morphology of emerged colonies was assessed based on colony characteristics and pigment production. The N4 strain was anaerobic, Gram-positive, with aerial mycelium. It also exhibited biochemical properties including hydrolytic activity on urea and nitrate reductase activity. The isolate was not able to grow well at 45°C (Table 1).

Table 1. Phenotypic characteristics of *N. soli* isolated from the soil

Tests	Results
Colony	
Soluble pigment	+
Aerial mycelium	+
Gram reaction	+
Acid-fast reaction	-
Partial acid-fast reaction	-
Enzyme production	

Nitrate reductase	+
Urease	+
Carbon source	
Citrate	+
Aesculin	+
Casein	-
Sorbitol	-
Tyrosine	-
Xanthine	-
Hypoxanthine	-
Tolerance to	
Lysozyme	+
Growth at temperature	
45 °C	-

The 16S rRNA nucleotide sequences of N4 isolate were compared with nucleotide sequence retrieved from the GenBank database. The phylogenetic analysis of 16S rRNA gene sequence of N4 strain showed the highest homology with *N. soli* (AF277191.1) (Figure 1).

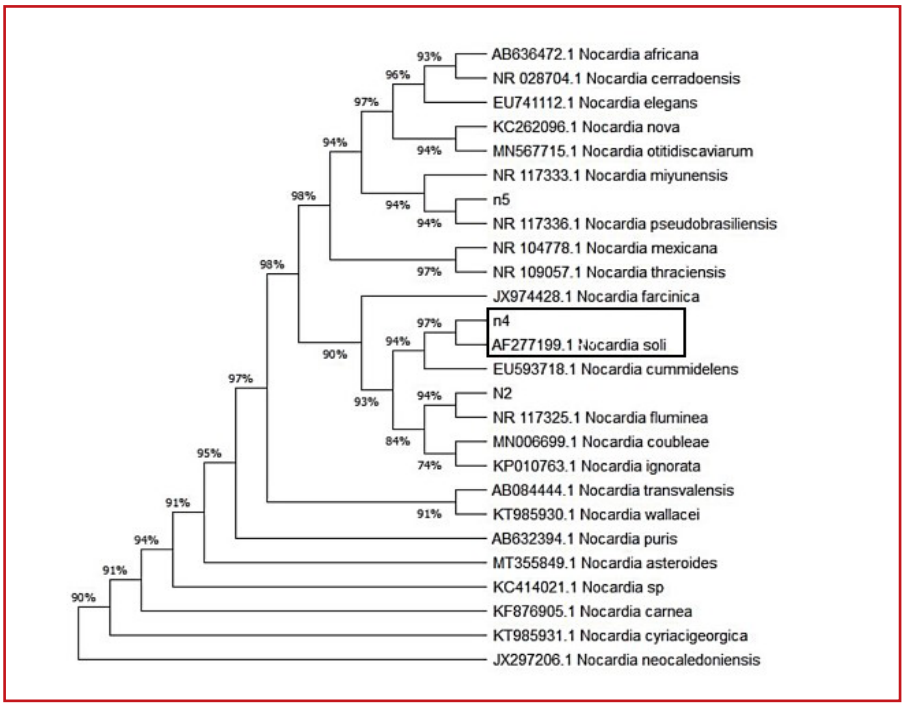

Figure 1. The relationship between N4 strain and other *Nocardia* strains was shown using 16SrRNA phylogenetic analysis and assessing was performed with MEGA 7.0 software. The phylogenetic tree was depicted by the neighbor-joining method

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Isolation and Determination of Antibacterial Effects of Metabolite

The preliminary assessment confirmed the antibacterial activity of the strain. Antibacterial activity of the extracted metabolite was investigated and showed potent antibacterial activity against Gram-negative strains.

The inhibition zone is shown in Table 2. No antibacterial activity was observed against *C. albicans* and *B.cereus.* investigated and showed potent antibacterial activity against Gram-negative strains.

The inhibition zone is shown in Table 2. No antibacterial activity was observed against *C. albicans* and *B.cereus.*

Table 2. The minimum inhibitory concentration (MIC) and zone of inhibition of *N. soli* metabolite against investigated strains

Bacteria strain	MIC (µg/ml ± SD*)	Zone of inhibition
E. coli ATCC 25922	4.67±3.055	+3
P. aeruginosa ATCC 27853	2.33±1.528	+3
K. pneumoniae ATCC 700603	3.33±1.155	+3
Salmonella Typhimurium ATCC 14028	0±4	+3
S. sonnei RI366	0±8	+3
E. faecalis (VRE) ATCC 51299	n	0
S. aureus ATCC 25923	n	0
MRSA ATCC 33591	n	0
B. cereus ATCC 11778	np	0
Aspergillus niger ATCC1015	np	+2
Candida. Albicans ATCC10231	np	0
Aspergillus fumigatus ATCC1022	np	+2

[n] no inhibitory effect was observed. np not performed

MIC values of N4 against all pathogen microorganisms are shown in Table 3. The highest and lowest MIC was recorded for *E. coli* (4.67 µg/mL) and *Salmonella* Typhimurium and *S. sonnei* (0 µg/mL) respectively. No antibacterial effect was observed against S. aureus, MRSA ATCC 33591, and *E. fecalis* (VRE). Furthermore, the percentage of fungal inhibition for different concentrations of the metabolite is shown in Table 3.

Table 3. The inhibitory effect (%) of different metabolite concentrations of fungal pathogens

Metabolite concentration (µl)	Control	3000	1500	750	375	187.5	93.8
Dry weigh of A. *niger* (mg)	0.0131	0	0.0047	0.0085	0.0097	0.01	0.0126
Percentage inhibition	-	100	64.12	35.11	25.95	23.66	3.8
Dry weigh of *A. fumigatus* (mg)	0.0152	0.0023	0.0062	0.0083	0.00895	0.0092	0.0109
Percentage inhibition	-	84.86	59.21	45.39	41.11	39.47	28.28

Chart 1 shows the bactericidal activity of the extracted metabolite and gentamicin in fresh rabbit serum. The metabolite inhibited the growth of bacteria for a longer time compared to gentamicin. The extract showed consistent bactericidal activity against *P. aeruginosa* for five hours.

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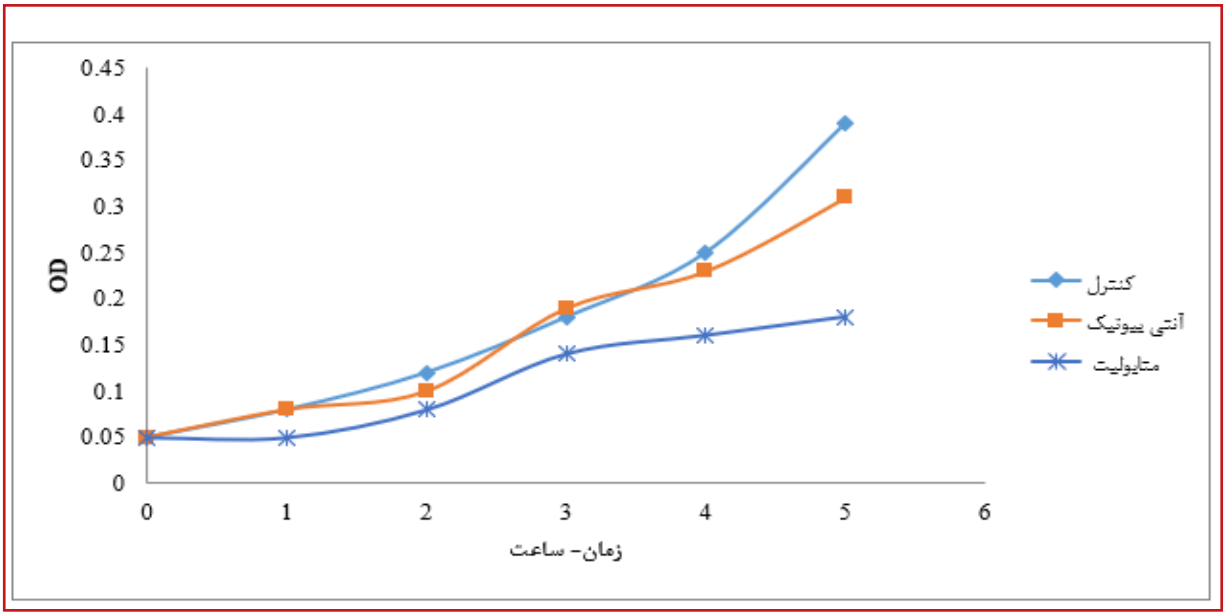

Chart 1. Antibacterial effect of *N. soli* metabolite supernatant against *P. aeruginosa* in comparison with gentamycin

Furthermore, the antibacterial activity remained stable at 60 °C and in the presence of proteinase K. (Table 4)

Table 4. The zone of inhibition of *N. soli* metabolite against human pathogens in presence of proteinase K and high-temperature conditions (60 °C)

Bacteria strain	Zone of inhibition
E. coli ATCC 25922	+3
S. aureus ATCC 25923	0
Aspergillus niger ATCC1015	+2

Structural Identification of Antibacterial Metabolite

We employed HPLC to investigate the antimicrobial compounds in the ethyl acetate extracts of the *N. soli*. The HPLC profile of the ethyl acetate extract was obtained at 264 nm. The mobile phase for purifying the extract was a 20:80 mix of water and methanol. A sharp single peak was observed at the retention time of 8.617 min with a frequency of 24.3% (except for the solvent peak of ethyl acetate at 2.96 minutes) which corresponded to the bioactive metabolite. The mass spectrum showed the last signal at 663 (m/z) corresponding to the molecular mass of the bioactive metabolite. Mass spectra database (https://www.mzcloud.org) was explored and the possible molecular structure was determined to be $C_{21}H_{28}N_7O_{14}$.

Discussion

Nocardia spp. serve as a valuable reservoir for the isolation of bioactive compounds with clinical significance (33, 34). The current study aimed to characterize a *Nocardia* strain isolated from soil and to analyze its antibacterial and antifungal metabolite production. Following a screening of the soil sample for possible *Nocardia* spp., a morphologically distinct isolate designated as N4 was

isolated using AIA medium and paraffin baiting techniques. According to morphological analysis, the strain was a typical *Nocardia* phenotype (35, 36).

Comparative analysis of the 16SrRNA gene sequence and its phylogenetic relationship revealed that the N4 strain is closely related to *N. soli* and shares a high degree of sequence similarity with it. Despite significant advances in the development of antibacterial agents, infectious disease remains the leading cause of death, posing a serious threat to public health (37). *Nocardia* spp. has demonstrated significant potential as a new source of novel bioactive metabolites in recent years. The genus contains nearly 113 species that are saprophytic soil microorganisms found worldwide (25).

Preliminary investigation of the N4 strain revealed promising antimicrobial activity against bacterial and fungal pathogens using the agar well diffusion method. We then examined the antibacterial activity of the N4 strain's ethyl acetate extract. Antibacterial and antifungal activity was demonstrated against a variety of bacterial and fungal pathogens, most notably *P. aeruginosa, K. pneumoniae, A. niger,* and *A. fumigatus. P. aeruginosa* strains have a high potential for developing resistance to a wide variety of antibiotics, making control and eradication extremely difficult. According to a World Health Organization (WHO) report, carbapenem-resistant *P. aeruginosa* is one of three bacterial species for which new antibiotics are urgently needed (38).

Kavitha et al. (2010) reported the antibacterial activity of 1-phenylbut-3-ene-2-ol from *Nocardia levis* against a wide range of bacterial and fungal pathogens (39). Sharma et al. (2016) *isolated* Nocardia strain designated as PB-52 with high sequence similarity with *Nocardia niigatensis IFM 0330*. The isolate showed high antimicrobial activity (40). Our findings are comparable with the study of Kumar P. S. et al. (2014) where the crude ethyl acetate extracted product of *Streptomyces lavendulae* strain SCA5 has shown remarkable antimicrobial activity with the MIC value of 125 and 31.25 µg/mL against bacterial and fungal pathogens (41). Nocardicins were isolated from the fermentation broth of *Nocardia uniformis* subsp. *tsuyamanensis* by Aoki et al (31). Nocardicins exhibit a high degree of antimicrobial activity against Gram (−) bacteria than Gram (+) bacteria (42). Wyche et al. (43), isolated peptidolipins B–F from A marine *Nocardia sp.* and found that they possessed moderate antibacterial activity against methicillin-resistant and methicillin-sensitive *S. aureus.*

Most secondary metabolites have complex structures with difficult synthesis procedures and high associated costs (44). Currently, various chromatographic techniques are used to fractionate and purify the biologically active compounds from the bacterial extracts. HPLC is one of the most widely used separation techniques employed to characterize organic chemicals. We successfully used this technique to characterize the metabolites from the N4 isolate, by monitoring chromatographic retention time. Numerous reports employed the HPLC method for the chemical analysis of microbial metabolites. Singh et al. (2018) identified antimicrobial agents isolated from *Streptomyces levis* using the HPLC method (45). A recent study conducted by Kurnianto et al. (2020), showed high antimicrobial activity in HPLC fractions containing amido-chromophoric groups with one or more peptide bonds in their molecules (46). Another study performed by Devi and Wahab (2012), showed that hexahydro-3-(2-methyl propyl)-pyrrolo[1,2-a]pyrazine-1,4-dione in endophytic fungi isolated from *Camellia sinensis* possess strong antimicrobial activity (47). Furthermore, Kavitha et al. (2010), reported the detection of bioactive compound 1-phenylbut-3-ene-2-ol, phenylethyl alcohol, dibutyl phthalate, and 1,2-benzenedicarboxylic acid, 3-nitro in the

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extract of *Nocardia levis MK-VL_113* with the help of HPLC (39). The N4 extract was subjected to HPLC analysis in the current study, and the identified compounds had a structure similar to $C_{21}H_{28}N_7O_{14}$. Additionally, future research will focus on the other biological activities of the metabolites produced by the N4 strain.

Conclusion

In the present study, it was discovered that *N. soli* isolated from soil produces a highly active bioactive compound with a molecular formula similar to $C_{21}H_{28}N_7O_{14}$ that exhibited remarkable antimicrobial activity. To our knowledge, this is the first report describing the metabolite isolated from this strain. Additionally, the present study demonstrated that the metabolite produced by *N. soli* is a promising agent for controlling infections caused by *P. aeruginosa*, *A. niger*, and *A. fumigatus*. Given that the structure of this metabolite has not been reported previously, additional research is recommended to fully characterize its structure and antimicrobial potential.

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Conflict of Interest

No conflict of interest declared.

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