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Antibacterial effects of bioactive metabolites extracted from *Nocardia pseudobrasiliensis*

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ABSTRACT

Our study was designed to examine the antimicrobial activity of an Actinobacteria strain previously isolated from soil. In the soil surrounding lemon beebrush (*Aloysia citriodora* L.), a *Nocardia* isolate was identified. This strain was evaluated using phenotypic, biochemical, and molecular methods and was distinguished as *Nocardia pseudobrasiliensis*. The structure of the metabolite was determined using mass spectrometry (MS). The antibacterial activity of this strain was detected during primary screening and was observed to exhibit strong antibacterial effects against important nosocomial infections. These bacterial strains were tested to determine their minimum inhibitory concentration (MIC). The metabolite was first distinguished in *N. pseudobrasiliensis*. Phylogenetic and biochemical analyses showed that the isolated strain is similar to *N. pseudobrasiliensis*. The possible molecular structure of the metabolite was distinguished as $C_{18}H_{36}O_2$. Based on these results, the metabolite appears to have strong antimicrobial activity both against Gram-positive and -negative bacteria. Despite the fact that *N. pseudobrasiliensis* was found in the soil surrounding the lemon beebrush roots, further investigations are necessary in order to determine whether it may be an endophyte or a soil microorganism. In the present study, the $C_{18}H_{36}O_2$ compound in the *N. pseudobrasiliensis* is reported for the first time.

Keywords: Antibacterial effects, Bioactive metabolites, *Nocardia pseudobrasiliensis*, Pathogens.
Article type: Research Article.

INTRODUCTION

The discovery of antibiotics was a great achievement for the control and treatment of infectious diseases (Ali *et al*. 2020; Rahman *et al*. 2022). In spite of the fact that there are multiple classes of antibiotics available, excessive use of antibiotics has led to the development of drug-resistant bacteria (Hutchings *et al*. 2019). Moreover, antibiotic resistance genes can be transmitted from a bacterium to other strains via different ways, such as plasmids, chromosomes, or conjugative transposons, which can lead to the spread of antibiotic-resistant infections (Lerminiaux & Cameron 2019, Goswami *et al*. 2020). In addition to its enormous economic burden, antibiotic resistance also leads to increased morbidity and mortality in patients with bacterial infections (Paul *et al*. 2010; Livermore 2012; Gandra *et al*. 2014). As a result, the identification of new and effective antibiotics that can inhibit the growth of antibiotic-resistant bacteria is necessary. Actinobacteria are one of the important groups of soil microorganisms that have an impressive role in the production of a variety of drugs that are highly essential for the health and nutrition of humanity (Dancer 2004). Hence, natural product drugs with new chemical structures made from these bacteria have beneficial biological activities. Studies have revealed that almost 80% of antibiotics

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belong to the Actinobacteria family (the Streptomyces, particularly *Micromonospora* genus; Jensen *et al*. 1991; Hassan *et al*. 2011). Studies on herbal medicine have also shown that endophytic actinomycetes surround the roots of many herbal plants. Furthermore, endophytic actinomycetes, especially in samples isolated from the surface of healthy sterilized tissues, are considered a potential source for the production of substances such as secondary metabolites, antimicrobial, antioxidants, and plant growth promoters (Tan & Zou 2001; Yu *et al*. 2010; Singh & Dubey 2018). An effective way to limit increased drug resistance in the human and plant pathogens is to isolate and identify endophytic actinomycetes functions. Beiranvand *et al.* (2017) reported that medicinal plants are reservoirs for biologically-active compounds in endophytic actinobacteria. Recently, Moazzen Rezamahalleh *et al.* (2019) also confirmed the presence of endophytic actinomycetes in Iranian medicinal plants. Nevertheless, many studies have assessed endophytes in plant structure, but limited information exists about the presence of actinobacteria producing antibacterial metabolites around the roots of medicinal plants. Therefore, in this study, the presence of a variation of actinobacteria in the soil around the roots of medicinal plants and its ability to produce antibacterial metabolites in several areas of Tehran, Iran were investigated.

MATERIALS AND METHODS

Nocardia strain isolation

Through the suspension of soil samples in physiological water (NaCl) and preparation of several different dilutions (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}), a strain of *Nocardia* was isolated from the surrounding soil of the medicinal plant root (Lemon Beebrush). In actinomycete isolation agar medium (AIA; Merck KGaA, Darmstadt, Germany), each dilution was cultured separately. All plates were incubated at a temperature of 28 °C for 2-4 weeks. There was a suspicion that the white colonies are *Nocardia* species.

Phenotypic assay

In order to culture the suspected colonies, Nutrient Agar, Sabouraud Dextrose Agar, and Blood Agar Medium (Merck, Germany) were used. Moreover, the colonies were isolated using the paraffin baiting technique. A period of 1-4 weeks at 37 °C was used for incubating the plates (Singh *et al*. 1987; Hayakawa & Nonomura 1987). Then, the morphology of actinomycete colonies was carefully scrutinized by a light microscope at 100X (Nikon, Japan).

Primary screening of antimicrobial metabolites

Primary screening of antimicrobial metabolites in *Nocardia* isolate was evaluated using the cross-streak method (Ramazani *et al.* 2013). The McFarland 0.5 turbidity standard (10^6 CFU mL^{-1}) was prepared for each human bacterial 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). Then, *Nocardia* was cultured on half a plate of Nutrient agar and a single streak of each pathogen was inoculated at a 90º angle on the plate and incubated at 37 °C overnight. Positive results were obtained with the absence of growth of pathogenic bacteria.

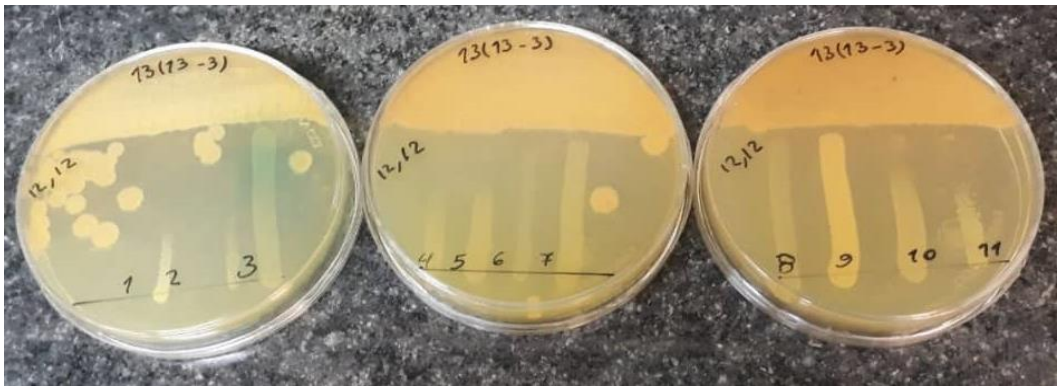

Fig. 1. Cross streak method of antimicrobial metabolites against human bacterial pathogens; (i) *S. aureus* ATCC 25923; (ii) *E. coli* ATCC 25922; (iii) *P. aeruginosa* ATCC 27853; (iv) *K. pneumoniae* ATCC 700603; (v) *S. dysentery* RI 366; (vi) *S. typhimurium* ATCC 14028; (vii) *S. saprophyticus* ATCC 15805; (viii) *E. faecalis* ATCC 51299; (ix) *B. cereus* ATCC 11778, (x) MRSA ATCC 33591; and (xi) *S. sonnei* ATCC 9290).

Biochemical assays

Biochemical testing was conducted to identify *Nocardia* isolate that was positive during the primary screening of antimicrobial metabolites. The following biochemical tests were performed: Gram, partial acid-fast, and acid-fast staining, growth in lysozyme broth (resistance to lysozyme), hydrolysis of casein, gelatin, tyrosine, urea, xanthine, and hypoxanthine, produce acid from carbohydrates, nitrate reduction test, and simmon citrate test (Merck, Germany; Kiska *et al*. 2002). The strain was inoculated on Brain Heart Infusion (BHI) broth with 10% glycerol and was maintained at 4 °C for further assay.

Phylogenetic analysis

Using the kit from Bioneer Corporation in Korea, the DNA of the *Nocardia* strain was extracted. In order to amplify the 16S rRNA gene, primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'; Bioneer, South Korea) were used (Miller *et al*. 2013). The polymerase chain reaction (PCR) mixture was composed of 25 mL master mix (Amplicon, Spanish), 2 mL DNA template, 21 mL deionised water (DW), and 1 mL each of forward and reverse primers. This PCR program consisted of the following steps: 94 °C for 5 min (first denaturation step); 35 cycles of 94C for 1 min, 50C for 1 min (annealing step), and 72C for 2 min (extension step); 72°C for 10 min (final extension step; Chun and Goodfellow, 1995). The 1480 bp PCR product was separated on a 1% agarose gel (Cinnagen Inc, Iran) and was observed by an LED transilluminator (ETS Vilber-Lourmat, France). Purified PCR products were sent to a sequencing company (Life BioScience, UK), along with five mL (0.1 concentration) forward primer. An analysis of the sequence results was conducted using version 1.4.0 of the Finch TV software. Mega 7 software was used for phylogenetic analysis. MEGA 7 software was used to plot a phylogenetic tree in order to determine the relationship between isolates using the Neighbor-Joining (NJ) approach.

Antimicrobial metabolite extraction

The isolates which were positive in the initial screening for antimicrobial metabolites were cultured in 250 mL brain heart infusion broth (BHI, Merck, Germany) and were kept in a shaker incubator at 30 ºC and 150 rpm for five days in an attempt to isolate antimicrobial metabolites. A 10% portion of this medium was transferred to a second flask with 250 mL yeast extract-malt extract (YEME) liquid medium and incubated for 7 days at 30 ºC and 200 rotations per min. It was then centrifuged for 4 min at 4 ºC at 12000 rpm. The supernatant was mixed with ethyl acetate at a ratio of 1: 1 for one hour. It was then necessary to separate and remove the phase of ethyl acetate after forming the two layers. The final precipitate containing the antibacterial metabolite was derived from the residual material. To improve the purification of columns, HPLC with a semi-preparative column, a specification of 250 × 10 mm, and methanol/water was used (Kavitha *et al*. 2010).

Analyses of the antibacterial activities of ethyl acetate extracts

Diffusion in agar wells

Ethyl acetate extract was tested for antimicrobial activity using the Agar well diffusion method. Inoculations of Mueller-Hinton agar plates were prepared using the McFarland 0.5 turbidity standard of human pathogenic bacteria. In the following step, a hole of 6 mm in diameter was punched in agar plates, and 100 µL of sediment was poured into the hole. Incubation of the agar plates was performed overnight at 37 °C. A clear zone around the pathogen colonies was recorded as a positive result (Borquaye *et al*. 2016).

MICs (minimum inhibitory concentrations)

A broth microdilution method was used to determine the MIC of antimicrobial metabolites in 96-well microtitre plates. Concentrations of 1024 mg mL^{-1} of concentrated metabolites were used as the stock solution. In each well, 100 µL sterile Mueller Hinton Broth were inoculated. Afterward, 100 µL stock solution was added to well 1 and mixed. Serial dilutions were performed for stock solutions to well 10, while 100 µL was discarded from well 10. A suspension of 10^5 pathogenic microorganisms was added to each well after dilution. Well 11 served as a negative and well 12 as a positive controls. Incubation at 37 °C was carried out for 24 h on the plates. Using a spectrophotometer (Jinoe, UK) to measure the turbidity of microorganisms at 620 nm, the MIC of the sample was found to be the lowest concentration that stopped all bacterial growth in it (no turbidity; BSSN *et al*. 2017).

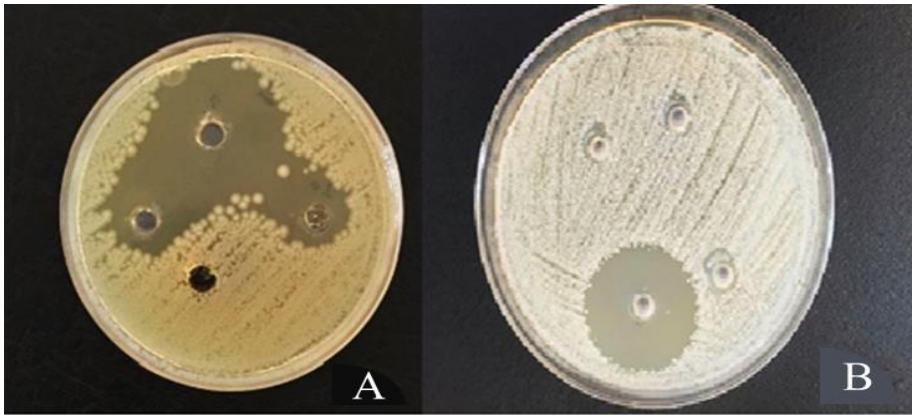

Fig. 2. Antimicrobial effects of metabolites extracted using agar well diffusion method (A: *E. coli*; B: *S. aureus*).

Analyzing rabbit serum for antimicrobial metabolites

The bactericidal activity of this metabolite was investigated in rabbit serum. An inoculum of 10^6 cells mL^{-1} of *P. aeruginosa* was inserted into Luria-Bertani medium containing 20% rabbit serum, antimicrobial metabolites (64 µg mL^{-1}) and gentamicin (64 µg mL^{-1}), and incubated for five days at 37 °C in a shaker incubator. As a control, a vial of LB broth medium that has been inoculated with bacteria was used. In the presence of gentamicin and three extracted metabolites, the absorbance of the bacteria at 600 nm (OD 600) was determined using a spectrophotometer, and the growth curve of the bacteria was plotted (Aoki *et al*. 1976).

Stability of the antimicrobial metabolites

The proteinase K enzyme (50 mg mL^{-1}) was used to assess antimicrobial metabolites' stability at around 60 ºC. The antibacterial activity of the metabolites was evaluated by measuring the inhibition zone diameter around the wells of pathogens which had an antibacterial effect on the primary screening (Rana & Salam 2014).

Chemical compound analysis metabolites using mass spectrometry

The presence of the organics in the sediment of the metabolites was determined using the mass spectrometry (MS) technique. As a final step, mass spectrometry analysis of the metabolites was conducted under suitable conditions (Table 1; Kavitha *et al.* 2009).

Table 1. Conditions of metabolite analysis using MS model 5973 (Agilent).

Direct Probe Temperature Program					
Parameter	Step 1	Step 2	Step 3	Step 4	Step 5
Initial Temperature (ºC)	50	---	---	---	---
Initial Time (min)	0	---	---	---	---
Program Rate (ºC/min)	70	---	---	---	---
Final Temperature (ºC)	350	---	---	---	---
Final Time (min)	5	---	---	---	---

RESULTS

Phenotypic and phylogenetic characteristics of *Nocardia pseudobrasiliensis*

The *Nocardia* suspected strain was isolated from the soil surrounding lemon beebrush (*Aloysia citriodora* L.) root. After 1-4 weeks of incubation, bacterial colonies were observed on the plate. The colony morphology of the strain was detected based on its shape, pigment production, density, and reaction to different strains (Table 2). The utilization of carbon sources such as citrate, aesculin, casein, sorbitol, tyrosine, xanthine, and hypoxanthine was used to distinguish the strain tolerant to lysozyme. Urease was produced, however, nitrate reductase activity was not detected in this strain. Phenotypic characteristics of the strain have been listed in Table 2. An analysis of the *Nocardia* strain, i.e., N5, was conducted based on the results of 16S rRNA gene amplification. The genome sequence of this strain was confirmed using NCBI blast and was analyzed by MEGA 7.0 software. The phylogenetic analysis showed that the strain is similar to *N. pseudobrasiliensis* (Fig. 3). The N5 strain formed a very tight monophyletic cluster with *N. miyunensis* and *N. pseudobrasiliensis*.

Table 2. Phenotypic characteristics of *N. pseudobrasiliensis.*

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	
45ºC	-

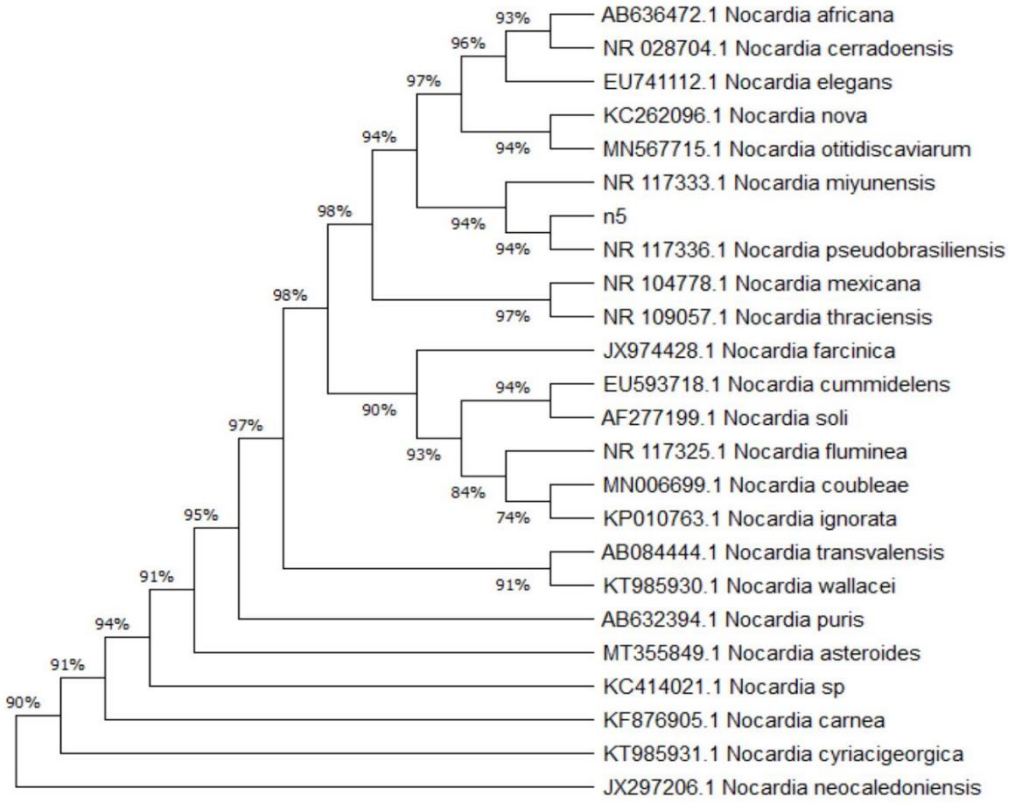

Fig. 3. The phylogenetic tree of the N5 strain and other *Nocardia* strains based on 16S rRNA gene analysis using 7.0 software.

Isolation and determination of antibacterial effects of metabolite

Antibacterial activity of this strain was observed during primary screening. Then the agar well diffusion method showed a strong antibacterial effect against *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. dysentery*, *S. typhimurium*, MRSA, *E. faecalis*, *B. cereus*, and *S. aureus*. Inhibition zone measurements were reported in millimeters (mm) for each well. Inhibition zones with a diameter of 0-5 mm were considered as no inhibition (0); 6-10 mm as weak antimicrobial activity (1+); 11-15 mm a moderate inhibition (2+); and more than 15 mm as strong antimicrobial activity (3+; Table 3). Minimum inhibitory concentration (MIC) of the metabolite was shown in Table 3 where the strongest effect of the metabolite was observed against MRSA (0 ± 33 μg mL^{-1}) and *S. typhimurium* (1.67 ±

0.577 µg mL^{-1}). As the bactericidal activity of gentamicin and the extracted metabolite is shown in Fig. 4, the ability to inhibit bacterial growth in the metabolite was more durable than that of gentamicin. Moreover, the bactericidal activity of the metabolite was stable.

Table 3. Analyzed strains and minimum inhibitory concentration (MIC) and inhibition zone for *N. pseudobrasiliensis* metabolites.

Bacteria strain	MIC (µg ml^{-1}± SD*)	Zone of inhibition
E. coli ATCC 25922	6±3.464	+3
P. aeruginosa ATCC 27853	5.33±2.309	+3
K. pneumoniae ATCC 700603	4.67±3.055	+3
Salmonella Typhimurium ATCC 14028	1.67±0.577	+3***
E. faecalis (VRE) ATCC 51299	-	0
S. aureus ATCC 25923	4.67±3.055	+3
MRSA ATCC 33591	32±0	+1**
B. cereus ATCC 11778	-	0

*SD = Standard deviation; ** = Weak antimicrobial activity (1+); *** = Strong antimicrobial activity (3+).

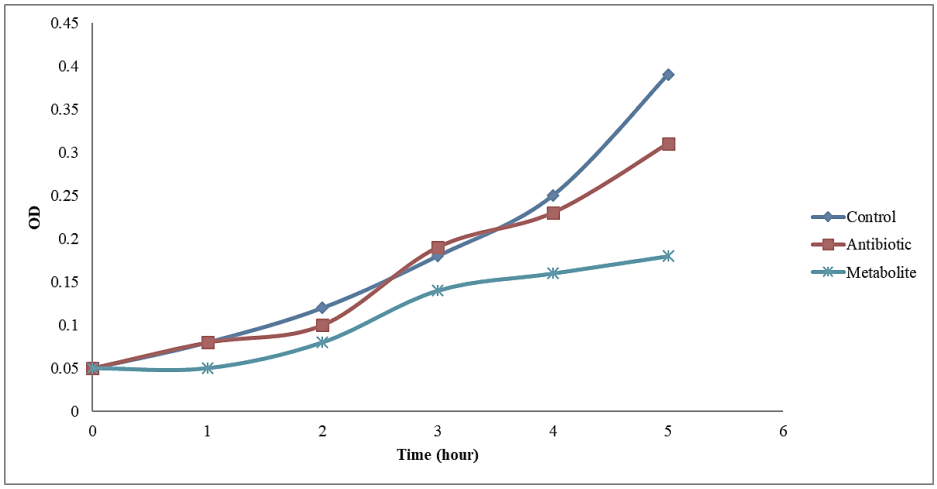

Fig. 4. A comparison of the antibacterial activity of *N. pseudobrasiliensis* metabolite supernatant with that of gentamycin against *P. aeruginosa*.

Structural identification of antibacterial metabolite

In high performance liquid chromatography (HPLC) at 228 nm, the metabolite components extracted from ethyl acetate were removed in the C18 column by washing the mobile phase with methanol and water at a ratio of 20:80. The bioactive metabolite was isolated at a time of inhibition of 5.133' with a frequency of 6.64%, except for the solvent peak of ethyl acetate (2.96 minutes with a frequency of 54.5%) (Fig 5). The last signal obtained from the mass spectrometer was observed at 284 m / z. The possible molecular structure was distinguished as $C_{18}H_{36}O_2$.

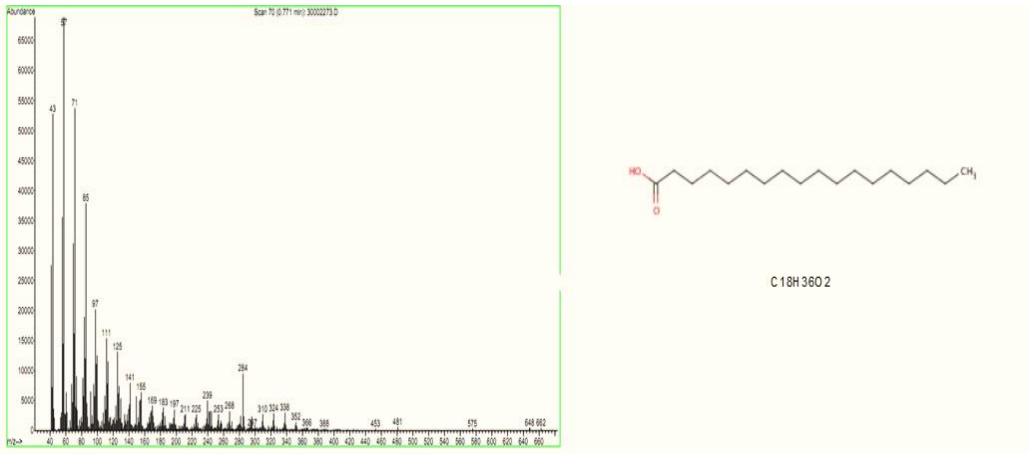

Fig. 5. HPLC analysis of *N. pseudobrasiliensis* metabolite.

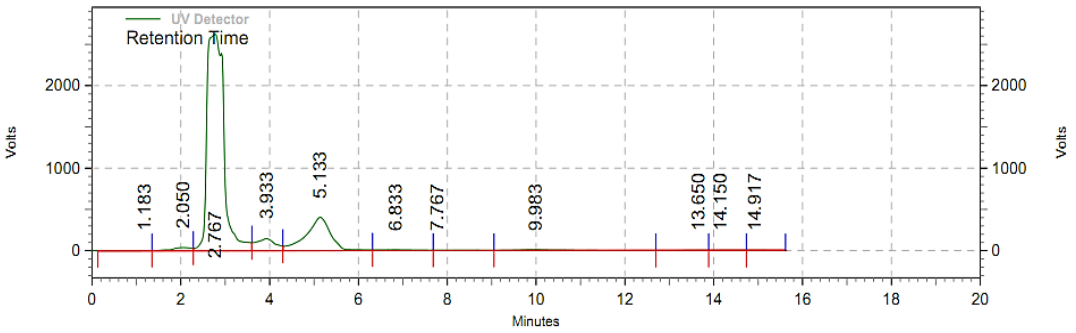

Fig. 6. Mass spectrometry analysis of *N. pseudobrasiliensis* metabolite.

DISCUSSION

In this study, the antibacterial activity of an Actinobacteria strain isolated from the soil was investigated. We detected a *Nocardia* strain which was considered as *N. pseudobrasiliensis* based on biochemical and phylogenetic analyses. In spite of the fact that actinomycetes are abundant in the soil, the majority of them remain unidentified at the moment (Davies 1999). *N. pseudobrasiliensis* was identified by Wallace *et al*. (Wallace *et al*. 1995) in 1995. Later, Ruimy *et al*. (1996) determined the relationship between the strain and other bacteria based on 16S rRNA gene sequence analysis and DNA-DNA hybridization. The biochemical characteristics of *N. pseudobrasiliensis* are similar to those of *N. brasiliensis.* However, the activity of nitrate reductase is not detected in *N. pseudobrasiliensis* which was isolated from the soil in some areas (Habibnia *et al*. 2015), However, it was recognized more frequently as a human pathogen (Sullivan & Chapman 2010; Javaid *et al*. 2012; Bell *et al*. 2014). We isolated the *Nocardia* strain in the surrounding soil of the lemon beebrush root. Several studies have proved its antibacterial effects on *E.coli*, *P. aeruginosa*, *S. aureus*, and many other bacteria (Ali *et al*. 2008; Hashemi *et al*. 2017). The metabolite extracted from the herbal was more effective on *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. dysentery*, *S. typhimurium*, *S. aureus*, and MRSA. In this study, the antimicrobial activity of metabolite extracts of the *Nocardia* strain was analyzed. Specifically, the metabolite showed the ability to inhibit the growth of Gram-negative (*E. coli*, *P. aeruginosa*, *K. pneumoniae*, *S. dysentery*, *S. typhimurium*, and Gram-positive (*S. aureus*) bacteria. Mukai *et al*. (2009) extracted antibacterial compounds (nocardicyclin, siderophore, and benzonoid) from *N. pseudobrasiliensis* that had a major effect on *Mycobacterium* and *Gordonia* species. Kavitha *et al*. (2009) detected bis-(2-ethylhexyl) phthalate and bis-(5-ethylheptyl) phthalate components from *N. levis* MK-VL_113 which exhibited antibacterial and antifungal activities. Minimum inhibitory concentration values were between 0 ± 32 and 6 ± 3.464 mg mL^{-1}. MIC values of the metabolite at low concentrations, i.e., 0 ± 33 µg mL^{-1} (for MRSA) and 1.67 ± 0.577 (for *S. typhimurium*) show that the *N. pseudobrasiliensis* can be one of the best sources of effective antibacterial agents for the treatment of antibiotic-resistant infections. Our findings are compared to the study conducted by Kavitha *et al*. (2009), where the range of MIC values was between 10 and 250 mg mL^{-1}, exhibiting a strong antibacterial effect on Gram-positive and Gram-negative bacteria. Celmer *et al*. (1980), showed that nargenicin A1, which is active against MRSA, is produced by *N. argentinensis*. The findings showed that the biochemical structure of the antibacterial metabolite is $C_{18}H_{36}O_2$. Luiza *et al*. (da Silva *et al*. 2008) extracted stearic acid from leaves and stems of *Stemodia foliosa* and showed the antibacterial effects on *B. cereus* and *B. subtilis*. Moreover, Oliveira *et al*. (2017) extracted saturated fatty acid lauric, and myristic, palmitic, stearic, oleic and linoleic acids from *Mauritia flexuosa* (Buriti) fruits with significant antibacterial effects on *E. faecalis*, *E. coli*, *P. aeruginosa*, and *S. aureus* (de Oliveria *et al*. 2017).

CONCLUSION

To the best of the authors' knowledge, $C_{18}H_{36}O_2$ compound is reported in the *N. pseudobrasiliensis* for the first time. These data suggest that the metabolite has strong antimicrobial activity against both Gram-positive and Gram-negative bacteria. Our detection showed strong activity against MRSA and *S. aureus* strains that should be considered as an important finding. Although *N. pseudobrasiliensis* was reported in the surrounding soil of the lemon beebrush root. Further investigations are required to explore whether the strain is endophytic or a soil microorganism.

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CONFLICT OF INTEREST

No conflict of interest declared

FUNDING

No sources of funding.

DATA AVAILABILITY

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

AUTHORS CONTRIBUTION

S.S.E. formed the presented idea. M.S. developed the theory, performed the computations, and was a major contributor in writing the manuscript. P.P., A.Z.R., and M.Y. performed and verified the analytical methods. All authors discussed the results and contributed to the final manuscript.

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