ORIGINAL ARTICLE

Potentials of Actinomycetes from Reserved Environments as Antibacterial Agents Against Drug-Resistant Clinical Bacterial Strains

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OPEN ACCESS

Citation: AHMED Risikat Nike, DANIEL Folake, GBALA Ifeoluwa Deborah[2], SANNI Alhassan. Potentials of Actinomycetes from Reserved nvironments as Antibacterial Agents Against Drug-Resistant Clinical Bacterial Strains. Ethiop J Health Sci. 2020;30(2):251. doi:http://dx.doi.org/10.4314/ejhs.v30i2.13

Received: August 19, 2019
Accepted: October 1, 2019
Published: March 1, 2020

Funding: Nil

Competing Interests: The authors declare that this manuscript was approved by all authors in its form and that no competing interest exists.

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ABSTRACT

BACKGROUND: *Increased antibiotic resistant properties of pathogens has led to a pronounced search for new effective antibiotics from microbes in diverse ecological niches. This study focused on isolating actinomycetes from soil of reserved areas and profiling them for antibacterial potentials.*

METHODS: *The isolates (IS-2, IS-4, IS-6, IS-10, IS-14) were assessed for antagonistic activity against ten multi-drug resistant bacterial strains (Gram positive and negative) by cross streak and well diffusion methods.*

RESULTS: *During the primary screening, four of the isolates showed good antagonistic activity against the test strains. Notably, all the bacterial strains except Pseudomonas aeruginosa responded sensitively to at least one of the actinomycetes screened. The crude extracts of the secondary metabolites of the four actinomycetes (IS-2, IS-4, IS-6, IS-10) with considerably significant antagonistic activities inhibited the growth of all the bacterial strains efficiently. All the test bacterial strains were sensitive to at least one of the extracts at a concentration of 100µg/ml. The minimum inhibitory concentration of the extracts against the isolates ranged from 12.5 - 25µg/ml. The crude extracts of IS-4 and IS-6 identified as Streptomyces glauciniger NBRC 100913 and Streptomyces griseoplanus NRRL-ISP 5009 by I6s rRNA sequencing, showed higher antibacterial activities against the bacterial strains. Significantly, the ethyl acetate crude extract of the actinomycetes demonstrated better antibacterial activities than the standard antibiotics (ofloxacin, amoxicillin/clavulanate, cefuroxime and ceftriaxone).*

CONCLUSION: *This study reports remarkable anti-MRSA activities as well as broad spectrum antibacterial potentials of extracts of Streptomyces spp. worthy of further exploration.*

KEYWORDS: *Actinomycetes, extreme environments, antibiotics resistance*

INTRODUCTION

Soil, a mixture of organic matter, minerals, gases and liquids, is the largest source of microorganisms. The soil habitat offers a great avenue for the isolation of microorganisms such as actinomycetes, for the production of newer metabolites. The actinomycetes, a group of physiologically multifaceted, are filamentous bacteria found in both terrestrial and aquatic habitats. They are capable of producing a wide variety of secondary metabolites as a survival strategy (1,2). These

metabolites possess unique properties including antagonistic activities against microorganisms. Among the actinomycetes, *Streptomyces* receives the most attention because of their abundance in soil, wide phylogenetic spread and diversity of bioactive secondary metabolites (3). Interestingly, the majority of the antibiotic-producing actinomycetes are found among these species. Microorganisms that thrive in extreme and diverse environments, including actinomycetes, are being investigated in recent researches. This focus is attributed to the array of physiological processes these organisms exhibit as adaptive measures for survivorship (4). Antimicrobial activities of secondary metabolites recovered from *Streptomyces* spp. isolated from reserved, unexplored or extreme environments have been reported (5,6,7,8).

The increase in the rate of antimicrobial resistance exhibited by bacteria, especially the Gram negative populace, is a threat to public health (9). Of great concern, also, is the unavailability and non-production of new classes of antibiotics which could present wider antimicrobial spectrum on resistant strains of bacteria. This increasing prevalence of antimicrobial resistance (AMR) coupled with the dry antimicrobial development pipeline threatens the success and continuation of clinical medicine as we know it. The search for new effective antimicrobial compounds is therefore inevitable. As such, This study is therefore aimed to discover actinomycetes in soils from rivers, hospital environment, farmland, forest reserve areas and rotten wood deposits; and also evaluate the antibacterial potentials of the isolates.

MATERIALS AND METHODS

Collection of soil samples: Soil samples were collected from five different locations within Ilorin metropolis, Nigeria - hospital environment, river sediments, farmland, forest reserve areas and rotten wood deposits. The samples were scooped at 5-15cm depth using a sterile hand trowel, transferred into sterile plastic containers and transported aseptically to the laboratory for physicochemical analysis and thereafter air-dried for 3 days at room temperature.

Physicochemical characteristics of soil samples: Temperature, pH, moisture content and soil organic matter of the soil samples were determined as described by (10,11,12,13). Soil parameters such as texture, particle size distribution and availability of macronutrients (Ca2+, Mg2+, K+) as well as exchangeable potassium and sodium were also determined (14).

Isolation of actinomycetes from soil samples: Prior to isolation, all soil samples were air-dried for 3 days. Dried soil samples were serially diluted in sterile distilled water up to 10−5 dilutions. Aliquots (0.1ml) from 10−3 and 10−5 dilutions were spread evenly over the surface of sterile starch casein agar, Mueller Hinton agar and sterile nutrient agar + calcium chloride agar plates, and incubated aerobically at 30oC for 7 days (8). The growth media were supplemented with amoxicillin (20µg/ml) and fluconazole (25µg/ml). The colonies were further grown on Yeast Extract-Malt Extract Agar (ISP-2 medium for clarity of pigmentation. Morphologically distinct colonies were further identified biochemically and by 16s rRNA sequencing.

Primary screening of actinomycetes against bacterial strains: Primary screening of the selected actinomycetes for antibiotics potential against *Klebsiella pneumoniae*, *Staphylococcus aureus* ATCC 25923, *Proteus mirabilis*, *Pseudomonas aeruginosa*, clinical MRSA, MRSA ATCC 43300, *Escherichia coli* ATCC 25922, methicillin-sensitive *Staphylococcus aureus* NCTC 6571, *Acinetobacter boumanii* and ESBL-producing *Escherichia coli*, were performed by cross streak method (15). Inoculum was prepared by growing cells in Mueller Hinton broth (Oxoid, UK) for 48h at 300C. A single vertical streak of the inoculum was inoculated on SCA (Oxoid, UK) and then incubated at 30o C for 48h. The plates were subsequently seeded by perpendicular streak of the overnight culture of the bacterial strains pre-adjusted to 0.5 McFarland cell densities. The plates were then incubated for another 48h at 30o C. The microbial interactions were analyzed by determining the distance of inhibition measured in mm.

Synthesis of metabolites from actinomycetes: Actinomycetes strains with considerable inhibitory activities were subjected to submerged fermentation. Pure cultures were inoculated into sterile fermentation medium (yeast extract, 3.0g; peptone, 3.0g; casein, 3.0g; soluble starch, 8.0g; glycerol, 3.0g; CaCO3, 0.75g; K2HPO2, 0.5g; MgSO4.7H2O, 0.5g; NaCl, 12g; 250ml of distilled water; pH 7.4). The broth was incubated at 30^{o}C for 7days on a

DOI: http://dx.doi.org/10.4314/ejhs.v30i2.13

rotary shaker (200±5 rpm). After incubation, the broth was centrifuged at 5000rpm for 20minutes then filtered. Clear filtrates were tested for antibiotics activities (16).

Extraction of active compounds: Equal volume (1:1) of filtrate and ethyl acetate (95%) solvent was mixed thoroughly on a rotary shaker for 24h and subsequently concentrated to crude with a rotary evaporator. The crude extracts were aseptically transferred into sterile sample bottles and refrigerated at 4°C until use (17).

Secondary screening of actinomycetes against bacterial pathogens: Screening of the actinomycetes crude metabolite was performed by agar diffusion method. The ethyl acetate crude extracts reconstituted to a concentration of 100µg/ml while DMSO was used as a control. Overnight cultures of the bacterial strains pre-adjusted to 0.5 McFarland were seeded on the solidified Mueller Hinton agar (Oxoid, UK) plates, and holes of 6mm were bored on the agar. Each hole was filled with 100µl of the crude extracts, left for 30 mins at room temperature for diffusion and then incubated at 37° C for 20h. Zones of inhibitions were observed and recorded following incubation. All tests were done in triplicates (8). The minimum inhibitory/bactericidal concentrations of the extracts were determined by varying the concentrations (6.25-100 µg/ml) in Mueller Hinton broth. The medium was then inoculated with 100µl of the standardized bacterial culture and incubated at 37°C for 24h. Negative and positive controls of broth without extracts or organisms were set up. After incubation, the least concentration of extract showing no growth (turbidity) was taken as the MIC for the respective organism. MBC was deduced by streaking aliquots from the concentrations without growth on MHA and incubated at 37°C for 24h. The least concentration without growth was taken as the MBC.

RESULTS

Based on the morphological characteristics of the colonies on Starch Casein agar, five actinomycetes isolates were presumptively selected.

Physicochemical parameters and elemental analysis of soil samples: The pH, temperature, organic matter and moisture content of the five samples showed similar values regardless of the variation in sampling sites, although the river sediments had much higher moisture content. The textural classes of the soils also varied as sandy loam (RS and RFA), loamy sand (HS), sand clay loam (VF) and loam (DWS). The most abundant element in all the soil samples was Phosphorus (1.81±0.02 - 3.05±0.04). Also, the soil samples from RFA and DWS were significantly richer in nutrients compared to other soil samples (Table 1).

Antibiotics susceptibility profile of the bacterial strains: Using the CLSI (2018) interpretative guideline, all the isolates were resistant to augmentin, cefuroxime and ceftriaxone. The methicillin-sensitive *Staphylococcus aureus* strain and the clinical MRSA were however sensitive to ofloxacin while MRSA 43300, and *Proteus mirabilis* were resistant to all the tested antibiotics (Figure 1).

Sample site	pH	Temp (°C)	Moisture content (%)	Organic Matter (%)	Ca →	Mg cmo/kg	K	Na →	P mg/kg	Zn cmo/kg	Fe → ←
H.S	7.17±0.12^{c,d}	30.33±0.58^{a}	2.98±0.17^{c}	29.01±1.28^{a,b}	1.35±0.21^{a}	0.39±0.78^{b}	0.18±0.02^{c}	0.09±0.01^{c}	1.82±0.10^{c}	0.02±0.01^{a}	0.34±0.04^{c}
R.S	8.47±0.12^{b}	30.00±0.50^{a}	33.63±1.2^{a}	19.21±0.36^{c}	0.70±0.00^{b}	0.35±0.01^{b}	0.39±0.01^{a}	0.80±0.01^{c}	1.86±0.06^{c}	0.01±0.01^{a}	0.22±0.04^{c}
V.F	8.73±0.06^{a}	30.67±0.58^{a}	12.32±0.69^{b}	27.38±0.01^{b}	0.85±0.07^{ab}	0.01±0.01^{c}	0.39±0.02^{a}	0.21±0.01^{b}	1.81±0.02^{c}	0.03±0.01^{a}	0.24±0.02^{c}
R.F.A	7.37±0.06^{c}	29.67±0.58^{a}	1.42±0.10^{c}	30.04±0.06^{a}	1.00±0.14^{ab}	0.57±0.02^{b}	0.27±0.02^{b}	0.04±0.00^{d}	3.05±0.04^{a}	0.02±0.01^{a}	1.15±0.07^{a}
D.W.S	7.13±0.06^{d}	29.33±0.58^{a}	1.46±0.10^{c}	31.28±0.00^{a}	1.15±0.07^{ab}	0.82±0.92^{a}	0.17±0.01^{c}	0.92±0.01^{a}	2.74±0.04^{b}	0.02±0.01^{a}	0.89±0.04^{b}

DOI: http://dx.doi.org/10.4314/ejhs.v30i2.13

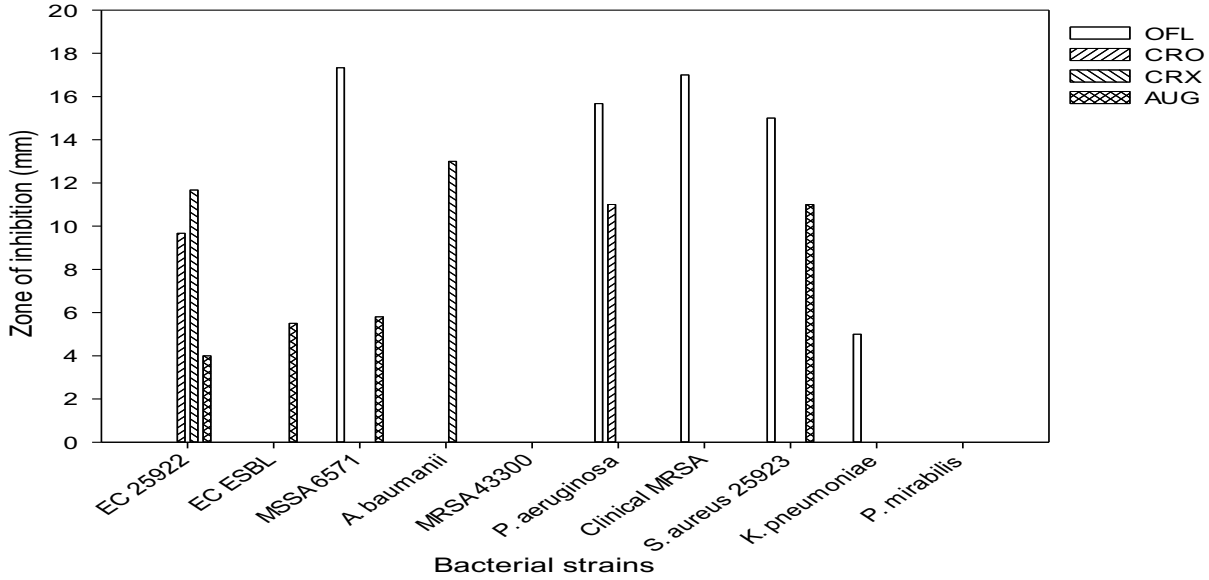

***Figure 1**: Antibiotics susceptibility profiling of bacterial pathogens*

Screening of actinomycetes against bacterial strains: The results of the primary and secondary screening of the actinomycetes and their secondary metabolites are presented in Figs. 2 and 3. All the bacterial strains except *Pseudomonas aeruginosa* responded sensitively to at least one of the actinomycetes screened. Out of the five actinomycetes isolates (IS-2, IS-4, IS-6, IS-10, IS-14) screened primarily for antibacterial potentials, IS-4 and IS-6 exerted more pronounced antagonistic activities against the test isolates while IS-14 showed the weakest activity. Notably, the Staphylococcus aureus strains and ESBL-producing E. coli strain showed considerable sensitivity to the actinomycetes with zones of inhibition ranging from 6 – 13mm. Also, IS-6 inhibited the growth of both Gram positive and negative bacteria with mean zones of inhibition of 8.5mm and 10.4mm, respectively (Figure 2).

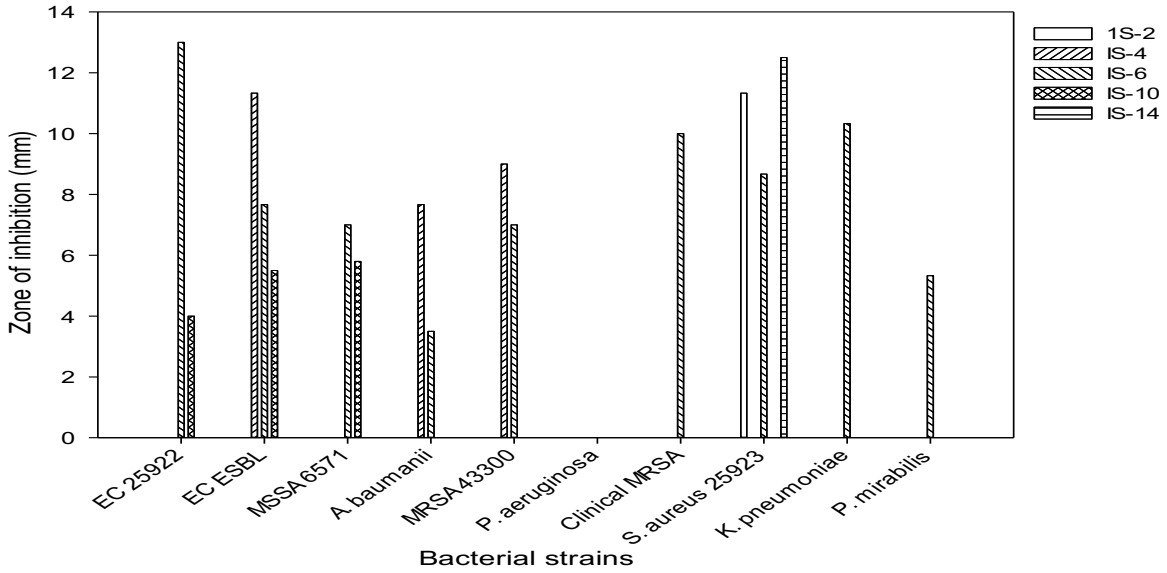

***Figure 2**: Primary screening of actinomycetes against bacterial strain*

The crude extracts of the secondary metabolites of the four actinomycetes (IS-2, IS-4, IS-6, IS-10) with considerably significant antagonistic activities inhibited the growth of all the bacterial strains efficiently. All the test bacterial strains were sensitive to at least one

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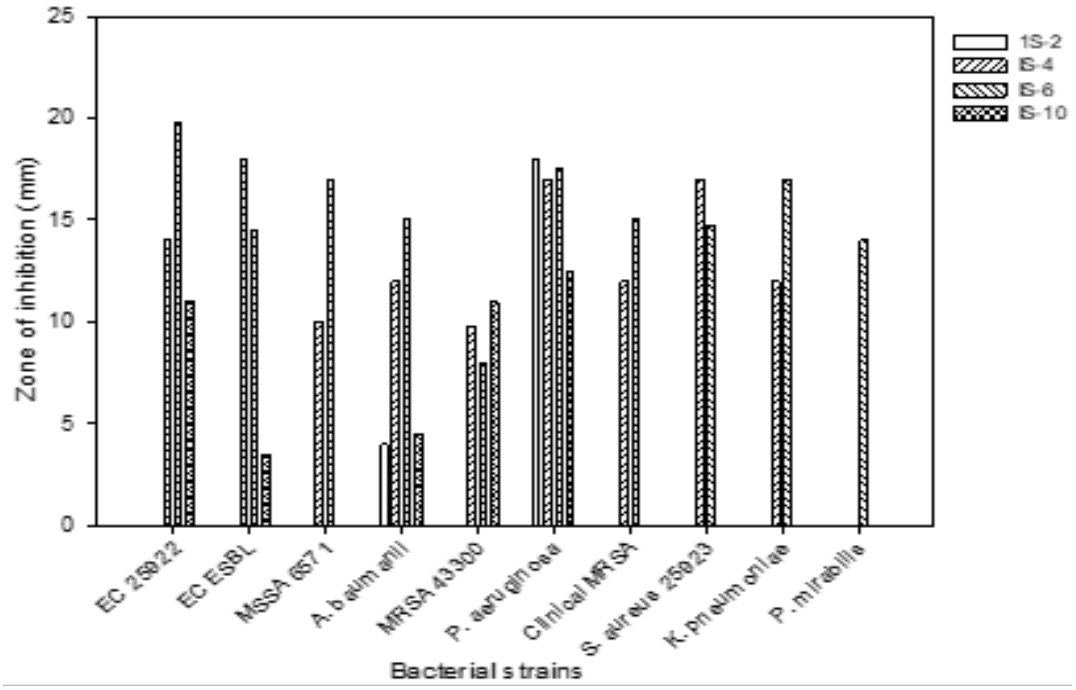
of the extracts at a concentration of 100µg/ml (Figure 3A). *Pseudomonas aeruginosa* which was previously resistant at primary screening was prominently inhibited by all extracts with inhibition zones range of 13 – 19mm. Similar to the results of the primary screening, the crude extracts of IS-4 and IS-6 exhibited the strongest antibacterial activities against the bacterial pathogens. IS-6 extract remarkably inhibited the growth of all the isolates with zone of inhibition ranging from 10-20mm. Likewise, IS-4 extract showed inhibitory effects on all the isolates but *Proteus mirabilis*. At 50µg/ml concentration, *Pseudomonas aeruginosa* consistently showed sensitivity to all the extracts with mean zone of inhibition of 7.25mm. IS-6 extract still maintained the highest efficacy on all the isolates while IS-2 and IS-10 displayed the least activities on the isolates at this concentration (Figure 3B).

Minimum inhibitory/bactericidal concentration: As shown in Table 2, the MIC for IS-2 extract against the two sensitive isolates, *A. baumanii and P. aeruginosa* was 25µg/ml. For IS-4 extract, all the isolates except E. coli 25922 and MRSA 43300 had MIC of 25µg/ml. For IS-6 extract, MSSA 6571, MRSA 43300, P. aeruginosa and clinical MRSA had MIC of 12.5µg/ml while the other isolates were least inhibited at 25µg/ml.

Despite the low MIC, IS-4 extract had no bactericidal effect on any of the isolates. However, the IS-6 extract exhibited bactericidal actions at 25 – 100µg/ml against the isolates except ESBL-producing *E. coli*, *A. baumanii*, MRSA 43300 and *Proteus mirabilis*.

Molecular characterization: The taxonomy and phylogeny of the isolates with the most pronounced activities, IS-4 ad IS-6, indicate that the isolates belong to the genus Streptomyces and designated as *Streptomyces glauciniger* NBRC 100913 and *Streptomyces griseoplanus* NRRL-ISP 5009, respectively. The partial 16s rDNA sequences showed 95% sequence similarity with *Streptomyces avellaneus*. The sequences were deposited in GenBank under the accession numbers NR11415 and NR041428. The phylogenetic tree shows the relationships between isolated actinomycetes and related strains.



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DISCUSSION

The urgent need for new effective antimicrobial compounds is escalated by the increasing dominance of antimicrobial resistance, globally. The consequential effects on public health also spirals exploring reserved environments as possible reservoirs of sources of antimicrobial compounds. This study assessed the antibacterial potentials of *Streptomyces* species isolated from soils of reserved forest areas and wood decay deposits against bacterial strains of clinical importance. The physicochemical parameters of the soil samples support the optimal growth of mesophiles including actinomycetes. All the bacterial strains used exhibited decreased susceptibility to beta-lactam and cephalosporins. Drug-resistant strains of clinical pathogens, especially the Gram negative bacteria, largely portend the risk of disease outbreak with increased fatality due to their propensity of dissemination (18). Extensive resistance to cephalosporins and beta-lactams is being increasingly reported in clinical isolates across the globe (19,20). This resistance pattern has been identified as indicators of beta-lactamase production in the bacteria, which confers resistance to a wide range of antibiotics on them (21,22).

Compared to the standard antibiotics, the five actinomycetes primarily screened inhibited the growth of the bacterial strains better although at varying extents. More significantly, the secondary screening of the ethylacetate extracts of the actinomycetes further affirmed the production of bioactive constituents in the isolates. Similar to the reports of Bhakyashree and Kannabiran (23), the extracts of the isolates, especially, *Streptomyces glauciniger* and *Streptomyces griseoplanus*, remarkably inhibited the growth of both methicillin resistant and sensitive *Staphylococcus aureus* strains. MRSA is one of the high priority pathogens causing life-threatening diseases in invalids and convalescents. The significant anti-MRSA activities displayed therefore suggests the presence of explorable compounds in the secondary metabolites of these isolates to tackle this pathogen. Besides the prominent anti-MRSA activities, the extracts also arrayed a good spectrum of inhibitory activities on Gram negative bacteria including MDR *P. aeruginosa, A, baumanii* and *P. mirabilis* which are renowned for substantial drug resistance.

The expansion of boundaries in the search for new antimicrobials with a focus on extreme and unusual environments largely targets actinomycetes. Not only because of their unique metabolic properties but also due to their adaptability to different environmental conditions such as neutral habitat which may gradually become saline, alkaline or acidic (4). The considerably low minimum inhibitory and bactericidal concentrations obtained for the extracts of *Streptomyces glauciniger* and *Streptomyces griseoplanus* in this study also reiterates the potency and broad-spectrum antagonistic properties of these metabolites. The limitation of this study is that we did not characterize the extracts of the isolates and as such cannot suggest the novelty of the bioactive compounds or compare them to existing ones. In future studies, we hope to further characterize the nature of these unknown antibiotics.

This study reported the broad-spectrum antibacterial activities of extracts of secondary metabolites from *Streptomyces glauciniger* and *Streptomyces griseoplanus* isolated from soil, which corroborates the fact that actinomycetes represents a hub of untapped resources of translational benefits. Conclusively, the soil ecosystems of less explored areas serve as rich sources of actinomycetes that may be important sources of antibiotics.

DOI: http://dx.doi.org/10.4314/ejhs.v30i2.13

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