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Research Article

Preliminary screening of selected soils in Ilorin, North-central Nigeria for antibiotic-producing actinomycetes

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Abstract

The present research aimed at screening various soils within Ilorin metropolis for antibiotic producing actinomycetes. The objectives of the study were to determine physiochemical parameters of soils, the occurrence of actinomycetes in soils, antibacterial potentials and identity of isolates. Soil parameters such as pH, temperature, moisture, organic matter and soil type were evaluated following standard procedures. Selective isolation to determine the occurrence of actinomycetes was performed by soil dilution using pour plate technique on starch casein agar. Preliminary antibacterial screening against 10 clinical test bacteria was performed using cross streak method. All isolates were initially identified based on morphological and biochemical characteristics, while the most bioactive isolates were further identified by molecular means. The soils were alkaline, with temperatures between 29 °C and 31 °C, moisture was in range of 0.72 ± 0.07^{c} and 6.62 ± 0.42^{b}. Highest organic matter content was 32.13 ± 0.20^{a} with soil types mostly loamy and sandy loam. Ten actinomycetes (SM1 - SM10) were isolated, with the most frequently occurring isolate being SM3 and SM5 (16.7%). SM5 was the most active, inhibiting 9 out of 10 tests, with the highest inhibition against *Staphylococcus aureus* 25923 ($24 \text{ mm} \pm 0.15^{a}$). All isolates were identified as *Streptomyces* by morphology and biochemical tests. Based on nucleotide similarity searches and phylogeny, two bioactive *Streptomycetes* were suggested as novel strains and thus named as *Streptomyces bottropensis* UIL RNA (SM5) and *S. flavoviridis* UIL RNA(SM7), which may serve as promising sources of antibiotics. Actinomycetes from Ilorin metropolis demonstrated broad spectrum of antibacterial activity against clinical test bacteria.

Keywords: antibacterial; bioactive; inhibition; physicochemical; *Streptomyces bottropensis*

Introduction

Human anthropogenic activities and constant emergence of newer strains of microorganisms have being the leading causes of microbial resistance to different antibiotics (Li and Webster, 2018). Similarly, the World Health Organization (WHO) has stated that there is presently a serious lack of new antibiotics to

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fight the increasing risk of antimicrobial resistant organisms, which represents a global health emergency (Ventola, 2015).

Antibiotic resistance among pathogenic bacteria is a well-documented phenomenon that has severe consequences on the treatment of infections caused by them in hospitals (Li and Webster, 2018). The problem of increasing drug-resistance is now even more disturbing owing to limited number of available conventional antibiotics and the slow rate at which they are released into the markets. To this end, it is imperative to comb the earth for alternative sources of antimicrobial agents (Edwards *et al*., 2018).

Antibiotics and other bioactive compounds are often sourced from different environments, mainly terrestrial and marine, from microorganisms, plants and soils (Newman, 2016). Actinomycetes represent a major group of bacteria largely dominant in soil, where they serve as vital origin of most currently used antibiotics (Sharma *et al*., 2014).

Among all the genus of actinomycetes, *Streptomyces* represents the best producers of a very broad range of secondary metabolites, notably antibiotics and are largely abundant in water and soil (Valli *et al*., 2012). However, their distribution largely depends on food stress, temperature, pH, moisture, salinity, soil texture and climate (Yan *et al*., 2015).

Soil is a natural habitat and reservoir of numerous microorganisms especially actinomycetes which are the major producers of antimicrobial metabolites; however, only very few of these have been commercially produced. More so, is the fact that soil diversity and several other ecological factors give rise to the production of different metabolites found within. This has often resulted in the presence of varying bioactive secondary metabolites found in these microorganisms.

The aim of the present study was to randomly screen different types of soils within Ilorin, North-Central Nigeria for the presence of actinomycetes and determine their possible antibacterial potentials against extensive bacterial pathogens of clinical concerns. Also, the paucity of information regarding the antibacterial potentials of actinomycetes present in soils from Ilorin metropolis has necessitated the present study, with the hope of isolating bioactive ones.

The objectives include determination of physiochemical parameters of soils, isolation of actinomycetes in the soils, screening for antibacterial activity and identification of the isolated actinomycetes.

Materials and Methods

Description of the study area

The locations for the study were within Ilorin metropolis, Nigeria. Ilorin is situated at 8.5° N latitude, 4.55° E latitude and 290 meters elevation above the sea level. The sampling sites were: a flower garden site, university of Ilorin Zoo, University of Ilorin park, a maize farm land and a refuse dump site.

Sample collection and preparation

Soil samples were collected at two different points each of the five locations of Ilorin metropolis, using a completely randomized design, from a depth of 10 cm (Ismail *et al*., 2015). They were air-dried for 5 days at room temperature prior to isolation of actinomycetes.

Physicochemical characteristics of soil samples

Physicochemical analysis (pH, temperature, moisture content, organic matter and soil type) of samples were determined as described by Makut and Owolewa (2011).

Determination of soil pH

Soil pH was measured by digital pH meter. A slurry containing 5 g of soil and 5 ml of distilled water in a beaker was stirred for few seconds, allowed to stand for 10 minutes and thereafter pH was measured. An average of three consecutive readings was recorded for each sample.

Determination of soil temperature

The temperature of soil was measured *in situ* with a mercury thermometer. Thermometer was allowed to remain into soil until a constant temperature was observed; three consecutive readings were recorded.

Determination of moisture content

A moisture analyzer was used to determine moisture content. Soil sample (2 g) was dispensed in the crucible of the analyzer and set at 120 °C. The average of three consecutive readings was recorded.

Determination of organic matter content

Dried soil obtained from determination of moisture content was used to determine soil organic matter. Two grams of the soil were dried in a furnace at 600 °C for 3 hours. This was allowed to cool and re-weighed. The percentage of organic matter in the sample was calculated as follows:

Loss in weight of sample/ Initial weight of sample × 100%

Determination of soil types

To determine the soil types, the triangular diagram of soil textural classes by the Soil Science laboratory, Faculty of Agriculture, University of Ilorin was used, in line with ASTM international (2013).

Selective isolation and enumeration of actinomycetes from soil samples

Isolation of actinomycetes was performed by soil dilution using pour plate technique on starch casein agar containing chloramphenicol and nystatin, in order to inhibit growth of unwanted bacteria and fungi (George *et al*., 2012; Ismail *et al*., 2015). Plates were incubated at 30 °C for 5-7 days. Following growth, colonies of actinomycetes were enumerated.

Determination of the frequency of actinomycetes isolates occurrence

Percentage frequency of occurrence for each species of Actinomycetes isolated was evaluated as described by Sampo *et al*. (1997).

Collection of test bacteria

A total of 10 test clinical bacteria (four Gram-positive and six Gram-negative) used for the study were obtained from the culture collection unit of University of Ilorin Teaching Hospital, Ilorin, Nigeria. These were: *Klebsiella pneumonia*, *Staphylococcus aureus* (25923), *Proteus mirabilis*, multidrug resistant *Pseudomonas aeruginosa* (MDRPA), methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* (25922), methicillin-sensitive *Staphylococcus aureus* (6571), *Acinetobacter boumanii*, extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* and methicillin-resistant *Staphylococcus aureus* (43330). They were verified for true identities and pure cultures were maintained on nutrient agar slants, stored at a temperature of 4 °C and routinely sub-cultured for the purpose of purity. Bacteria were standardized using McFarland (0.5%) and suspensions were prepared for antibacterial screening.

Screening of actinomycetes for preliminary antibacterial activity

Preliminary antibacterial screening was performed using cross streak method as described by Gulve and Deshmukh (2012). Seven (7) day old pure culture of actinomycetes isolate was inoculated as a vertical

straight line on Mueller Hinton agar and incubated at 30 °C for 3-5 days after which test bacterium was inoculated perpendicularly to the straight line and incubated at 37 °C for 24 h. Plates were observed for clearance or absence of growth at site of test bacterium. Diameter of zone of inhibition was recorded to the nearest millimeter.

Identification and characterization of actinomycetes

Pure isolates were identified based on morphological and biochemical characteristics following the methods described by Fawole and Oso (2007) and result was compared with Bergey's manual of determinative bacteriology, 9th edition (2000).

Molecular identification of most bioactive actinomycetes

The identities of two most active isolates were further characterized by genomic DNA extraction, polymerase chain reaction (PCR) and DNA sequencing using 16S rRNA primers. Isolation of DNA was performed using QIAamp DNA Mini Kit (250) (cat No. 51306) according to the manufacturer's instructions. The small sub unit of 16S rRNA genes were amplified from isolated genomic DNA with two sets of primers 16SF (GTGCCAGCAGCCGCGCTAA) and 16SR (AGACCCGGGAACGTATTCAC) to obtain an amplicon size of 1,500 bp. Amplification was carried out in 25 µl reaction mixture consisting of 10x PCR buffer, 2.5 µl; 2.0 µl; 2.5 Mm DNTPs, 0.1 µl; 5.0 u/µl Taq DNA polymerase, 0.2 µl; 10 ng/µl of each primer, 3.0 µl; template DNA, 1.0 µl and sterilized distilled water 13.4 µl in a Biorad (USA) thermal cycler using the PCR conditions of 94 °C for 5 minutes (initial denaturation), 94 °C for 30 sec (denaturation), 72 °C for 45 sec (extension) and 72 °C for 7 minutes (final extension). The total number of cycles was 36, with the final extension of 72 °C for 7 minutes. The amplified products (50 µl) were size separated on 1% agarose gel prepared in 1% TAE buffer containing 0.5 µl/ml ethidiumbromide and photographed with transilluminator (Biorad, USA). A 1,000 bp DNA ladder (Genei) was used as molecular weight size markers. PCR product (1,500 bp) was purified by electro elution of gel slice containing the excised desired fragments with Qiaquick gel extraction kit (Qiagen, USA). The elution was carried out in 300 µl of nuclease free water in order to enhance the purification of PCR product. PCR amplicons obtained after amplification was diluted in Tris buffer (10 mM, pH 8.5) at 1:1,000 in order to obtain the DNA concentration required for sequencing (30 ng/µl), the sequencing reaction required 8 µl DNA. Sequencing was performed using automated sequencer (ABI PRISM 310, Applied Biosystems, USA). Nucleotide sequences coding for each isolate were analyzed for similarities by BLASTN tool (*www.ncbi.nlm.nih.gov:80/BLASTN/*). The sequences were aligned and bio-straped using MAAFT (Berkeley Software Distribution). The phylogeny of the organisms was compared with those of related species from standard laboratories available in GenBank of National Centre for Biotechnology Information (NCBI) to reveal the identity of each bacterium up to strain level.

Results

Physicochemical parameters of soil

Table 1 reveals the physicochemical characteristics of soils from the five different locations within Ilorin metropolis. All soils were alkaline in pH, with temperatures between 29 °C and 31 °C, moisture content was highest in the flower garden soil with a value of 26 ± 1.97^{a}, while University Zoo had the highest content of organic matter (32.13 ± 0.20^{a}). Soils were mostly loamy in texture.

Isolation of actinomycetes

A total of ten actinomycetes were isolated from the different sampling sites and designated as SM1 - SM10. It was observed that SM3 and SM5 were the most frequently occurring isolates with a percentage frequency of occurrence of 16.7 as shown in Table 2.

Table 1. Physicochemical characteristics of soils from different locations of Ilorin metropolis

Location	pH	Temperature (°C)	Moisture content (%)	O M C (%)	Textural class
F G	9.2±0.17^{b}	29±1.00^{b}	26±1.97^{a}	19.57±0.31^{c}	Loamy
U Z	9.2±0.05^{b}	28±0.58^{ab}	0.51±0.02^{c}	32.13±0.20^{a}	Loamy
M F	8.7±0.12^{c}	30±1.16^{ab}	1.73±0.03^{c}	31.30±0.31^{a}	Sandy loam
D W	9.9±0.15^{ab}	30±1.29^{ab}	6.62±0.42^{b}	26.62±0.19^{b}	Sandy loam
U P	7.5±0.06^{a}	31±0.58^{a}	0.72±0.07^{c}	30.65±0.66^{a}	Loamy

Key: Values represented are means of triplicates ± SEM. Means with the same superscript in the same row are not statistically different from each other. (p≤0.05); FG = flower garden, UZ = University Zoo, MF = maize farm, DW = refuse dump site, UP = University Park

Table 2. Percentage frequency of occurrence of Actinomycetes isolated from locations in Ilorin metropolis

	Sampling sites					
Actinomycetes isolates	FG	UZ	MF	DW	UP	Frequency of occurrence (%)
SMI	+	-	+	+	-	12.5
SM2	-	-	+	-	+	8.3
SM3	+	+	-	+	+	16.7
SM4	-	-	+	-	-	4.2
SM5	+	+	+	-	+	16.7
SM6	+	+	-	-	-	8.3
SM7	+	-	-	+	-	8.3
SM8	-	-	+	-	+	8.3
SM9	+	+	-	+	-	12.5
SM10	-	+	-	-	-	4.2

Key: FG = flower garden, UZ = University Zoo, MF = maize farm, DW = refuse dump site, UP = University Park; + means Present, - means Absent

Antibacterial activity of actinomycetes

Evaluation of antibacterial activity of the isolated actinomycetes showed that five (SM1, SM3 SM5, SM7 and SM9) of these isolates demonstrated activity in the assay against the clinical test bacteria, at varying degrees. It was also observed that methicillin-sensitive *Staphylococcus aureus* (6571), *Acinetobacter boumanii* were the most susceptible, and their growth were inhibited by the action of four actinomycetes. Interestingly, drug resistant bacteria such as methicillin resistant *S. aureus* and MDR *P. aeruginosa* demonstrated significant resistance. However, all test bacteria showed susceptibility to at least one of the 5 active actinomycetes. This is presented in Figure 1. It is worthy of mention that SM5 was the most active, with a zone of inhibition of 24 mm against *S. aureus* 25923.

Morphological and biochemical characterization of actinomycetes

Morphological and biochemical identification suggested all 10 isolates to be *Streptomyces* species. All isolates were Gram positive with pigmentation varying from white to dark brown and green as presented in Table 3.

The biochemical characteristics are shown in Table 4. All the isolates were oxidase and urease positive. Citrate utilization was also confirmed positive.

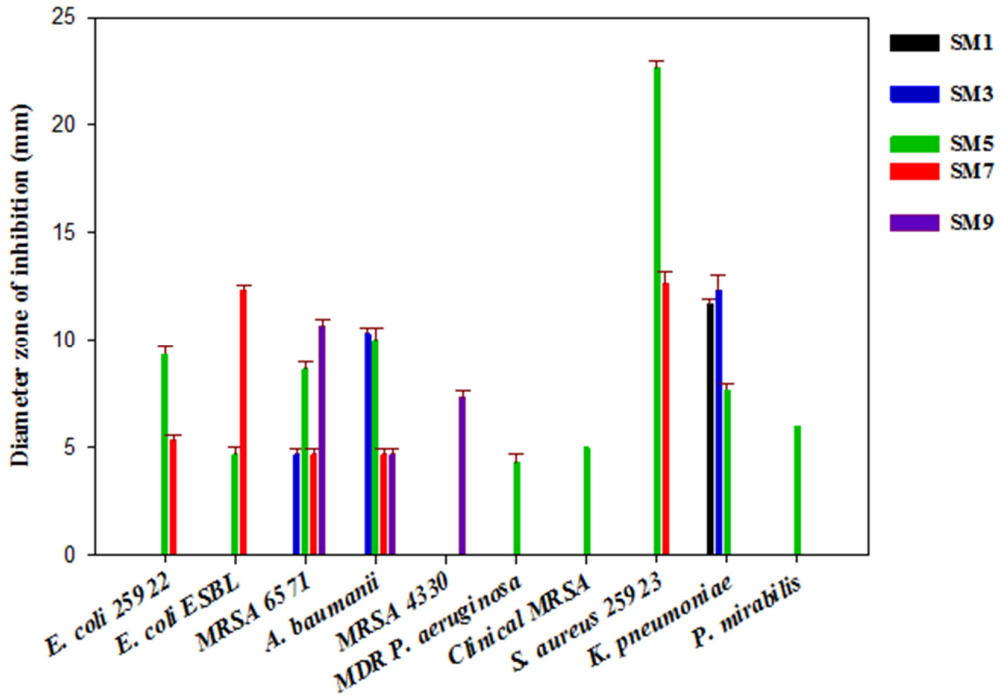

Figure 1. Sensitivity pattern of test bacteria to Actinomycetes isolated from Ilorin metropolis soils

Table 3. Morphological and cellular identification of Actinomycetes isolates

Character	SMI	SM2	SM3	SM4	SM5	SM6	SM7	SM8	SM9	SM10
Gram reaction	+	+	+	+	+	+	+	+	+	+
Acid fast stain	-	-	-	-	-	-	-	-	-	-
Colour	White	White	Brownish	Dark-brown	Cream	Greenish-brown	Light yellow	Brownish	Green	White
Morphology form/ surface	Smooth woolly	Rough, hard	Smooth, dusty	Smooth, dusty	Smooth woolly	Smooth, dusty	Smooth, chalky	Rough, Powdery	Smooth	Smooth hard
Elevation	Raised	Flat	Raised	Raised	Flat	Raised	Flat	Raised	Raised	Flat
Consistency	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry	Dry
Margin	Filament-ous	Round	Filament-ous	Filament-ous	Round	Filament-ous	Round	Filament-ous	Filament-ous	Round
Reverse side	Cream	Pale yellow	Dark-brown	Pale yellow	Dark brown	Cream	Cream	Cream	Cream	Cream
Aerial mycelium	White	White	Cream	Light brown	Light brown	Light green	Pale yellow	Brown	Green	White
Substrate mycelium	Cream	Light yellow	Light brown	Dark brown	Light brown	Brown	Pale yellow	Brown	Green	Cream

Table 4. Biochemical characterization of isolates

Isolates	Indole	MR	Oxi	Cata	S H	Cit	Casein hydro.	Nitrate Red.	V P	Gelatin liq.	Ure			TSI			Org
												A	B	Glu	Suc	Lac	
SMI	-	+	+	-		+	-	-	-	+	+	-	-	+	+	+	*S.vellaneus*
SM2	-	+	+	+		+	-	-	+	-	+	-	-	+	+	+	*S.malayensis*
SM3	-	+	+	-	-	+	+	+	-	+	+	-	-	+	+	+	*S.bikiniensis*
SM4	-	+	+	-	+	+	-	+	+	+	+	-	+	+	-	-	*S. sp.*
SM5	-	+	+	+	+	+	+	+	-	-	+	-	-	+	+	+	*S. sp.*
SM6	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	*S. sp.*
SM7	-	+	+	-	-	+	+	+	+	+	+	-	-	+	-	-	*S. rutgersensis*
SM8	-	+	+	-	-	+	+	+	+	-	+	-	-	+	+	+	*S. sp.*
SM9	-	+	+	+	+	+	-	+	+	-	+	+	-	+	+	+	*S. cinnamoneus*
SM10	-	+	+	-	+	+	+	+	-	+	+	-	-	+	+	+	*S. specie*

Keys: + = Positive, = Negative, MR = Methyl red test, Oxi = Oxidase test, Cata = Catalase test, SH = Starch hydrolysis test, Cit = Citrate test, Casein Hydro = Casein Hydrolysis, Nitrate Red = Nitrate Reduction, VP = Voges Proskauer, Gelatin Liq. = Gelatine Liquefaction, Ure = Urease, TSI = Triple Sugar Iron test, (A = Gas production, B = H_2S Production, Glu = Glucose, Suc = Sucrose, Lac = Lactose)

Molecular characterization of the two most potent isolates

Based on their ribosomal RNA analysis, nucleotide similarity searches and phylogenetic analysis, SM5 and SM7 were suggested to be novel strains of S. *bottropensis* and S. *flavoviridis* respectively as shown in Figure 2. They were subsequently designated as *Streptomyces bottropensis* UIL RNA and *S. flavoviridis* UIL RNA. The gene sequences of these isolates have been submitted to GenBank to obtain accession number, while respective pure cultures of these strains have been deposited at the culture collection center of the Department of Microbiology, University of Ilorin under the same name as identified. Further analysis is ongoing to establish the novelty of the isolates.

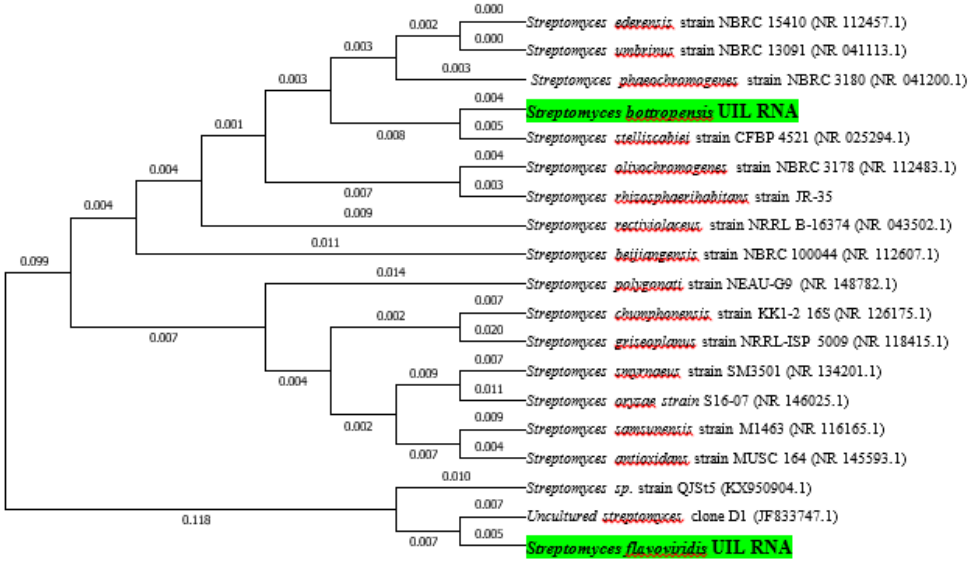

Figure 2. Maximum likelihood tree of the sequenced 16S rRNA gene of the isolated *Streptomyces bottropensis* strain UIL RNA and *Streptomyces flavoviridis* strain UIL RNA. The numbers in the branches of the phylogram indicate boostrap value in percentage by 1,000 replication multiples

Discussion

The screening of soils from various environments for antibiotic producing-actinomycetes has gained much attention in the last few decades due to the potentials exhibited by this group of microorganisms against pathogens. However, Ilorin, Nort-Central Nigeria is yet to be explored in this regard.

According to Yan *et al*. (2015) soil parameters such as temperature, pH, moisture, organic matter, salinity and vegetation are crucial factors influencing distribution of soil actinomycetes. In the present study it was observed that all soil samples were alkaline. According to McCauley *et al*. (2017) soil pH affects the activity of soil microorganisms. Acidic soils prevent break down of organic matter by bacteria, thereby preventing nutrient availability in soils, suggesting that alkaline soils are more preferred. This report is also in agreement with Makut and Owolewa (2011) reporting that actinomycetes thrive better in alkaline soils. In a study by Muhammad *et al*. (2016) optimum growth of actinomycetes was in a range of 25-35 °C hence promoting their growth. This is in consonance with the values of this study with temperatures between 28 and 31 °C. Actinomycetes thrive in soil samples with low levels of moisture as observed in most of the study area in the present work. They have been established to grow even in arid conditions of little moisture unlike other soil bacteria (Ghorbani-Nasrabadi *et al*., 2013) and this may be due to their sporulation ability in response to drought. However, this was not the case with soil from flower garden used in the hereby study, which had very high moisture content (26 ± 1.97^{a}) and still supported the growth of the highest number of isolates. This may be because the presence or absence of water does not prevent soil actinomycetes from thriving as there are several other parameters such as organic matter which are contributory factors affecting their distribution. Similarly, actinomycetes are heterotrophic organisms that prefer decomposable organic matter rich in plant and animal residues as of those obtained at the flower garden site. Hence, the presence of more isolates in FG site is an indication that high moisture aided by other physicochemical parameters supported growth of actinomycetes. Soil organic matter has a fundamental impact on soil microorganisms since it provides necessary nutrients for the soil microbial community (Jacoby *et al*., 2017). The values

obtained in the current study were high enough to have supported the growth of actinomycetes. In general, it was demonstrated in the present work that all physicochemical parameters studied were related to each other and they supported the isolation of the actinomycetes. The results indicated that the studied soils in Ilorin metropolis were mostly loamy and possess necessary characteristics that can support the growth of actinomycetes.

In the present study, higher number of actinomycetes were observed in soils from flower garden and maize farm. This observation agrees with Ian and Terry (2015) that actinomycetes are heterotrophic organisms that prefer decomposable organic matter rich in plant and animal residues. Hence suggesting that the two sampling sites where higher number of actinomycetes were isolated may have been richer in organic matter due to plant and flower debri. Ghorbani-Nasrabadi *et al*. (2013) also reported that actinomycetes population is higher in agricultural land compared to forest soils and this may be as a result of high organic matter content.

The antibacterial activity revealed that only five of the ten actinomycetes were active in the inhibition of the clinical bacteria. This uneven antibacterial activity suggests that antimicrobial biosynthetic genes are determined by the genetic composition of the genomes of actinomycetes (Ranjita *et al*., 2018). This implies that the ten isolates of actinomycetes in the present study even though are all Streptomyces, possess varied genetic composition of their genomes, which of course may have influenced the type of antibacterial genes producing the active metabolites. Interestingly, SM5 which was suggested as a likely novel strain (*Streptomyces bottropensis* UIL RNA) inhibited nine of the ten clinical pathogens tested, unlike the other four active actinomycetes. This may imply that SM5 possesses several antibacterial biosynthetic genes coding for the production of a broad spectrum of antibacterial metabolites active against the test bacteria. The reason for the observed resistance of some of the test bacteria to SM1-SM4 may be due to the fact that their biosynthetic genes did not code for broad spectrum antibacterial metabolites or it may be that such multi-drug resistant pathogens possess certain features which confer resistance to antibacterial agents. From literature, multi-drug resistant pathogens have efficient resistance mechanisms which enables them to resist the activity of antimicrobial agents. For instance, Staphylococci, which was one of the test bacteria used in this study have been shown to express beta lactamase enzyme which hydrolyzes the beta lactam rings of antibiotics and rendering them inactive (Kong *et al*., 2010). Even more, means of resistance by these organisms is the acquisition of genes encoding a modified antibiotic binding protein (Kong *et al*., 2010). These factors may have been responsible for the resistance demonstrated by the drug resistant test bacteria.

The morphological and biochemical characteristics observed with the *Streptomyces* isolated in the hereby study were similar with those observed by Afifi *et al*. (2014). Molecular characterization of the two most active isolates based on their ribosomal RNA analysis, nucleotide similarity searches and phylogenetic analysis suggest both as novel strains of actinomycetes. These strains were subsequently designated as *Streptomyces bottropensis* UIL RNA and *S. flavoviridis* UIL RNA because their levels of similarity did not meet the standard to match them with existing strains in the gene bank.

Conclusions

The emergence of resistance to antibiotics in Gram-positive pathogens has become a major international concern, as there are often very few or no effective antimicrobial agents available for the treatment of infections caused by them. This is especially true for Staphylococci which have developed different mechanisms to neutralize existing conventional antibiotics. However, the present study has proven that actinomycetes isolated from Ilorin metropolis soils inhibited the growth of methicillin resistant *S. aureus*, extended spectrum beta-lactamase (ESBL) producing *Escherichia coli*, as well as other multidrug resistant bacteria of clinical concern. Further investigations are necessary on the seemingly new strains of

Streptomyces; *Streptomyces bottropensis* UIL RNA and *S. flavoviridis* UIL RNA were isolated in the study in order to determine the metabolites' nature and possible mechanisms of their actions which may have accounted for such remarkable antibacterial activity. Thus, *Streptomyces bottropensis* UIL RNA may be a promising source of antibacterial agent.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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