*Asian Journal of Research in Medical and Pharmaceutical Sciences*

*4(2): 1-13, 2018; Article no.AJRIMPS.39742*
*ISSN: 2457-0745*

# Characterization of Antibiotics Inhibitory to Methicillin Resistant *Staphylococcus aureus* (MRSA) from Soil Actinomycetes

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author UA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author JSO managed the analyses of the study. Author SMJ managed the literature searches. All authors read and approved the final manuscript.*

***Article Information***

DOI: 10.9734/AJRIMPS/2018/39742
Editor(s):
(1) Lokendra Bahadur Sapkota, Assistant Professor, Department of Biochemistry, Chitwan Medical College, Bharatpur, Nepal.
Reviewers:
(1) Bamidele Tajudeen, Nigerian Institute of Medical Research, Nigeria.
(2) Gokben Ozbey, Firat University, Vocational School of Health Services, Turkey.
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(4) Charles Nnadi, University of Nigeria, Nigeria.
Complete Peer review History: http://www.sciencedomain.org/review-history/25550

***Original Research Article***

***Received 29th January 2018***
***Accepted 15th June 2018***
***Published 14th July 2018***

## ABSTRACT

Actinomycetes are subgroup of actinobacteria which include *Streptomyces,* a source of many important antibiotics. Methicillin-resistant *Staphylococcus aureus* is one of the emerging infectious diseases which pose a significant health and occupational risk worldwide. The aim of this work was designed to isolate and characterize antibiotic producing actinomycetes from soil capable of inhibiting methicillin-resistant *Staphylococcus aureus*. Soil samples collected from rhizosphere of plants, agricultural soil and hospital dump soil cultured on starch casein agar yielded only one (1)

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isolate showing antibacterial activity against a diameter of zone of inhibition for Methicillin-resistant *Staphylococcus aureus* (12 mm), *Bacillus subtilis* (*16* mm), *Escherichia coli* (15 mm) and *Pseudomonas aeruginosa* (14 mm). Ethyl acetate was used to extract antibacterial compounds from the culture filtrate. Thin Layer Chromatography was done on silica gel using Methanol: Chloroform (9.4:0.6). The extract was analyzed with Fourier Transform-Infrared Red spectroscopy with a view to establish the chemical identity of the compound. The isolate showed good growth and white aerial mycelium on starch-casein agar and whitish-brown substrate mycelium on starch-casein broth. The isolate also utilized glucose, sucrose and fructose during sugar fermentation and were positive on amylolytic activity, hydrogen sulfide production, and nitrate reduction, milk Coagulation and Peptonization and gram staining. Results also suggest that the actinomycete isolate belongs to the genus *Micromonospora*. A candidate ($R_f$: 0.8) with antibiotic potential against methicillin-resistant *Staphylococcus aureus* was isolated from soil habitat. The chemical finger prints identified in the extract included aldehydes, alkynes, 2 aromatic rings, alkanes and alkynes. The extract ($R_f$: 0.8) has antibiotic potential against methicillin-resistant *Staphylococcus aureus*. The chemical finger prints identified in the extract included aldehydes, alkynes, 2 aromatic rings, alkanes and alkynes. Based on biochemical and morphological characterization, the isolate (A2) belongs to the genus *Micromonospora* of the family Micromonosporoceae.

*Keywords: Actinomycetes; MRSA; antibiotics; TLC and FT-IR.*

## 1. INTRODUCTION

Actinomycetes are a subgroup of the actinobacteria that are gram positive organisms with a high G+C ratio ($> 55\ mol\ \%$) in their DNA. This group is very ancient, and branched off from the other groups very early in the evolutionary sequence. The actinomycete group include many familiar and important bacteria, including *Mycobacterium* (the causal agent of tuberculosis and leprosy), *Corynebacterium* (a common commensal on human skin and, therefore often recovered in bacterial air samples) and *Streptomyces* (the source of many antibiotics as well as the pleasant odour of freshly turned soil). In general, the optimal temperature for their growth is between 25-30°C (50°C) for the thermophilic actinomycetes) and most are aerobic and neutrophilic. The bacteria do not grow in non- selective media, but instead require the use of specialized and nutritionally complex media. Furthermore, incubation needs to be in the absence of oxygen (anaerobically) and the growth of the bacteria is quite slow. Solid growth medium may need to be incubated for up to 14 days to achieved visible growth [1].

The actinomycetes are rod shaped or filamentous. Those that are rod shaped may form long, branching, chains of cells. Many actinomycetes form true filaments that branch and form colonies that look like fungi, although the diameter of the filaments is much smaller than that of the fungi. Filamentous form produce spores that may be single, in short chains, or in very long chains that may form beautiful spirals. There are both anaerobic and aerobic actinomycetes. Actinomyces are an important anaerobic genus. The truly filamentous forms are predominantly aerobic in nature.

Methicillin was first introduced in 1959– 1960, and, within a year, methicillin-resistant isolates were reported [2]. Methicillin resistance is conferred by the mecA gene, which encodes a penicillin-binding protein (PBP2A) with decreased affinity for β-lactam antibiotics. mecA is part of a mobile genetic element called the "staphylococcal cassette chromosome (SCC) mec." SCCmec is flanked by cassette chromosome recombinase genes (ccrA/ccrBor ccrC) that permit intra and interspecies horizontal transmission of SCCmec. The initial reservoir of SCCmec is unclear but may have been a coagulase-negative staphylococcal species [3,4, 5]. A limited number of MRSA lineages have emerged from the transfer of SCCmec into successful methicillin-susceptible *S. aureus* (MSSA) clones. Using multi locus sequence typing (comparing the internal sequences of 7 housekeeping genes), Enright, et al. [6] demonstrated that MRSA clones evolved from 5 different groups of related genotypes or clonal complexes, each arising from a distinct ancestral genotype. The earliest MRSA isolates evolved from sequence type (ST) 8-MSSA, which, after a point mutation, evolved into ST250-MSSA. This MSSA was likely the first recipient of SCCmec specifically, type I) to yield the first MRSA, labelled ST250-MRSA-I as in the works of Enright, et al. [6]). Crisostomo et al. [7] identified

probable recipient MSSA strains for early MRSA trains in another collection of isolates.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Soil Samples

Soil samples were collated from various locations (9°22''N-9°16'45''N and 12°26'45''E-12°33''E) in Jimeta, Girei and Yola. Several diverse habitats were considered for the isolation of different strains. These habitats include rhizosphere of plants, agricultural soil, preserved areas and hospital dump soils. The samples were taken from a depth of 20 cm after removing approximately 3 cm of the soil surface. The samples were placed in a polythene bags, closed tightly and stored in a refrigerator.

The soil samples were dried separately at 37°C for 1 hour in hot air oven. Then they were cooled to room temperature. One gram of each soil sample was added to a conical flask containing 100 ml of sterile water. All flasks were shaken for 30 min. in orbital shaker incubator at 27°C. These flasks were considered as stock cultures [8].

### 2.2 Isolation of MRSA

Wound swab samples were collected from hospitalized patients. Mannitol salt agar was used for inoculation. Biochemical test such as coagulase and catalase were performed and antibiotic susceptibility screening was done. The antibiotics included: Ampicillin (10 µg), Chloramphenicol (10 µg), Cloxacillin (5 µg), Gentamicin (10 µg), Streptomycin (10 µg), Tetracycline (10 µg), Ceftriaxone (30 µg) and Cephalexin (30 µg) as described by Akindele, et al. [9]. Samples were codified, cultured on selective and differential media (Mannitol Salt Agar, Phenol red Mannitol Salt Broth, Himedia, India), and incubated at 37ºC for 48 hours. Grown colonies on these media were identified as *Staphylococcus aureus* with morphology, gram staining, and catalase, coagulase, DNase tests. Then, disk diffusion test based on Clinical and Laboratory Standard Institute (CLSI) method was used for the isolates to evaluate their susceptibility to methicillin (Cloxacillin). For MRSA detection, first bacterial suspensions equal to 0.5 McFarland tube were made from *Staphylococcus aureus* isolate which were grown on TSB (Trypticase soy broth**)** medium (Himedia, India). The suspension was cultured with swab on Muller- Hinton Agar (Himedia, India) that contained 4% NaCl and 6 µg/mL oxacillin (Himedia, India), and then incubated at 35ºC for 18 to 24 hours and examined for evidence of growth.

### 2.3 Physiochemical Parameters of the Soil Samples

Soil pH was measured in 1:2.5 soils: water suspensions using a glass electrode digital pH meter (Digital PH Meter, Mifa system Private Ltd, India). $Ca^{+}$ and $Mg^{+}$ were determined by titration and $K^{+}$ and $Na^{+}$ by flame photometer (Esico International, India). The organic carbon content of the soil samples was determined by the method of Walkley and Black (1934). The total nitrogen content of the soil samples was determined by the Macro-Kjeldah method [10].

### 2.4 Screening of Soil Samples for Actinomycetes by Crowded Plate Technique

A series of culture tubes containing 9 ml of sterile water were arranged in a test tube rack (Serial dilution). From the stock culture, 1 ml suspension was transferred aseptically to the first tube ($10^{-1}$). From the first tube, 1 ml of suspension was transferred into the second tube ($10^{-2}$) and also mixed well. Similarly, dilutions of up to $10^{-5}$ were made. 0.1 ml of suspension from each culture tube was spread on sterile starch– casein agar (SCA) plates. The plates were incubated for 5 days at 37°C; colonies with clear zone of inhibition were selected. The pinpoint colonies with inhibitory or clear zone of inhibition were purified into starch-casein agar slants and stored at 4°C in the refrigerator. The actinomycetes colonies isolated from the crowded plate were designated as A1, A2, A3 ….and so on [11].

### 2.5 Screening of Crude Extract for Antibiotics

The microbial sensitivity of the soil isolates were analyzed by agar streak method. Each of the isolates was streaked as a straight line on starch-casein medium (HiMedia, India) and incubated at 27°C for about 6 days. After 6 days, methicillin resistant *Staphylococcus aureus* was streaked at right angle, but not touching the crude extract and incubated at 37°C for 24 hours. After 24 hours incubation, the zone of inhibition was observed and recorded [11]. Chloramphenicol (30 ug) was used as positive control.

## 2.6 Tests for Nitrate Reduction

The soil isolates were evaluated by using 'organic nitrate broth'. 10 ml of sterile organic nitrate broth was prepared. A loopful of soil isolate was added into the broth aseptically and incubated at 27°C for 5 days. Its nitrate reduction property was observed by adding 2 drops each of Naphthalene solution and Sulfonilic acid solution to the broth under examination. A positive reaction showed a pink-red color [12].

## 2.7 Milk Coagulation and Peptonization

All the soil isolates were inoculated aseptically into a different sterile culture tubes containing pasteurized skimmed milk and incubated at 37°C for 48 hours. The tubes were observed daily for 48 hours. The tubes were observed for the following reaction; (a) Reduction of litmus paper and (b) Change in medium color. These changes take place due to the digestion of milk proteins and change in pH of the medium [12].

## 2.8 Tests for Amylolytic Activity by Starch Hydrolysis

A sterile starch agar slant was prepared. The soil isolates were streaked on the slants by simple streak method aseptically, and incubated for 5 to 7 days at 28°C. Amylolytic activity was observed by using iodine solution which indicates the hydrolysis of starch as described by Kokare, et al. [12].

## 2.9 Hydrogen Sulfide ($H_2S$) Production

A sterile slant of kligler's iron agar (KIA) was prepared, streaked with soil isolates and incubated at 37°C for 4 days. After the incubation period, $H_2S$ production was observed by rotten egg smell and change in color of the medium to greenish brown, bluish black or black color [11].

## 2.10 Gram Staining

A smear of the isolate was made and heat fixed. The fixed smear was covered with crystal violet stain for 30-60 seconds and washed off with clean water. The smear was tipped off all the water and covered with lugol's iodine for 30-60 seconds and washed off also with clean water. The smear was decolorized with acetone for few seconds and immediately washed with clean water. The smear was stained with safranin for 2 minutes and washed off with water. The slide was placed in a draining rack for the smear to air-dry and was examined with oil immersion under x100 objective lens [13].

## 2.11 Fermentation

The isolate which have shown broad spectrum activity during the primary screening was selected for secondary screening. The isolate was seeded into Starch-casein agar and grown into mass. Harvested spore of active isolate was inoculated in a 500 ml Erlenmeyer flask containing 100 ml Starch-casein broth and incubated at 28°C for 7 days under the standard condition of aeration and agitation. Then, the fermentation process was terminated [11].

## 2.12 Isolation of Anti-microbial Metabolites

From the fermented broth, the mycelium was removed by filtration and the cleared filtrate was used for further isolation. The anti-microbial compound was isolated from the filtrate by solvent extraction method. Antibacterial compound was recovered from the filtrate by solvent extraction with ethyl acetate. Ethyl acetate was added to the filtrate in the ratio of 1:1 (v/v) and shaken vigorously for 1 hour for complete extraction. The ethyl acetate phase that contains an antibiotic was separated from the aqueous phase. It was evaporated to dryness in water bath (Narang Scientific, NSW-128, India) at 80-90°C and the extract obtained was used for Thin-layer chromatography.

## 2.13 Purification of the Active Compound

Thin layer chromatography was used to purify the extract. The chromatographic parameters used in thin-layer chromatography are as follows: The Stationary Phase is made of Silica gel while the mobile phase is solvent mixture of methanol: chloroform (9.4: 0.6). The TLC plates (F254, 20X20 cm, Merck, Germany) were prepared and spotted with the sample and standards. The standards and sample were run with the mobile phase and observed under UV light at 254 nm. The separated spots were developed with iodine vapor as described by Sharma et al. [14].

### 2.14 Determination of Chemical Finger prints of the Extract

The instrument used for the IR spectroscopy was Nicolet IR 100 FT-IR (Cole-Parmer, USA). Two drops of the cell free extract of sample was placed on one of the KBr plates and spread sensibly around the top of the plate into a thin capillary film by enclosure with a second plate on top of it. The plates were gently inserted into the plate holder which was then introduced into the IR spectrometer machine after being screwed tight. The background was collected and then followed by the IR of the sample.

## 3. RESULTS

### 3.1 Activity of Different Isolates on Crowded Plate and MRSA

All the Fourteen (14) isolates showed zone of inhibition on crowded plate but only A2 was active against methicillin-resistant *Staphylococcus aureus* (MRSA) (Table 1).

### 3.2 Physiochemical Parameters of the Soil Samples

The samples analyzed for texture and colour were dark-brown and clay loam, pH averaged 5.6. Exchangeable bases are 3.78, 0.74, 0.36, 0.22 me/ 100 g of soil for $Ca^{2+}$, $Mg^{2+}$, $K^{+}$ and $Na^{+}$ respectively. Organic carbon and total nitrogen ranged from 1.2-1.7 and 0.1-0.14% respectively (Table 2).

### 3.3 Activity Profile of Crude Extract of Actinomycete Isolate on *E. coli, B. subtilis, P. aeruginosa* and MRSA

Isolate A2 showed zone of inhibition (12 mm) against MRSA. *E. coli, B. subtilis* and *P. aeruginosa*. Chloramphenicol (standard) was active against *E. coli, B. subtilis* and *P. aeruginosa* but not active against *MRSA* (Table 3). The isolate showed 12 mm zone on MRSA (Fig. 1).

### 3.4 Biochemical Characterization of the Isolate

Isolate A2 was positive for amylolytic activity, hydrogen sulfide production, nitrate reduction, milk coagulation and peptonization and Gram staining see (Table 4).

### 3.5 Fermentation of Sugars by the Isolate

Isolate A2 utilized glucose, sucrose, and fructose but unable to utilized inositol and mannitol (Table 5).

### 3.6 Cultural and Microscopic Characterization of the Isolate

Isolate A2 showed good growth and white aerial mycelium on starch-casein agar and whitish-brown substrate mycelium on starch-casein broth while the microscopy showed branching mycelia (Table 6).

**Table 1. Activity of different isolates against MRSA**

| Isolates | Zone of inhibition without test organism | Zone of inhibition on MRSA |
|---|---|---|
| A1 | + | _ |
| A2 | ++ | + |
| A3 | + | _ |
| A4 | + | _ |
| A5 | + | _ |
| A6 | + | _ |
| A7 | + | _ |
| A8 | + | _ |
| A9 | + | _ |
| A10 | + | _ |
| A11 | + | _ |
| A12 | + | _ |
| A13 | + | _ |
| A14 | + | _ |

*+ : Minimum inhibition*
*_ : No inhibition*
*++: Maximum inhibition*
*A1-A14 (Actinomycetes)*
*MRSA: Methicillin-Resistant Staphylococcus aureus*

**Table 2. Physiochemical parameters of the soil samples**

| Samples | Color/ Texture | pH | $Ca^{2+}$ | $Mg^{2+}$ | $K^{+}$ | $Na^{2+}$ | % O.C | % T.N |
|---|---|---|---|---|---|---|---|---|
| J1 | DB/ CL | 5.73 | 4.1 | 0.82 | 0.14 | 0.26 | 1.20 | 0.102 |
| J2 | DB/ CL | 5.70 | 4.0 | 0.80 | 0.39 | 0.25 | 1.40 | 0.118 |
| J3 | DB/ CL | 5.60 | 3.8 | 0.76 | 0.37 | 0.23 | 1.30 | 0.111 |
| J4 | DB/ CL | 5.26 | 2.3 | 0.46 | 0.24 | 0.18 | 1.30 | 0.110 |
| J5 | DB/ CL | 5.63 | 3.6 | 0.72 | 0.35 | 0.20 | 1.50 | 0.127 |
| J6 | DB/ CL | 5.41 | 2.3 | 0.46 | 0.24 | 0.15 | 1.20 | 0.101 |
| J7 | DB/ CL | 5.26 | 3.0 | 0.62 | 0.31 | 0.18 | 1.50 | 0.128 |
| J8 | DB/ CL | 5.74 | 4.0 | 0.82 | 0.40 | 0.23 | 1.30 | 0.111 |
| J9 | DB/ CL | 5.38 | 2.9 | 0.58 | 0.28 | 0.16 | 1.30 | 0.110 |
| J10 | DB/ CL | 5.84 | 4.4 | 0.89 | 0.43 | 0.27 | 1.70 | 0.145 |
| J11 | DB/ CL | 5.79 | 4.1 | 0.82 | 0.40 | 0.25 | 1.40 | 0.119 |
| J12 | DB/ CL | 5.42 | 3.1 | 0.62 | 0.31 | 0.18 | 1.50 | 0.128 |
| J13 | DB/ CL | 5.94 | 4.5 | 0.90 | 0.41 | 0.28 | 1.40 | 0.120 |
| J15 | DB/ CL | 5.73 | 4.1 | 0.83 | 0.26 | 0.25 | 1.60 | 0.136 |
| J16 | DB/ CL | 5.38 | 2.7 | 0.54 | 0.44 | 0.15 | 1.50 | 0.127 |
| J17 | DB/ CL | 5.88 | 4.4 | 0.89 | 0.45 | 0.26 | 1.30 | 0.111 |
| J18 | DB/ CL | 5.96 | 4.6 | 0.92 | 0.45 | 0.27 | 1.30 | 0.110 |
| J19 | DB/ CL | 6.43 | 3.1 | 0.62 | 0.31 | 0.19 | 1.40 | 0.119 |
| J20 | DB/ CL | 5.81 | 4.4 | 0.88 | 0.44 | 0.26 | 1.20 | 0.101 |
| J21 | DB/ CL | 5.97 | 4.7 | 0.94 | 0.46 | 0.28 | 1.40 | 0.120 |
| J22 | DB/ CL | 5.63 | 3.9 | 0.79 | 0.39 | 0.24 | 1.20 | 0.104 |
| J23 | DB/ CL | 5.55 | 3.5 | 0.70 | 0.34 | 0.20 | 1.40 | 0.119 |
| J24 | DB/ CL | 5.56 | 3.5 | 0.71 | 0.35 | 0.20 | 1.60 | 0.137 |
| J25 | DB/ CL | 5.44 | 3.1 | 0.61 | 0.31 | 0.19 | 1.20 | 0.100 |

*Key: DB: Dark-brown, CL: Clay-loam, O.C: Organic carbon*
*T.N: Total nitrogen*

**Table 3. Antibacterial Activity of Extract on *E. coli, B. subtilis, P. aeruginosa* and MRSA Zone of inhibition (mm)**

| Isolates | *MRSA* | *E. coli* | *P. aeruginosa* | *B. subtilis* |
|---|---|---|---|---|
| A2 | 12 | 15 | 14 | 16 |
| CH (30 µg/ml) | 00 | 20 | 19 | 23 |

*Key: CH: Chloramphenicol*
*A2 (Actinomycete)*
*MRSA: Methicillin-Resistant Staphylococcus aureus*

**Table 4. Biochemical characterization of the actinomycete isolate**

| Isolates | AA | $H_2SP$ | NR | MCP | GS |
|---|---|---|---|---|---|
| A2 | + | + | + | + | + |

*Key: +: Positive*
*AA: Amylolytic activity, NR: Nitrate reduction, MCP: Milk coagulation and peptonization*
*GS: Gram Staining, $H_2SP$: Hydrogen Sulfide Production*
*A2 (Actinomycete)*

**Table 5. Fermentation of sugars by actinomycete isolate**

| Isolates | Glucose | Sucrose | Fructose | Inositol | Mannitol |
|---|---|---|---|---|---|
| A2 | + | + | + | _ | _ |

*Key: +: Positive, -: Negative*
*A2 (Actinomycete)*

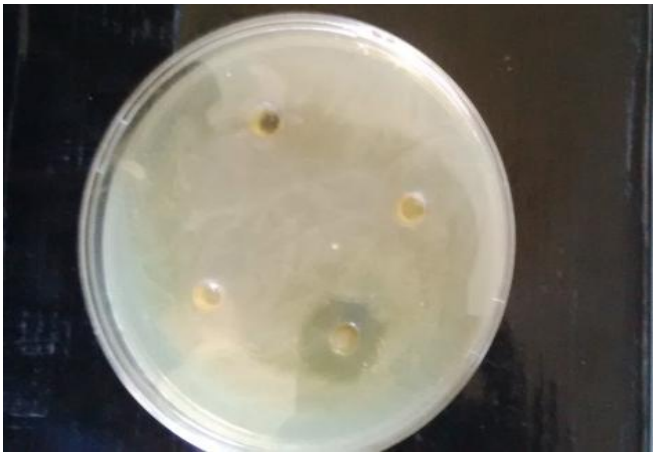

**Fig. 1. The zone of inhibition of A2 against MRSA on Mueller-Hinton Agar**
*Key: A2: Actinomycete*
*MRSA: Methicillin-Resistant Staphylococcus aureus*

**Table 6. Cultural and microscopic characterization of the actinomycete isolate**

| Isolate | Growth | AM | SM Microscopy |
|---|---|---|---|
| A2 | Good | White | whitish-Branching-mycelia brown |

*Key: A2 (Actinomycete)*
*AM: Aerial mycelium*
*SM: Substrate mycelium*

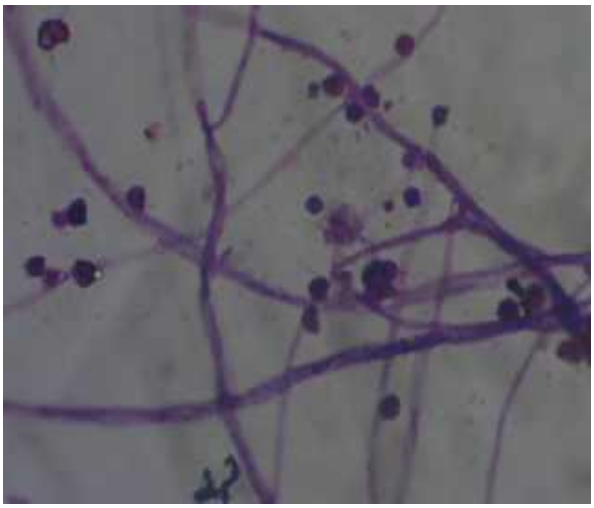

**Plate I. The microscopy of the actinomycete isolates (100 x using oil immersion)**

### 3.7 TLC of the Extract, Chloramphenicol and Streptomycin

The extract showed a single spot with $R_f$ =0.8 while both standard (chloramphenicol and streptomycin) showed double spots with $R_f$ = 0.4, 0.61 for chloramphenicol and $R_f$ =0.35, 0.55 for Streptomycin see (Table 7 and Fig. 2).

### 3.8 Fourier Transform-Infra Red Chemical Fingerprints of the Extract

Fourier Transform-Infra Red spectrum of the extract A2 showed 6 absorption peaks in the regions of 2364.95 $cm^{-1}$ for H-C=O:C-H (Aldehydes), 2076.67 $cm^{-1}$ for -C≡C- (Alkynes), 1648.13 $cm^{-1}$ for –C-C- (Aromatics in rings),

1459.68 $cm^{-1}$ for –C-C- (Aromatics in rings), 1390.22 $cm^{-1}$ for C-H (Alkanes) and 1043.09 $cm^{-1}$ for =C-H (Alkenes) (Table 8 and Fig. 1).

**Table 7. Thin layer chromatography of the extract, chloramphenicol and streptomycin**

| Isolate | $R_f$ values |
|---|---|
| Sample (A2) | 0.8 |
| Chloramphenicol | 0.4, 0.61 |
| Streptomycin | 0.35, 0.55 |

## 4. DISCUSSION

Soil samples were analysed for texture and colour which were dark-brown and clay loam, pH averaged 5.6. Exchangeable bases ranged from 3.78, 0.74, 0.36, 0.22 me/ 100 g of soil for Ca, Mg, K and Na respectively. Organic carbon and total nitrogen ranged from 1.2-1.7 and 0.1-0.14 % respectively.

According to McCarthy and Williams [15], organic matter, temperature and pH are important factors which control abundance of actinomycetes in soil. A comparison of the mean values of the chemical and physical soil properties as well as the actinomycetes population showed significant differences in pH value, organic carbon (OC), total nitrogen (TN), exchangeable bases ($Na^+$, $K^+$, $Mg^+$ and $Ca^+$) and actinomyctes in different soil ecosystem studied.

**Table 8. Fourier transform-infra red chemical fingerprints of the extract**

| Wavelength ($cm^{-1}$) | Functional groups |
|---|---|
| 2364.95 | Aldehydes |
| 2076.67 | Alkynes |
| 1648.13 | Aromatics (in rings) |
| 1459.68 | Aromatics (in rings) |
| 1390.22 | Alkanes |
| 1043.09 | Alkenes |

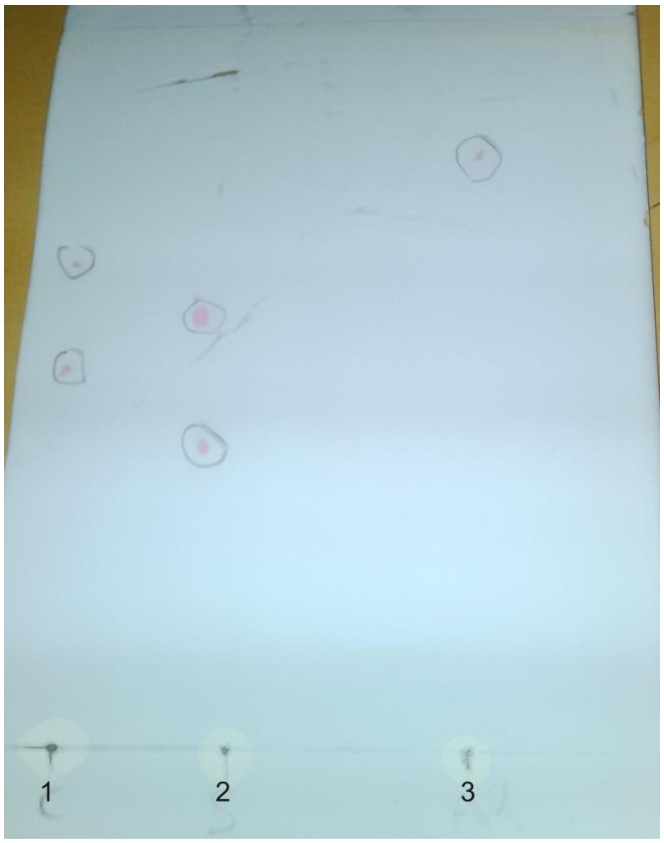


**Fig. 2. TLC of the extract, chloramphenicol and streptomycin**

*Key: 1: Extract*
*2: Chloramphenicol*
*3: Streptomycin*

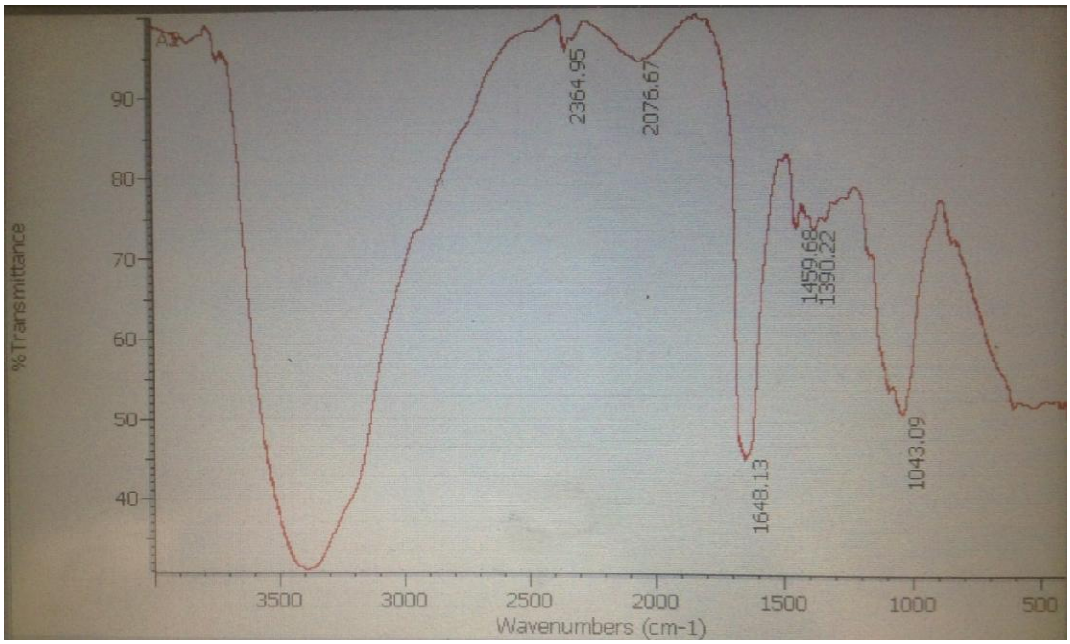


**Fig. 3. The FT-IR chemical fingerprints of the extract**

The pH value of the forest soil was significantly lower (pH 5.6). The pH values found in this study are in good agreement with those reported by Ghorbani, et al. [16]. The apparently lower pH values observed in agricultural soils are due to a downward movement of basic ions in these soils. Natural processes such as carbon dioxide evolution from plant roots or soil microbial respiration are believed to be responsible for controlling soil pH. Lauber, et al. [17] discussed that among other vegetation, soil type and soil management can alter soil pH.

Soil organic matter has a key role on beneficial biological processes as well as on the chemical and physical properties of soils. It provides energy for soil microbial community, increases cation exchange capacity and ameliorates soil aggregate and structure [18].

A survey of actinomycetes from several diverse habitats including the rhizospheres of plants, agricultural soils and hospital dump soil yielded a total of [14] bioactive actinomycetes coded A1-A14 that wereanalyzed for antimicrobial activity. Among these isolates, only one sample showed antibacterial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA).

Antimicrobialactivity of cell free extract of the isolated actinomycete was tested on *MRSA, E. coli, B. subtilis* and *P. aeruginosa*. Only A2 showed a zone of inhibition on*MRSA: 12* mm*; E. coli: 15* mm*; B. subtilis: 16* mm and *P. aeruginosa*: 14 mm while A3, A9 and A12 showed no zones of inhibition on any of the test organisms. A standard antibiotic chloramphenicol (30 µg) was used as a control, the zone of inhibition were *MRSA: 00* mm*, E. coli: 20* mm*,B. subtilis: 23* mm and *P. aeruginosa*: 19 mm.

The zone of inhibition on *MRSA* (12 mm) for A2 isolate agrees with those of Sharamugaraju et al. [19] which revealed that partially purified and characterized anti-MRSA peptide from marine *P. aeruginosa* showed a zone of 12 mm. Bashir, et al. [20] who isolated actinomycete from soil that inhibited multi-drug resistant human pathogen also recorded a zone of 12 mm against MRSA. The result of *E. coli* (15mm) agrees with that of Bello, et al. [21] which showed that *Streptomyces* species isolated from soil inhibited *E. coli* with 15 mm zone of inhibition. As well, the inhibitory zone of *Bacillus subtilis* (16 mm) agrees with that of Dattu, et al. [22] which revealed that biologically active silver nanoparticles from *Streptomyces* showed a 16 mm zone of inhibition against *B. subtilis*.

Biochemical tests conducted on A2 were nitrate reduction, milk coagulation and peptonization, starch hydrolysis, gram staining and hydrogen sulfide production. The sample showed positive result for starch hydrolysis. The starch hydrolysis

results agree with those of Parthasarathi et al. [23], Attimarad, et al. [11] and Preeti, et al. [24]. Actinomycetes are known to possess the ability to produce amylase that breaks starch into maltose. The amylase is an extracellular enzyme which is released from the cell of the microorganism [11].

In the nitrate reduction test, the sample reduced nitrate to nitrite. The results agree with those of Parthasarathi, et al. [23] and Attimarad, et al. [11]. The reduction of nitrate to nitrite has been used as one of the criteria for species differentiation of actinomycetes [11]. Nitrate and Nitrite serve as sources of nitrogen for the synthesis of organic nitrogenous compounds or they may function as $H^+$ acceptors. Nitratase converts $NO_3^-$to $NO_2^-$.

The A2 isolate showed positive results on milk coagulation and peptonization. The finding agrees with that of Parthasarathi, et al. [23] who stated that actinomycetes were positive on milk coagulation and peptonization. The digestion of coagulated milk by the action of proteolytic enzymes is termed peptonization. Some species such as *Streptomyces* peptonized milk without coagulation and if caseinase is not present in the microorganisms, peptonization however, does not follow precipitation [12].

The A2 isolate showed positive result for $H_2S$ production. Actinomycetes obtained energy by oxidizing organic compounds or molecular hydrogen ($H_2$) while reducing sulphate ($SO_4^{2-}$) to hydrogen sulphite ($H_2S$) [25].

The A2 isolate showed positive result for Gram stain. The result agrees with those of Attimarad, et al. [11].

In the Gram stain reaction, an insoluble crystal violet-iodine complex is formed inside the cell and this complex is extracted by alcohol from Gram negative but not from Gram positive bacteria. Gram positive bacteria, which have very thick cell wall made up of several layers of peptidoglycan, become dehydrated by the alcohol. This causes the pores in the walls to close, preventing the insoluble crystal violet-iodine complexes from escaping. In the gram negative bacteria, the solvent readily dissolves in and penetrates the outer layer, and the thin peptidoglycan layer also does not prevent solvent passage. However, the gram reaction is not related directly to bacterial cell wall chemistry, since yeasts, which have a thick cell wall but of an entirely different chemical composition, also stain Gram-positively. Thus, it is not the chemical constituents but the physical structure of the wall that confers gram positivity [26].

The A2 isolate showed good growth and white aerial mycelium on starch-casein agar and whitish-brown substrate mycelium in starch-casein broth.

Type of carbohydrate source utilized by actinomycetes is an important biochemical property for the identification of actinomycetes [11].

Phenol red broth was used as a fermentation media comprising Tryptone, NaCl, Phenol red and a carbohydrate sources such as glucose, sucrose, fructose, inositol and mannitol. The Tryptone provides amino acids, vitamins, minerals and other nitrogenous substances making a nutritious medium for the isolates. NaCl helps in maintaining the osmotic balance and provides the essential electrolytes for the transport into the cell while the carbohydrates act as a source of energy. The phenol red is the pH indicator. The A2 isolate ferments glucose, sucrose, and fructose but unable to utilized inositol or mannitol. The results of glucose and sucrose fermentation agree with those of Attimarad, et al. (2003), but disagree with the result of fructose, inositol and mannitol fermentation by Parthasarathi, et al. [23].

Thin layer chromatography of the A2 isolate revealed only one spot with $R_f$value of 0.8, Methanol and Chloroform (9.4:0.6) were used as a solvent system. Chloramphenicol and streptomycin were run as a control on the same plate with the extract of A2. Chloramphenicol revealed 2 spots with a distance of 0.4, 0.61 while streptomycin revealed 0.35, 0.55 respectively. The $R_f$ value of 0.8 for A2 isolate agrees with those of Attimarad, et al. [11], Preeti, et al. [24] and Parthasarathi, et al. [23].

The larger the $R_f$ value of a compound, the larger the distance it travel on the TLC plate and it is less polar because it interacts less strongly with the polar adsorbent on the TLC plate. A compound of low polarity will have a larger $R_f$ value than a polar compound run on the same plate. The A2 extract $R_f$ value of 0.8 is less polar than the 2 controls.

Fourier transform infrared spectroscopy (FT-IR) offers qualitative and quantitative analyses for organic and inorganic samples. FT-IR identifies chemical bonds in a molecule by producing an infrared absorption spectrum. The spectra produce a profile of the sample, a distinctive molecular fingerprint that can be used to screen and scan samples for many different components. IR exploits the fact that molecules absorb specific frequencies (wavelength) that are characteristics of their structure. This absorption is resonant frequencies, that is, the frequency of the absorbed radiation matches the transition energy of the bond or group that vibrates [23]. FT-IR spectrum of the sample showed the presence of H-C=O: C-H (Aldehydes), -C≡C- (Alkynes), –C-C- (Aromatics in rings), –C-C- (Aromatics in rings), C-H (Alkanes) and =C-H (Alkenes).

Based on the biochemical, cultural and microscopic characterization of the isolate (A2), the extract is suggested to belong to the genus *Micromonospora* of the family Micromonosporoceae. The various species are sources of aminoglycoside antibiotics , which spellings often ends with *–micin* , unlike the most other aminoglycosides names that ends with *–mycin* to highlight the very different species from which they originate (e.g Neomycin and Streptomycin ).

## 5. CONCLUSION

The present study reemphasizes the promise of soil and river bank as source of antimicrobial actinomycetes.

Although, soil habitats have been ignored for exploration, several recent reports corroborate the importance of such ecosystems for the search of antibiotic producing actinomycetes. A *Micromonospora, Norcadia and Streptomyces* spp., with broad-spectrum antimicrobial activity, was isolated from water sample of the Nambul River in India by Debanda, et al. [27]. Himabandu, et al. [28] reported Micromonosporaechinospora, a Gentamicin producing strain which was active against both Gram positive and Gram negative bacteria. Pranav, et al. [29] recently stressed the significance of soil as source of bioactive actinomycetes. They obtained actinomycete belonging to *Micromonospora*spp.Crude extracts of these strains were found to exhibit potent antibacterial activity against both Gram positive and Gram negative bacteria.

## CONSENT

It is not applicable.

## ETHICAL APPROVAL

It is not applicable.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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