Antimicrobial Potential of a Rare Actinomycete Isolated from Soil: *Crossiella* sp.-EK18

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Authors’ contributions

This work was carried out in collaboration between both authors. Author AAO designed the study. Author OMA the performed experiments, the statistical analysis, wrote the first draft of the manuscript and managed the literature searches. Authors AAO and OMA managed the analyses of the study, read and approved the final study.

Article Information

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ABSTRACT

Aim: The aim of this work was to screen for important broad-spectrum antimicrobial metabolites-producing species of Actinomycetes from the uncultivated soil of Ekiti State, Southwestern, Nigeria.

Methodology: Ten uncultivated or barren soil samples each of 20 g were collected across Ekiti state between April and June 2014 for the isolation of Actinomycetes. The isolate was identified culturally as well as molecularly using 16S rDNA analysis. The influence of production parameters on antimicrobial activity was performed using standard method. The antimicrobial metabolites were produced by submerged fermentation. Partial purification was carried out by column chromatography. Chemical characteristics of the metabolites were determined by Fourier transformed infra-red spectrometer (FTIR) and gas chromatography coupled with mass spectrometer. The antimicrobial activity was carried out by agar well diffusion and macro broth dilution.

Results: Out of ten actinomycetes isolated, Isolate EK18 possessed broad-spectrum antimicrobial activity and it was identified to be a strain of *Crossiella* based on its 16S rDNA gene sequence.

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The best antimicrobial activity against indicator strains was observed at pH 7.0, at 28 degree Celsius after 15 days of incubation, in a medium that contained starch, casein+KNO₃ supplemented with sodium chloride of 0.6% (w/v). Unsaturated aliphatic alkene, carboxylic acids, amides, hydroxyl, and carbonyls were functional groups detected in the synthesized antimicrobial metabolites by the strain while eleven antimicrobial metabolites were identified and characterized. The Minimum Inhibitory Concentration (MIC) against indicator strains was between 3.12 mg/L to 12.5 mg/L while Minimum Bactericidal Concentration (MBC) ranged between 12.5 mg/L and 25.0 mg/L. *Crossiella* sp.-EK18 exhibited broad-spectrum activity against indicator strains.

**Conclusion:** The results indicated that potential broad-spectrum antimicrobial metabolites-producing strains of *Actinomycetes* could be isolated from the soil of southwestern Nigeria. These strains could be used in the production of antimicrobials that may find usefulness in combating emergent and re-emerging pathogens.

**Keywords:** *Actinomycetes*; Isolation and identification; broad spectrum antimicrobial activities; *Crossiella* sp.-EK18; submerged fermentation.

1. **INTRODUCTION**

Actinobacteria are prolific producers of a wide range of secondary metabolites including bioactive peptide [1,2]. It is estimated that these group of bacteria produce more than seven thousand metabolites [3]. Many of these secondary metabolites have found applications in the treatment of human infections. They are antibacterial, antifungal, anti-tumour as well as growth and plant protection agents [4].

Bioactive compounds have a tremendous impact on human health during the second half of the century [5]. 1-phenylbut-3-ene-2-ol, an antimicrobial metabolite produced by a strain of *Nocardia* was reported to be inhibitory against a wide range of pathogenic bacteria and fungi [6], other examples of antimicrobial metabolites such as phenol 2, 4-bis (1, 1-dimethylethyl and dibutyl phthalate had been reported to possess antimicrobial property [7,8]. In addition, aliphatic compounds such as nonadecene and tetradecene have been identified in the solvent extract of different strains of *Streptomyces* [9]. Furthermore, Silber et al., [10] showed that bis (2-ethylhexyl) phthalate possessed inhibitory property against *Micrococcus luteus*,*Vibrio harveyi* and *Pseudoaltermones piscida*.

Hence, the objectives of this study were to screen for antimicrobial-producing actinomycetes from uncultivated tropical rain forest of Ekiti state, southwestern Nigeria, to profile antimicrobial metabolites produced by *Crossiella* sp.-EK18 and to assess its inhibitory activity against some indicator strains.

2. **MATERIALS AND METHODS**

2.1 SOIL Samples Collection

Ten barren or uncultivated soil samples were collected at a depth of 10–15 cm at different sampling coordinates points of 7°36’15”N 5°29’15”E, 7°35’20”N 5°18’10”E, 7°31’20”N 5°13’14”E, 7°40’42”N 4°45’48”E, 7°39’15” 4°47’10”E, 7°46’01”N 5°28’10”E, 7°45’10”N 5°26’15”E, 7°37’20”N 5°30’18”E, 7°36’10”N 5°15’17”E and 7°38’40”N 5°20’05”E of Ekiti State, southwestern, Nigeria. The soil samples were transported in a sterile polythene bag to the Microbial Physiology and Biochemistry Laboratory, Department of Microbiology, University of Ibadan, Ibadan.

2.2 Isolation of Actinomycetes

Isolation of the Actinomycetes was carried out using ten-fold serial dilution following the procedure of Harrigan and McCance [11] and by pour plate technique using glycerol asparagine medium, tyrosine medium and starch casein medium [12,13]. The plates were incubated at 28°C for seven days (Memmert 854, Schnabach, Germany).

2.3 Screening for Antimicrobial Activities of the Actinomycetes

The inhibitory activity of the actinomycete isolates against bacterial test strains was done by cross-streaking method following the procedure of Oskay [14]. The plates were incubated at 37°C (Memmert 854, Schnabach, Germany).
2.3.1 Test strains

Agar well diffusion was done using the following test strains *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9077, *Enterococcus faecalis* ATCC 29212 and *Campylobacter jejuni* ATCC 33291 while *Bacillus cereus* ATCC 10876, *Staphylococcus aureus* ATCC 700699, *Escherichia coli* ATCC 35218, *Salmonella typhimurium* ATCC13311, *Klebsiella pneumoniae* ATCC 8309 and *Pseudomonas aeruginosa* ATCC 9077 were used for the determination of MIC and MBC.

2.4 Morphological Studies of the *Crossiella* sp.-EK18

The morphological characteristics of the selected actinomycete isolate were done following the procedures described in International Streptomyces Programme ISP by Shirling and Gottlieb [13].

2.5 Molecular Identification Using the 16S rRNA Gene Sequence

Chromosomal deoxyribonucleic acid (DNA) was isolated using the versatile quick-prep method for genomic DNA with some modifications [15]. The 16S rRNA analysis of the strain was carried out by the method described by Pospiech and Newmann [15]. The amplification of the 16S rRNA gene was done using universal primers FC27 (5’ to 3’) AGAGTTTGATCCTGGACTT and RC1492 (3’ to 5’) ACGGCTACCTTGTTACGACTT, one forward and one backwards. The phylogenetic tree was constructed using MEGA version 5.0 [16].

2.6 Effect of Culture Conditions on the Antimicrobial Activity of *Crossiella* sp.-EK18

The basal medium used was starch casein broth (g/l) (soluble starch 15.0, potassium phosphate dibasic 2.0, Potassium nitrate 2.0, Sodium chloride 2.0, Casein 0.30, Magnesium sulphate heptahydrate 0.05, Calcium carbonate 0.02, iron II sulphate heptahydrate 0.01 at pH 7.2 [17].

Five hundred millilitre (500 mL) of basal medium was inoculated with 5.0% (v/v) 24 h old culture of *Crossiella* sp.-EK18. This was followed by incubation at 30°C. The inoculated flasks were placed on an Orbital Shaker (Platform Shaker MSZ-100A) at 150 rpm for seven days. The fermented broth was filtered using Whatmann No. 1 and treated with 50% (w/v) ammonium sulphate, so as to remove interfering metabolites from the antimicrobial extract. The mixture was centrifuged at 5000 rpm for 20 min (Wincom 80-2). Furthermore, equal volume (1:1) of the supernatant and ethyl acetate (Loba Chemie PVT LTD, India) were vigorously shaken together for 30 min in separating funnel to effect the extraction of the antimicrobial metabolites by the solvent. The separating funnel with the mixture was further allowed to stand for another 30 min. The supernatant was collected and concentrated at 60°C using Rotary Evaporator (Rotary Evaporator, RE-52A) [7]. The crude antimicrobial extract was used for agar well diffusion assay. However, the best supporting cultural condition of a previously determined parameter was kept constant in subsequent parameter under study.

2.6.1 Effect of pH

The medium of fermentation was adjusted to different pH values of 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0.

2.6.2 Effect sodium chloride concentrations (w/v)

Different concentrations of sodium chloride was added to the starch casein broth and inoculated with a 24-h growth inoculum of *Crossiella* sp.-EK18. Similar production medium without sodium chloride served as control.

2.6.3 Effect of temperature

The influence of temperature on the antimicrobial compounds synthesize and activity was carried out by incubating the production medium at 4°C, 28°C, 35°C, 45°C, 55°C and 60°C.

2.6.4 Effect of carbon, nitrogen sources and the control

The effects of different carbon (glucose, galactose, maltose, sucrose, glycerol, starch and control) and nitrogen sources (ammonium sulphate, peptone, yeast extract, malt extract, casein, potassium nitrate, casein+potassium nitrate and control) were carried out on the antimicrobial activity of *Crossiella* sp.-EK18. However, fermentation set up without any carbon source and nitrogen source were respectively used as the control.
2.6.5 Effect of incubation period

The influence of different incubation period in days (3, 5, 7, 10 and 15) on the antimicrobial activity of Crossiella sp.-EK18 was studied.

2.7 Agar Well Diffusion

Agar well diffusion technique was used to assess the effect of culture conditions on the inhibitory activity of the Crossiella sp.-EK18 against Bacillus subtilis ATCC 6633, Enterococcus faecalis ATCC 29212, Campylobacter jejuni ATCC 33291 and Pseudomonas aeruginosa ATCC 9077 [17]. A well of 6.0 mm was made at the center of petri dishes containing Mueller Hinton medium, which had been earlier swabbed with a 24 h culture of the indicator strain but had their turbidity adjusted to 0.5 McFarland standards (1.5x10^8 CFU/mL). Each well was filled with crude extract (50.0 μL) and the plates were incubated at 37°C (Memmert 854, Schnabach, Germany). The zones of inhibition were measured in triplicate using ruler. The 6.0 mm of the well was subtracted from the readings.

2.8 Partial Purification and Characterization of the Antimicrobial Compounds Produced Crossiella sp.-EK18

Autoclaved ten litres starch casein broth was inoculated with 5.0 % (v/v) 24 h seed inoculum of Crossiella sp.-EK18. The fermented broth was filtered using Whatman No1 filter paper so as to remove the mycelial debris. The filtrate was treated with 50 % (w/v) ammonium sulphate. The treated filtrate was centrifuged at 5000 rpm for 20 min. The supernatant was mixed with equal volume (1:1) of ethyl acetate (Loba Chemicals, India) and it was shaken vigorously in a separating funnel for 30 min for the extraction of the antimicrobial metabolites. The separating funnel was allowed to stand for another 30 min. Thereafter, the supernatant containing antimicrobial metabolites was pooled together and concentrated at 60°C using a rotary evaporator (Rotary Evaporator, RE-52A) [7]. The crude antimicrobial concentrate was then transferred to a water bath at 50°C to dry off the remaining solvent.

Furthermore, one gram of the crude antimicrobial concentrate was mixed with 3.0 mL ethyl acetate. The dissolved crude antimicrobial extract was loaded onto a silica gel (100-200 mesh) column chromatography, column dimension 2.0 cm inner diameter x 25 cm length. The column was eluted with a mixture of n-hexane and ethyl acetate (1:4 v/v). Thirty fractions, 3.0 mL each was collected and they were screened for antimicrobial activity and fractions that exhibited antimicrobial activity were pooled together and concentrated at 60°C using rotary evaporator (Rotary Evaporator, RE-52A). The partially purified antimicrobial extract was used for chemical characterization minimum inhibitory concentration (MIC) assay against indicator strains.

2.9 Chemical Characterization of the Antimicrobial Compounds Produced by Crossiella sp.-EK18

The chemical characterization of the purified antimicrobial metabolite synthesized by Crossiella sp.-EK18 was carried using Fourier transformed infrared (FTIR) spectrophotometer. The antimicrobial extract was mixed with potassium bromide (KBr). The spectra of the purified antimicrobial compounds were recorded on Shimadzu AUX220 spectrophotometer that was in the range of 4000 cm⁻¹ to 400 cm⁻¹ [18]. The chemical structures, formulae and the names of the compounds were determined using gas chromatography-mass spectrometer (GC-MS) Shimadzu QP 2010 [19].

2.9.1 Determination of MIC and MBC of the partially purified antimicrobial compounds

The MICs of the partially purified antimicrobial extract was done using macro broth dilution method as described by Andrews [20]. The tubes without turbidity after MIC was recorded were streaked on nutrient agar. The plates were observed for growth after 24 h and the plates without growth inoculated with the lowest concentrations of the antimicrobial extract were recorded as the minimum bactericidal concentrations (MBCs).

2.9.2 Statistical analysis

Data were collected and recorded in triplicates and subjected to Duncan Multiple Range Test for Mean±S.D.

3. RESULTS

3.1 Isolation and Identification of the Actinomycetes

Seventy actinomycetes were isolated from ten soil samples collected purposively across Ekiti
Effect of pH on production and activity by All the carbon sources gave good antimicrobial activity. However, isolate EK18 had broad-spectrum antimicrobial activity.

Table 1 showed that isolate EK18 grew abundantly on all the four media used, the aerial colour was white while the reverse side colour was brown. It produced no diffusible pigment and it was Gram-positive. The 16S rRNA nucleotide sequence of the isolate showed that it has 98% similarity to Crossiella equi. The 16S rRNA gene sequence has been deposited at NCBI GenBank and assigned accession number KU934250.

3.2 Effect of pH on Antimicrobial Activity

The effect of pH presented in Table 2 showed that pH 7 was the best for antimicrobial synthesis and activity by Crossiella sp.-EK18 with a zone of inhibition that was between 30.3 mm and 35.7 mm. Low antimicrobial activities (16.3 mm and 18.3 mm) were recorded at extreme pH 4 and 9 respectively.

3.3 Effect of Sodium Chloride on Antimicrobial Activity

The influence of sodium chloride concentration as shown in Table 3 revealed that a concentration of 0.6% gave the highest antimicrobial activity against indicator strains, with Enterococcus faecalis ATCC 29212 most sensitive with a zone of inhibition of 28.7 mm and Pseudomonas aeruginosa ATCC 9077 least sensitive with a zone of inhibition of 20.0 mm.

3.4 Effect of Temperature on Antimicrobial Activity

The effect of temperature on the antimicrobial activity of Crossiella sp.-EK18 indicated that a temperature between 28-30°C was most suitable for the antimicrobial production and activity of Crossiella sp.-EK18 against indicator strains (Table 4). Bacillus subtilis ATCC 6633 was most inhibited with a zone of 33.3 mm. Low inhibition was observed at extremes temperatures of 4°C and 60°C.

3.5 Effect of Carbon Source on Antimicrobial Activity

All the carbon sources gave good antimicrobial production and activity by Crossiella sp.-EK18 against test strains except glucose and sucrose (Plates 1a, 1b). Interestingly, medium without any carbon source also supported the production of antimicrobial compounds and activity with zones of inhibition that was between 27.7 mm and 33.0 mm (Table 5).

3.6 Effect of Nitrogen Source on Antimicrobial Activity

The importance of nitrogen source on the synthesis and antimicrobial activity of Crossiella sp.-EK18 as presented in Table 6 showed that the combination of potassium nitrate and casein gave the best antimicrobial activity. The antimicrobial activity against indicator strains measured in the zone of inhibition with casein+KNO₃ ranged between 29.3 mm and 38.3 mm. The activity was very poor in the absence of nitrogen source.

3.7 Effect of Incubation Period on Antimicrobial Activity

The observed incubation period showed that Crossiella sp.-EK18 had the highest antimicrobial activity against test strains by the fifteen days of incubation (Table 7). The zone of inhibition ranged between 29.3 mm and 35.3 mm by the fifteenth day of fermentation.

3.8 Chemical Characterization of the Partially Purified Antimicrobial Compounds

The IR spectral analysis for the antimicrobial compounds produced by Crossiella sp.-EK18 had peaked at wave numbers 447.50 cm⁻¹, 611.45 cm⁻¹, 705.97 cm⁻¹, 742.62 cm⁻¹ showed the presence of aromatic rings in the compounds. Also, aliphatic compounds at wave numbers 1039.67 cm⁻¹, 1074.39 cm⁻¹ and 1122.61 cm⁻¹, unsaturated alkyl group wave number 1600.97 cm⁻¹, ketone and aldehyde functional groups at wave number 1728.28 cm⁻¹ and amide at wave numbers 3493.20 cm⁻¹ (Fig. 2).

The antimicrobial metabolites profile of Crossiella sp.-EK18 according to the GC-MS spectral analysis showed that seven different compounds were identified in the partially purified solvent antimicrobial extract (Table 8). These antimicrobial compounds include the aliphatic alkenes such as Penta-1-decene, Hepta-1-decene and Nona-1-decene (Table 9). Other are...
### Table 1. Culture characteristics of *Crossiella* sp.-EK18

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth</th>
<th>Aerial colour</th>
<th>Reverse side colour</th>
<th>Diffusible pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ISP2</em></td>
<td>Abundant</td>
<td>White</td>
<td>Golden brown</td>
<td>Nil</td>
</tr>
<tr>
<td>ISP4</td>
<td>Abundant</td>
<td>White</td>
<td>Yellowish brown</td>
<td>Nil</td>
</tr>
<tr>
<td>ISP5</td>
<td>Abundant</td>
<td>White</td>
<td>Light grey</td>
<td>Nil</td>
</tr>
<tr>
<td>SCNA</td>
<td>Abundant</td>
<td>White</td>
<td>Golden yellow</td>
<td>Nil</td>
</tr>
</tbody>
</table>

*ISP2: Yeast extract malt extract agar  
ISP4: Inorganic starch salt agar  
ISP5: Glycerol asparagine agar  
SCNA: Starch casein Nitrate Agar  
Nil: No pigment production

### Table 2. Effect of pH on the antimicrobial activity of *Crossiella* sp.EK18 against test strains

<table>
<thead>
<tr>
<th>Test strains</th>
<th>pH / Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> ATCC 29212</td>
<td><em>20.3±0.5&quot;</em></td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> ATCC 33291</td>
<td>18.3±0.2&quot;</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 6633</td>
<td>16.3±0.4&quot;</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 9077</td>
<td>18.3±0.2&quot;</td>
</tr>
</tbody>
</table>

*Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan’s Multiple Range Test*

### Table 3. Effect of sodium chloride concentration on the antimicrobial activity of *Crossiella* sp.EK18 against test strains

<table>
<thead>
<tr>
<th>Test strains</th>
<th>Sodium chloride concentration (% w/v) / Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> ATCC 29212</td>
<td><em>14.0±0.5&quot;</em></td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> ATCC 33291</td>
<td>5.3±0.4&quot;</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 6633</td>
<td>6.7±0.2&quot;</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 9077</td>
<td>0.0±0.0&quot;</td>
</tr>
</tbody>
</table>

*Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan’s Multiple Range Test*
Table 4. Effect of temperature on the antimicrobial activity by Crossiella sp.-EK18 against test strains

<table>
<thead>
<tr>
<th>Test strains</th>
<th>Temperature (°C) / Zone of inhibition (mm)</th>
<th>4</th>
<th>28</th>
<th>35</th>
<th>45</th>
<th>55</th>
<th>60</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC 29212</td>
<td></td>
<td>*16.7±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.7±0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.7±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.7±0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.3±0.6&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>10.3±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Campylobacter jejuni ATCC 33291</td>
<td></td>
<td>14.7±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.7±0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.3±0.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.7±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.7±0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.0±0.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bacillus subtilis ATCC 6633</td>
<td></td>
<td>8.7±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.7±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.7±0.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.3±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.7±0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>10.3±0.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 9077</td>
<td></td>
<td>14.3±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.7±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.7±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.3±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.3±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.7±0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan’s Multiple Range Test

Table 5. Effect of carbon source on the antimicrobial activity of Crossiella sp.-EK18 against test strains

<table>
<thead>
<tr>
<th>Test strains</th>
<th>Control</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Maltose</th>
<th>Sucrose</th>
<th>Glycerol</th>
<th>Starch</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC 29212</td>
<td>*33.0±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.7±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.7±0.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.7±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.7±0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34.3±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>35.3±0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Campylobacter jejuni ATCC 33291</td>
<td>27.7±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.3±0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.7±0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.7±0.9&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.3±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.3±0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.7±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bacillus subtilis ATCC 6633</td>
<td>30.7±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.7±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.3±0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25.7±0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.7±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.7±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.3±0.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 9077</td>
<td>30.6±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.7±0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30.7±0.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>26.7±0.6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22.7±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.3±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.0±0.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan’s Multiple Range Test

Table 6. Effect of nitrogen source on the antimicrobial activity of Crossiella sp.EK18 against test strains

<table>
<thead>
<tr>
<th>Test strains</th>
<th>Control</th>
<th>KNO&lt;sub&gt;3&lt;/sub&gt;</th>
<th>NH&lt;sub&gt;4&lt;/sub&gt;NO&lt;sub&gt;3&lt;/sub&gt;</th>
<th>Yeast extract</th>
<th>Malt extract</th>
<th>Peptone</th>
<th>Casein</th>
<th>Casein+KNO&lt;sub&gt;3&lt;/sub&gt;</th>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis ATCC 29212</td>
<td>*32.3±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.7±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.3±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.7±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.7±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.7±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.3±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Campylobacter jejuni ATCC 33291</td>
<td>18.7±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.7±0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.7±0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.3±0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.7±0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.0±0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.3±0.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.3±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bacillus subtilis ATCC 6633</td>
<td>16.0±0.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.7±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.0±0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16.3±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>26.7±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.0±0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.7±0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.7±0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC 9077</td>
<td>18.3±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>21.7±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.3±0.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18.3±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.0±0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.3±0.7&lt;sup&gt;d&lt;/sup&gt;</td>
<td>22.7±0.5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>34.3±0.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan’s Multiple Range Test
Table 7. Effect of incubation period on the antimicrobial activity of *Crossiella* sp.EK18 against test strains

<table>
<thead>
<tr>
<th>Test strains</th>
<th>Incubation period / Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day3</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> ATCC 29212</td>
<td><em>23.3±0.5</em>a*</td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em> ATCC 33291</td>
<td>27.7±0.7<em>a</em></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> ATCC 6633</td>
<td>25.7±0.4<em>b</em></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 9077</td>
<td>24.7±0.5<em>b</em></td>
</tr>
</tbody>
</table>

*Values are means of triplicate determinations ± Standard Error. Means with different letters within each column differ significantly (p≤0.05) using Duncan’s Multiple Range Test.*

Table 8. MIC and MBC of partially purified antimicrobial compounds produced by *Crossiella* sp.-EK18 and Gentamicin (control)

<table>
<thead>
<tr>
<th>Indicator strain</th>
<th>MIC (mg/L)</th>
<th>MBC (mg/L)</th>
<th>Gentamicin (Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em> ATCC 10876</td>
<td>3.12</td>
<td>12.5</td>
<td>0.78</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> ATCC 700699</td>
<td>3.12</td>
<td>12.5</td>
<td>0.78</td>
</tr>
<tr>
<td><em>Escherichia coli</em> ATCC 35218</td>
<td>6.25</td>
<td>12.5</td>
<td>3.12</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> ATCC 8309</td>
<td>6.25</td>
<td>25.0</td>
<td>0.78</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> ATCC 9077</td>
<td>12.5</td>
<td>25.0</td>
<td>0.78</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> ATCC 13311</td>
<td>12.5</td>
<td>25.0</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table 9. GC-MS analysis of antimicrobial metabolites profile produced by *Crossiella* sp.-EK18

<table>
<thead>
<tr>
<th>Peak</th>
<th>R. Time</th>
<th>Area</th>
<th>Area %</th>
<th>Height %</th>
<th>Molecular weight</th>
<th>Chemical formula</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.242</td>
<td>906847</td>
<td>0.90</td>
<td>1.07</td>
<td>210</td>
<td>C15H30</td>
<td>1-pentadecene</td>
</tr>
<tr>
<td>2</td>
<td>10.044</td>
<td>1193054</td>
<td>1.18</td>
<td>1.41</td>
<td>206</td>
<td>C14H22O</td>
<td>Phenol, 2, 4-bis (1, 1- dimethylethyl</td>
</tr>
<tr>
<td>3</td>
<td>10.626</td>
<td>1366980</td>
<td>1.35</td>
<td>1.56</td>
<td>238</td>
<td>C17H34</td>
<td>1-Heptadecene</td>
</tr>
<tr>
<td>4</td>
<td>12.798</td>
<td>1413010</td>
<td>1.40</td>
<td>1.58</td>
<td>266</td>
<td>C19H38</td>
<td>1-Nonadecene</td>
</tr>
<tr>
<td>5</td>
<td>15.831</td>
<td>5066312</td>
<td>5.00</td>
<td>4.20</td>
<td>278</td>
<td>C16H22O4</td>
<td>1, 2 benzenedicarboxylic acid, bis (2 methylpropyl ester</td>
</tr>
<tr>
<td>6</td>
<td>16.356</td>
<td>1215645</td>
<td>12.01</td>
<td>11.66</td>
<td>278</td>
<td>C16H22O4</td>
<td>Dibutyl phthalate</td>
</tr>
<tr>
<td>7</td>
<td>16.514</td>
<td>3608182</td>
<td>3.56</td>
<td>3.30</td>
<td>292</td>
<td>C17h24O4</td>
<td>Phthalic acid, isobutyl 2-pentyl ester</td>
</tr>
<tr>
<td>8</td>
<td>16.834</td>
<td>1048514</td>
<td>10.36</td>
<td>10.42</td>
<td>278</td>
<td>C16H22O4</td>
<td>Dibutyl phthalate</td>
</tr>
<tr>
<td>9</td>
<td>16.990</td>
<td>6460352</td>
<td>6.38</td>
<td>6.06</td>
<td>334</td>
<td>C20H30O4</td>
<td>Benzene dicarboxylic acid, butyl octyl ester</td>
</tr>
<tr>
<td>10</td>
<td>17.264</td>
<td>2986890</td>
<td>2.93</td>
<td>2.46</td>
<td>296</td>
<td>C18H30O2</td>
<td>9-Octadecenoic acid, methyl ester</td>
</tr>
<tr>
<td>11</td>
<td>20.056</td>
<td>3918931</td>
<td>3.87</td>
<td>3.25</td>
<td>281</td>
<td>C18H35NO</td>
<td>9-Octadecenamide (Z)-</td>
</tr>
<tr>
<td>12</td>
<td>20.095</td>
<td>51700801</td>
<td>51.07</td>
<td>53.06</td>
<td>390</td>
<td>C24H38O4</td>
<td>Bis (2-ethylhexyl) phthalate</td>
</tr>
</tbody>
</table>

*R. Time: Retention time*
Plate 1 (a-c) Inhibition of A1, A2 and A3 by crude antimicrobial compounds produced by Crossiella sp.-EK18


B: Antimicrobial extract, C: Zone of inhibition
Fig. 1. The phylogenetic tree of Crossiella sp.-EK18
Fig. 2. FTIR chromatogram of partially purified antimicrobial compounds produced by *Crossiella* sp.-EK18

Fig. 3. GC-MS spectrum of antimicrobial compounds produced by *Crossiella* sp.-EK18
3.9 MIC and MBC

The MIC and MBC of the partially purified antimicrobial extract against *Bacillus cereus* ATCC 10876 and *Staphylococcus aureus* ATCC 700699 indicator strains was 3.12 mg/L and 12.5 mg/L respectively. Additionally, *E. coli* ATCC 35218, *Salmonella typhimurium* ATCC 13311 had a MIC of 6.25 mg/L and 12.5 mg/L respectively while *Klebsiella pneumoniae* ATCC 8309 and *Pseudomonas aeruginosa* ATCC 9077 had MIC 12.5 mg/L and MBC 25.0 mg/L respectively (Table 8).

4. DISCUSSION

The morphological and cultural characteristics of *Crossiella* sp.-EK18 are similar to the report of Oskay [13] (Table 1). The partial sequence of the 16S rRNA gene of the isolate OY62 showed that the isolate had 98% similar nucleotide sequence.
to other strain of *Crossiella* sp.-EK18 (Fig. 1). The genetic and physiological factors of the strains could be responsible for the similarity observed.

The pH 7 agrees with the previous reports of Bundale et al. [21], Sarad et al. [22] who had earlier reported pH 7 as the best supporting pH for antimicrobial production and activities for the *Streptomyces* strains used for their study. The similarity could be attributed to the structural stability of the enzymes and antimicrobial metabolites at pH 7. Hydrogen ion concentration has been reported to play a key role in the activity of enzymes and secondary metabolites production [23]. Sodium chloride has been reported to exert osmotic pressure on the production strain that result to increase in the amount of metabolites excreted into the production medium. The observation is in agreement with the report of Reddy et al. [24].

The temperature of 28°C for optimum antimicrobial activities by *Crossiella* sp.-EK18 observed in this study (Table 4) is similar to the previous report of Khattab et al. [25]. The similarity could be due to the stability of the enzymes which enhance microbial metabolism. Furthermore, starch, glycerol and absence of carbon source supported good antimicrobial activity against indicator strains (Table 5). Starch and glycerol have been previously reported to support good antimicrobial production and activity [22,26]. There is possibility that the *Crossiella* sp.-EK18 might have utilized other organic nutrients in the medium as carbon and energy sources. The use of amino acids such as alanine and valine for instance, has been reported to supply carbon skeleton needed in antimicrobial biosynthesis by actinomycetes [27]. The combined casein and potassium nitrate was observed to be the best nitrogen source for antimicrobial production and activity. This differs from previous reports of Bundale et al. [21] and Sarad et al. [22]. Both authors had previously mentioned peptone, casein and combined peptone and yeast extract respectively as nitrogen sources. The differences observed could be attributed to preference for a particular nitrogen source that optimally supports antimicrobial metabolites biosynthesis. An incubation period of fifteen days shown in Table 7 was recorded as the best for antimicrobial production and activity for the strain *Crossiella* sp.-EK18. However, this is different from previous reports of Bundale et al. [21] and Khattab et al. [25] who had earlier mentioned five and ten days respectively. The difference obtained in this study could be as a result of differences in the strains used and the nature of the nitrogen and carbon sources used for fermentation.

The IR spectrum analysis of the partially purified antimicrobial compound synthesized by *Crossiella* sp.-EK18 had peaks at various wave numbers indicating the presence of functional groups such as unsaturated aliphatic alkenes, aromatic rings, amide, hydroxyl as well as carboxyl (Fig. 2). These are in agreement with earlier reports of Ayari et al. [28]. However, the differences observed in wave numbers even, though indicating the same functional groups could be attributed to the spatial arrangement of the molecules and their stretch during vibration [29]. Antimicrobial metabolites such as Bis (2-ethylhexyl) phthalate, dibutyl phthalate, 1, 2-benzene dicarboxylic acid, bis (2-ethylhexyl) ester, phenol, 2, 4-bis (1, 1-dimethylethyl, 9-Octadeceneamide, (Z), 9-Octadecenoic acid methyl ester (E)-, 1-pentadecene, 1-heptadecene and 1-nonadecene were observed in the partially purified solvent extract (Table 3). Most of these compounds have been reported previously to possess inhibitory properties [3,6].

The results of the MIC and MBC of partially purified antimicrobial compounds produced by *Crossiella* sp.-EK18 against the test strains ranged between 3.12 mg/L and 12.5 mg/L and 12.5 to 25.0 mg/L respectively (Table 4). These results are higher than the previous report of Al-Bari et al. [30] and Arasu et al. [31]. The observed values of MIC and MBC of the partially purified antimicrobial extract are higher than the control (Gentamicin). The MIC and MBC of the control (Gentamicin) ranged between 0.78 mg/L to 3.12 mg/L and 1.56 mg/L to 6.25 mg/L respectively. The differences observed could be due to different strains of indicator organisms and level of purity of the antimicrobial compounds.

5. CONCLUSION

The research work established that *Crossiella* sp.-EK18 a rare actinomycete strain was isolated from soil of tropical rainforest of Ekiti state, southwestern Nigeria. *Crossiella* sp.-EK18 possessed broad-spectrum inhibition against some selected test strains. Therefore, the isolate may find usefulness in the production of antimicrobial agents. These agents could be useful in the tackling the emerging pathogens.
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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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