An Investigation into the Antibacterial Activity of Pigmented Secondary Metabolites Isolated from *Streptomyces* from Windhoek Soil

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Abstract

There is an increase in mortality from infectious diseases due to an increase in antibiotic resistance worldwide. Hence, this study aimed at investigating the antibacterial activity of *Streptomyces* that produces pigmented secondary metabolites. The soil samples were collected from 3 different sites in Windhoek. *Streptomyces* were isolated on starch casein agar. Biochemical and physiological tests were used to identify *Streptomyces* species. Casein slant agar method was used for preliminary antibacterial test. Chloroform and n-Hexane were used to extract secondary metabolites by solvent-liquid extraction method. Thin layer chromatography plates were used for antibacterial activity of the isolated secondary metabolites. Minimum inhibitory concentration of isolated secondary metabolites was determined by the broth dilution method at concentrations ranging from 50 to 1.5 μg/ml. Six out of nine *Streptomyces* isolates showed antibacterial activity against at least one of three test bacteria. *Streptomyces* isolate 1 exhibited potent activity of 19.5 mm $\pm$ 0.056 against *M. avium*. Different pigmented secondary metabolites were produced and showed antibacterial activity against at least against one of three test organism. *Streptomyces* isolate 4 and 6 showed the lowest minimum inhibitory concentration of 6.25 μg/ml against *M. avium* and *E. faecialis*. The results showed that Windhoek soil contain antibiotic producing *Streptomyces*.

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1 Introduction

Streptomyces are filamentous gram positive aerobic bacteria with a high guanine-plus-cytosine content (GC) in their genome (Ceylan, Gulten & Ugur, 2008). They belong to the phylum of actinobacteria that predominantly inhabit the soil. Members of the genus have saprophytic, free-living lifestyles, and contend for resources with several other organisms in oligotrophic environments (Cruz-Morales et al., 2013). *Streptomyces* grow as hyphae, which frequently branch to form an intricate vegetative mycelium (Cruz-Morales et al., 2013). *Streptomyces* are important decomposers and they are well known for producing pigments that have antimicrobial activities (Moustafa, 2007; Mathura, Paliwala, Sharmaa, Kumara, Bhatnagar, 2012). *Streptomyces* have a complex multi-cellular development, in which their germinating spores form hyphae, with multinuclear aerial mycelium, which forms septa at regular intervals, creating a chain of uninuclearated spores (Procópioa, Silvaa, Martinsa, Azevedoa & Araujo, 2012; James et al., 2010). The genus is made up of more than 500 species. Their life cycle goes from germination, vegetative growth to aerial growth and then sporulation (Procópioa et al., 2012).

Streptomyces are well-documented for their ability to produce bioactive secondary metabolites with antifungals, antivirals, antitumor, anti-hypertensives, antibacterial and immunesuppressive properties (Jameset al., 2010). Actinomycetes produce approximately 45% of the 23 000 bioactive secondary metabolites produced by microorganisms (Valli et al., 2012). Today, 75% of the clinically useful antibiotics are obtained from the genus *Streptomyces* (Kieser et al., 2000; Valliet al., 2012). *Streptomyces* are known to produce pigments such as the blue actinorhodin and red undecylprodigiosin produced by *S. coelicolor* and *S. lividan* respectively. The first macrolide antibiotic, erythromycin, was isolated in 1952 from *Streptomyces erythreus*. Antibiotics such as streptothricin, actinomycin, streptomycin and tetracycline as well as other medically-relevant compounds such as anti-fungal agents (e.g. nystatin), anti-cancer agents (e.g. doxorubicin) and immunomodulatory agents (e.g. rapamycin) are produced by Streptomyces species (Isenberg, 1992; Baltz & Faber, 2006; Keiser et al., 2000).

Despite the success of the discovery of antibiotics, and advances in the process of their production; infectious diseases still remain the second leading cause of death worldwide (Procópio et al., 2012). Bacterial infections cause approximately 17 million deaths annually, affecting mainly children and the elderly (Procópio et al., 2012). This is partly due to an

increase in microbial resistance and emergence of new diseases (World Health Organization, 2016). Bacteria such as multidrug resistant *Enterococcus faecialis, Mycobacterium avium* and *Escherichia coli* highly contribute to infectious deaths globally (Isenberg, 1992; Baltz & Faber, 2006). *E. faecialis* and *M. avium* are common nosocomial bacteria that have developed resistance inpatients with Acquired Immune Deficiency Syndrome (AIDS). Many strains of *E. coli* and *E. faecialis* are unresponsive to fluoroquinolones and cephalosporins and vancomycin (Isenberg, 1992).

Namibian soil is colonized by unique microorganism including *Streptomyces*, which can be used as a source of new antibiotics. Windhoek which is in central Namibia, is a rocky, mountainous and dry area. Hence, bacteria need specific mechanisms to enable them survive this harsh conditions. In extreme environments; *Streptomyces* compete with other microorganisms by producing antibiotics as their defense mechanism. This study investigated whether *Streptomyces* from soils in Windhoek produce pigmented secondary metabolites with antibacterial activity against *M. avium, E. coli* and *E. faecialis*.

2 Materials and Methods

2.1 Isolation and characterization of Streptomyces isolates

The soil samples were collected using purposive sampling from 3 different habitats (Sample 1 was collected from a high vegetative area, sample 2 was collected from a mountainous area and sample 3 was collected from an unadorned area) in Windhoek. The samples used were taken at a 4 cm depth and collected in sterile bags. The soil samples were sieved to remove small pieces of stones and organic matters. One gram of the soil sample was mixed with 9 ml of distilled water to forma stock solution. Serial dilution method was used to prepare dilutions of 10-2, 10-4 and 10-6 from the stock solution as described by Collingnon (2008). Two μl of the suspension of the 10-6dilution was inoculated on prepared starch casein agar plates that were supplemented with nalidixic acid and cyclohexamide. Inoculated plates were incubated at 37°C for 14 days. Dry colonies of *Streptomyces* were selected and subculture on new starch casein agar plates at 37°C for 14 days (Collingnon, 2008).

Culture characterization for each isolate was determined as described by Shirling & Gottlieb (1966). Physiological criteria and biochemical tests for *Streptomyces* identifications such as: morphology, aerial mycelium, substrate mycelium, spore morphology, production of diffusible pigments, case in degradation, utilization of gelatin and xylan as growth substrates and tolerance to 7% sodium Chloride (NaCl) were used in identifying the isolates. Moreover the production of melanine pigments was studied on starch casein agar and in starch casein broth in order to reach a possible classification to species as described by Nanjwade, Chan-

drashekhara, Shamarez, Goudanavar & Manvi(2010); Buchanan & Gibbons(1974); Taddie, Rodriguez & Castelli (2006). Gram staining was performed and the prepared slides were viewed under light microscope at 100 × magnification to confirm the spore-chain morphology and to observe the formation of filamentous structures of the isolates as described by Korn-Wendisch & Kutzner (1992).

2.2 Preliminary antimicrobial screening

All the test bacteria used in this study were purchased from Biodynamic in Windhoek. Antibacterial activity of the isolated strains was evaluated against *M. avium, E. coli* and *E. faecialis* by double layer agar method on starch casein agar medium.Pure *Streptomyces* strains were spot inoculated on starch casein agar media for six days at 28°C. After the colonies had grown to a 6 mm diameter, they were overlaid with 7 ml of sloppy-nutrient (0.6%) containing 100 μl (1.5 × 105 CFU/μl) of seeded microbes. The inoculated plates were incubated for 48 hours at 37°C. Zones of inhibition around the colonies were measured and recorded after 48 hours at 37°C. The assay was done in triplicate (Das, Bhattacharya, Mohammed & Rajan, 2014). Amoxicillin, azithromycin and ampicillin (30 mg each) were used as positive controls and distilled water was used as a negative control. Zones of inhibition were interpreted as follow: $\geq$11 strong, $\geq$ 5 moderate and $<$ 5 inactive.

2.3 Fermentation and production of secondary metabolites

Streptomyces isolates that showed antibacterial activity during the preliminary antibacterial activity test were cultured in starch casein broth. Fermentation was carried out in 1000 ml Erlenmeyer flasks containing 200 ml casein broth inoculated with 100 μl of *Streptomyces* isolates' spore suspensions (1.5 × 105CFU/μl). The flasks were incubated on a shaker at 37^{c}ircC for 14 days (Das et al., 2014; Dezfully & Ramanayaka, 2015).

2.4 Extraction of pigmented secondary metabolites and antibacterial screening

Antibacterial secondary metabolites were extracted from *Streptomyces* isolates that showed antibacterial activity during the preliminary antibacterial activity test. Solvent-liquid extraction method for extraction of secondary metabolites. Chloroform and n-hexanewere added to the mycelium - free supernatant in a 1:1 ratio (v/v)and incubated on a shaker at 37°C for 2 days. Chloroform and n-hexane containing antibacterial compounds were sepa-

rated from the broth culture by using Whatman No. 1 filter papers and centrifuged at 5000 rpm as described by Das et al., (2014); Dezfully & Ramanayaka (2015). Chloroform and n-hexane were used to extract pigmented secondary metabolites from the broth.

Thin layer chromatography (TLC) plates were used to detect the antimicrobial activity of the isolated pigmented secondary metabolites against *M. avium, E. coli* and *E. faecialis*. TLC plates were cut into 4 cm strips and 1 μl of the extracted secondary metabolites were placed on the center of each TLC plate. The plates were left to dry for 15 minutes. The TLC plates were inoculated with 1.5 $\times$ 105 CFU/μl of *M. avium, E. coli* and *E. faecialis* and incubated in sealed containers at 37°C for 24 and 48 hours. The TLC plates were flooded with 3-(4,5-Dimethylthiazol-2-yl)-2) and incubated for 5 minutes. The TLC plates were observed for the inhibition zones in the center where the secondary metabolites were spotted. Chloroform and n- hexane were also assayed to confirm that they did not have any antimicrobial activity.

2.5 Determination of dry weight of Streptomyces isolates' mycelia

The mycelia was collected on Whatman No. 1 filter as residue after 14 days of incubation in casein broth. The mycelia was dried in the oven at 60°C to determine the growth mass(dry weight of mycelia) of the isolates after 14 days of incubation. The biomass was recoded in milligrams (mg) and compared to the optical density (OD) of the isolated pigmented secondary metabolite at 480 (Dezfully & Ramanayaka, 2015).

2.6 Minimum inhibitory concentration (MIC) on isolated secondary metabolites

Minimum inhibitory concentration (MIC) of isolated secondary metabolites was determined against *M. avium, E. coli* and *E. faecialis*. Broth two-fold dilution method was used at concentrations ranging from 50 to 1.5 μg/ml at 37°C; for 24 and 48 hours as described by Al-Hulu, Charrakh & Jarallah (2012). The assay was done in triplicate.

3 Results and Discussion

3.1 Isolation and characterization of Streptomyces isolates

Based on physiological and morphological characterization as well as on biochemical tests, fifteen isolates of actinomycetes were recovered and 60% were identified as Streptomyces species. The results on physiological characterization and biochemical tests of the 60% *Streptomyces* isolates are depicted in table 1. The isolates were slow growing, aerobic, chalky, heaped, folded and with aerial and substrate mycelia of different colours. *Streptomyces* isolate 3, 6 and 9 colonies appeared chalky while isolates 1, 2, 4, 5, 7 and 8 appeared white on the surface of the starch casein agar. In addition, all colonies possessed an earthy odour. These characteristics were consistent with the physiological and biochemical characteristics used in identifying *Streptomyces* (Shirling & Gottlieb, 1966; Nanjwade et al., 2010; Buchanan & Gibbons, 1974; Taddie, Rodriguez & Castelli, 2006). A microscopic view of the isolates under light microscope and macroscopic view on starch casein agar plates are depicted in figure 1 and 2.

Most of isolates tend to grow in alkaline soil which is an important characteristic feature of *Streptomyces* species due to their ability to tolerate salt as indicated in table 1.

Table 1: Summary of biochemical tests and physiological characterization of different *Streptomyces* isolates

Characteristics	Isolate 1	Isolate 2	Isolate 3	Isolate 4	Isolate 5	Isolate 6	Isolate 7	Isolate 8	Isolate 9
Mycelia color	White	White	Chalky	White	White	Chalky	White	White	Chalky
Starch degradation	+	+	+	+	+	+	+	+	+
Gelatin degradation	+	+	+	+	+	+	+	-	+
Casein hydrolysis	+	-	+	-	+	+	+	+	+
7% NaCl tolerance	+	+	+	+	+	+	+	+	+
Xylan degradation	-	+	-	-	+	+	+	-	+

Key: +: Positive results - : Negative result

3.2 Preliminary antimicrobial screening

Only six of the nine *Streptomyces* isolates showed antibacterial activity against *E. coli*, *M. avium* and *E. faecialis* during preliminary antimicrobial activity screening. Antimicrobial activities of nine *Streptomyces* isolates are recorded in Table 2. *Streptomyces* isolates one and four showed strong antimicrobial activity against *E. coli* (18.5 mm ± 0.058) and M. avium (19.5 mm ± 0.056) respectively. These results on antibacterial activities of *Streptomyces*

species against *E. coliwere* consistent with the findings of Al-Hulu et al.,(2012) in which growth of *E. coli* was inhibited by *Streptomyces gelaticus*. Several *Streptomyces* isolates are reported to produce chemical substances such as tetracycline, macrolides, quinocycline and meroparamycin that are used today as pharmaceuticals agrochemical products (Al-Hulu et al., 2012).

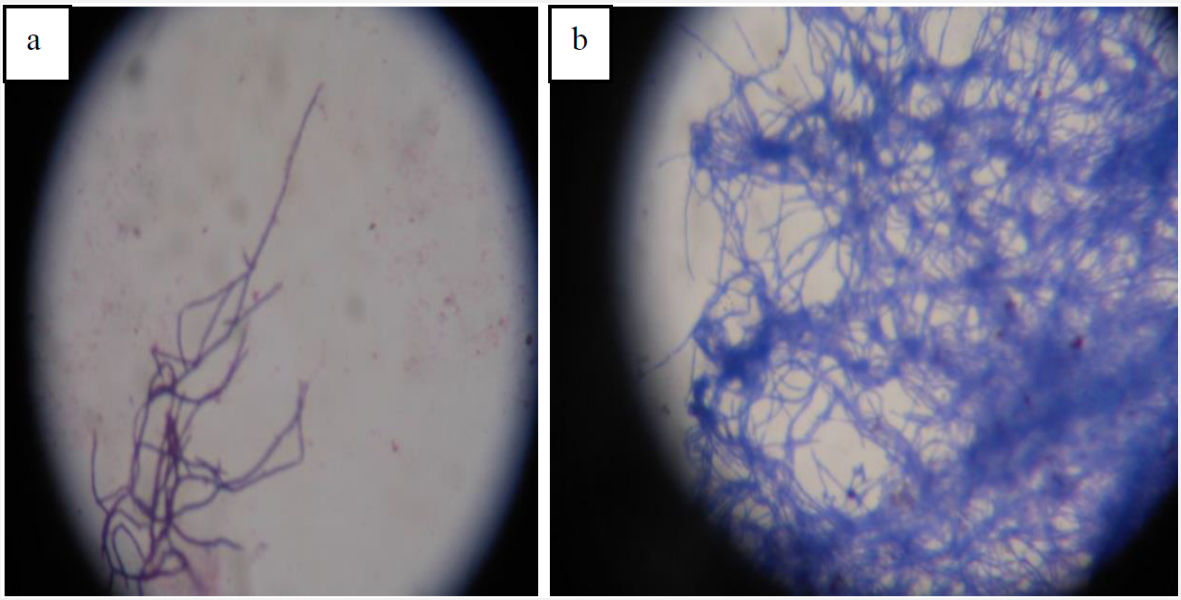

Figure 1: Microscopic appearance of *Streptomyces* isolates 1 (a) and 2 (b) at 100 ×.

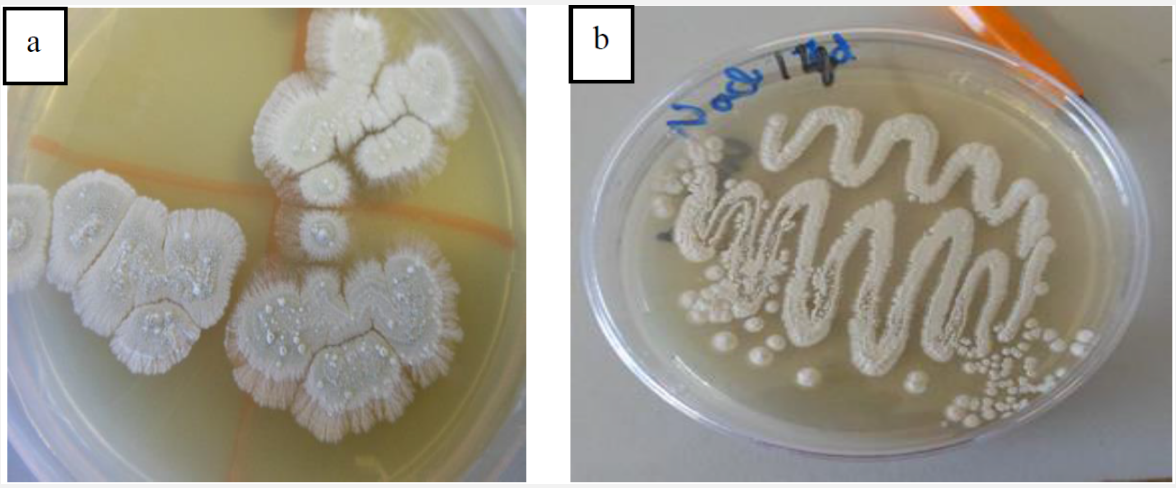

Figure 2: Macroscopic appearance of *Streptomyces* isolates 1 (a) and 2 (b).

Studies such as Al-Huluet al., 2012 and Ramazani, Moradi, Sorouri, Javani & Garshasbi (2013) showed that gram positive bacteria are more susceptible to *Streptomyces* antimicrobial agents than gram negative bacteria due to the lack of a double membrane barrier trans membrane effluxin gram positive bacteria. However, the findings of this study are on the contrary, since *E. faecalis* (gram positive) showed resistance to most *Streptomyces* isolates and only showed moderate susceptibility against *Streptomyces* isolate 5 and 6 (5±0.056 and 6.5 mm ± 0.056) respectively. Distilled water that was used as a negative control did not show any antibacterial activity against *E. coli, M. avium* and *E. faecialis*. All the positive controls showed antibacterial activity ranging between 13 and 18 mm.

Table 2: Average inhibition zones from preliminary antimicrobial activity screening of *Streptomyces* isolates

Streptomyces Isolates	Inhibition zone of against *E. coli* (mm)	Inhibition zone of against *M. avium* (mm)	Inhibition zone of against *E. faecalis* (mm)
Isolate 1	18.5± 0.058	NAA	NAA
Isolate 2	10.5± 0.056	11.5± 0.056	NAA
Isolate 3	16± 0.057	NAA	NAA
Isolate 4	16. 5± 0.057	19.5± 0.056	NAA
Isolate 5	7.5± 0.058	NAA	5±0.056
Isolate 6	NAA	NAA	6.5± 0.056
Isolate 7	NAA	NAA	NAA
Isolate 8	NAA	NAA	NAA
Isolate 9	NAA	NAA	NAA
Positive controls			
	Amoxicillin	Azithromycin	Ampicillin
	15± 0.056	18± 0.057	13±0.055

Key: a ± b: average inhibition zone standard deviation; NAA: No Antibacterial Activity

3.3 Melanoid pigments production by Streptomyces isolates

Streptomyces isolates produced different melanoid pigments as shown in table 3 and figure 3. The colour of produced melanoid pigments on starch casein agar and in starch case in broth were similar. *Streptomyces* isolates 2, 3 and 5 produced green melanoid pigments. Amal, Keera, Samia, Nadia, Ahmed & El-Hennawi (2011) reported on the production of different pigments by *Streptomyces* isolates.

3.4 Antibacterial activity of extracted secondary metabolites

All the pigmented secondary metabolites showed similar antimicrobial activity in the primary antimicrobial screens against *E. coli, M. avium* and *E. faecalis* as depicted in figure

Table 3: Melanoid pigments produced by *Streptomyces* isolates

Streptomyces Isolates	Pigment production on Solid media	Pigment production in broth
Isolate 1	Dark pink	Pink
Isolate 2	Dark green	Green
Isolate 3	Dark Green	Green
Isolate 4	Yellow	Yellow
Isolate 5	Dark green	Dark green
Isolate 6	Dark brown	Brown

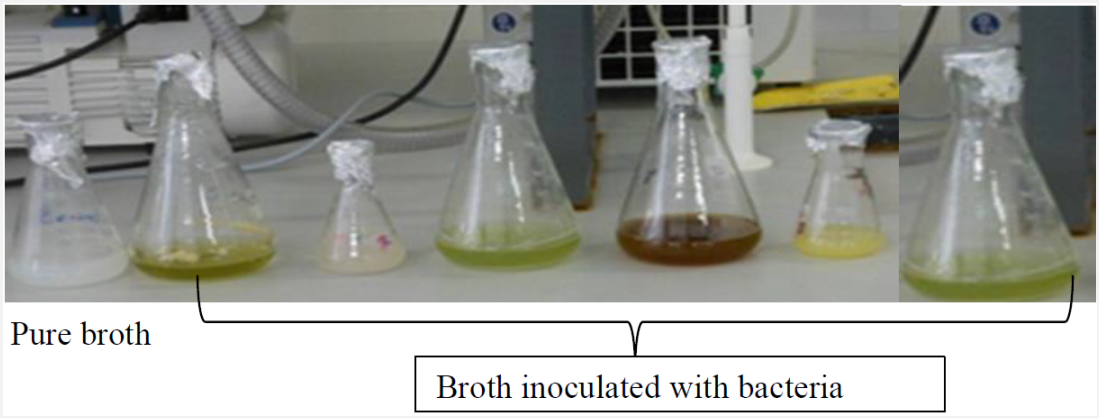

Figure 3: Pigments produced by *Streptomyces* isolates in starch casein agar after 72 hours of incubation.

4. This supports the inference that the susceptibility observed during primary screening was due to the production of secondary metabolites released from the bacteria cells. Secondary metabolites extracted with chloroform showed potent antibacterial activity than n-hexane extracts. Studies on the antibacterial activity of secondary metabolites extracted from *Streptomyces* species are well documented globally.Antibacterial activities of different *Streptomyces* isolates against *M. avium, E. coli* and *E. faecialis* have been reported and documented worldwide (Zhu, 2014; Jeya, Kiruthika & Veerapagu, 2013; Ganesan, Reegan, Davis, Gandhi, Paulraj, Al-Dadi & Ignacimuthu, 2016). However, the findings of this study are the first to report on the potency of Streptomyces species and their pigmented secondary metabolites in Namibia.

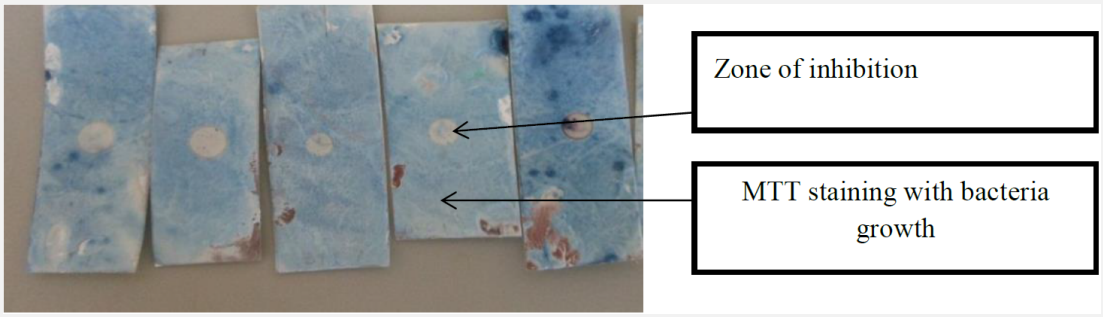

Figure 4: Antimicrobial activities of secondary metabolites produced by chloroform extracted secondary metabolites from *Streptomyces* isolates.

3.5 Minimum inhibitory concentration (MIC) of isolated secondary metabolites

Findings on MIC of isolated pigmented secondary metabolites are depicted in table 4. Pigmented secondary metabolites from Streptomyces isolate 4 and 6 showed the lowest MICs of 6.25 *u*g/ml against *M. avium* and *E. faecialis* respectively. The lower MIC against *E.coli* was observed in pigmented secondary metabolite of isolate one and two (12.5μg/ml). Although *E. coli* was susceptible to most of the isolated secondary metabolites, it required a higher concentration of the secondary metabolites for effective antibacterial activity. This could be because Gram negative bacteria are inherently more resistant to anti-microbials than Gram positive organisms due to their double membrane barrier and trans-membrane efflux (Al-Huluet al., 2012).

The findings on antibacterial activity of secondary metabolites and MIC provide useful information on the potential of these isolates in treating infectious diseases when the etiological agents are *M. avium, E. coli* and *E. faecialis*. This will help to address the multidrug resistance emerging crisis described by Tanwar, Das, Fatima & Hameed (2014); World Health Organization (2016).

3.6 Comparison of Pigment production with the mass (mg) of the Streptomyces isolates

When the growth mass of the culture on solid media was compared to the optical density (OD) of the isolated pigmented secondary metabolite, the higher the mass of the culture, the higher the optical density of the pigmented secondary metabolite. This could mean the growth mass is directly proportional to the pigment production. These findings are

Table 4: The Minimum inhibitory concentrations (MIC) of isolated secondary metabolites against *M. avium, E. coli* and *E. faecialis*

Isolated pigmented secondary metabolites	MIC against *E. coli* (μg/ml)	MIC against *M. avium* (μg/ml)	MIC against *E. faecialis* (μg/ml)
Isolate 1	12.5	NAO	NAO
Isolate 2	12.5	12.25	NAO
Isolate 3	25	NAO	NAO
Isolate 4	25	6.25	6. 25
Isolate 5	25	NAO	NAO
Isolate 6	NA	NAO	12.5

Key: NA - No Antibacterial activity Observed

in compliance with those of Dastager, Dayan and, Tang, Tian, Zi, Xu & Jiang (2006). Moreover, isolate 1 and 4 showed the showed the highest optical density of 0.60 and 0.5 respectively. This correlates with their efficacy against *E. coli* and *M. avium*. Findings on pigment production OD and growth mass are depicted in table 5.

Table 5: Comparison of Pigment production with the mass (mg) of the *Streptomyces* isolates

Streptomyces Isolates	Growth mass (mg)	Pigmentation OD at 480
Isolate 1	1.26	0.60
Isolate 2	1.12	0.21
Isolate 3	1.28	0.25
Isolate 4	1.25	0.50
Isolate 5	1.12	0.12
Isolate 6	0.27	0.25

4 Conclusions and Recommendations

The findings of this study provide significant information and add value to the existing knowledge on antibiotic production by *Streptomyces*. Further analysis of this isolates could help address the morbidity and mortality caused by *E. coli, M. avium* and *E. faecialis*. Secondary metabolites from this study can also help address the issue of microbial resistance. Hence these strains should be further studied to determine the physicochemical properties of the pigments through analytical chemistry and identify the *Streptomyces* isolates using molecular techniques such as PCR.

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