ANTIMICROBIAL ACTIVITY OF A STREPTOMYCETE ISOLATE FROM WHEAT FARM SOIL IN MAU FOREST COMPLEX, KENYA

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ABSTRACT
The study involved isolation of *Streptomyces* spp. from the soil of various sites in the Mau Forest Complex in Kenya. The isolates were screened for antimicrobial activity against selected bacterial and fungal plant pathogens including; *Fusarium moniliforme*, *Ascochyta rabiei*, *Erwinia carotovora*, *Xanthomonas campestris* pv. *campestris*, *Pseudomonas savastonoi* pv. *phaseolicola* and reference cultures which were: *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus subtilis* ATCC 6633. One isolate from a wheat farm designated as WHF2B16 tested positive as a *Streptomyces* species through cultural, morphological, biochemical and molecular characterization. It was found to have antimicrobial activity against the fungal pathogens with a zone of inhibition >20 mm; one plant bacterial pathogen i.e. *Pseudomonas savastonoi* pv. *phaseolicola* with an inhibition zone of 15.5 ± 1.2 mm and was active against the Gram positive bacteria i.e. *Bacillus subtilis* and *Staphylococcus aureus*. Ethyl acetate extracts compared to culture filtrates of the isolate were found to produce significantly higher growth inhibitory effects in the test microorganisms in a t test (t-value $P > 0.05$). The isolate was further subjected to 16S RNA analysis and confirmed to be a *Streptomyces* species assigned as *Streptomyces* mau 1 (Accession No. KR780774) from the NCBI database. This study has revealed that a streptomycete from a wheat farm in the Mau Complex in Kenya has the potential to be used as an antifungal and antibacterial agent.
Keywords: Antimicrobial activity, Plant pathogens, *Streptomyces*

INTRODUCTION

Streptomyces is a genus of Gram positive bacteria belonging to the order Actinomycetales in the Actinobacteria phylum (Kampfer, 2006). About 80% of all known antibiotics are derived from this genus (Kieser *et al.*, 2000). These include a diverse array of antibiotics including aminoglycosides, anthracyclins, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines. *Streptomyces* are the producers of more than 5,000 known bioactive compounds (Anderson and Wellington, 2001). This genus is known to produce antibiotics, anti-tumor agents, immunosuppressive agents and enzymes (Hamid *et al.*, 2012; Changhyun and Chan-Kyu, 2014; Laishram *et al.*, 2014). Many antibiotics from streptomycetes today have found practical application in human and veterinary medicine, agriculture and industry (Madigan and Martinko, 2007; de Lima Procopio *et al.*, 2012). Members of this genus are ubiquitous in nature and are mostly soil inhabitants (Madigan and Martinko, 2007). Plant diseases caused by fungal, bacterial and viral pathogens have resulted in epidemics, famines and hunger. In Kenya, for example, rice blast disease by the fungus *Magnaporthe oryzae* has resulted in great losses in rice farms (Kihoro *et al.*, 2013). There is a need to look for an alternative means to manage these plant diseases other than the classical known chemical pesticides which have a negative effect on the environment, humans and animals. This necessitates investigating alternative strategies to control fungal and bacterial plant pathogens. The Mau Forest Complex is found in the western side of the Mau Escarpment in Kenya. It is an important water tower in Kenya with many rivers originating here. It is the home of different indigenous flora and fauna (Kanyinke, 2005). The microbial diversity of the Mau complex today remains un-investigated. Like other natural environments, it may serve as a repertoire of many useful microbial resources. Due to increasing population pressure, the Mau Complex has encountered many anthropogenic activities in the recent past that have greatly affected this ecosystem (Gichuhi, 2013). The Government of Kenya is making efforts to protect and conserve the biodiversity of this area. To support these efforts there is need to carry out research geared towards conservation and bio-prospecting of useful microbial resources. This study isolated and studied antibiotic producing *Streptomyces* from the Mau Complex that are potentially useful for biological control of plant pathogens affecting food crops in Kenya.

MATERIALS AND METHODS

Study Area

The Mau Forest Complex is the largest closed-canopy forest ecosystem in Kenya and the largest indigenous forest in East Africa, stretching across 400,000 hectares (1,544 square miles) (Kanyike, 2005). The latitude is

between 0º 20′ 60″S and the longitude is between 35º 27′ 32″E. It lies between 2,000 m and 2,600 m above the sea level, on the Western slope of the Mau Escarpment. The soil for the current study was collected in a wheat farm (35º 89´ 21.37E, 0º 36´ 21.48N) near a place called Mwisho wa Lami on the Eastern side of the Mau complex.

Soil Sampling and isolation of *Streptomyces*

Soil samples from the wheat farm were collected randomly using a sterile Soil Auger (20 cm in depth, 2.5 cm in diameter). Soil sampling was done from a depth of 10-20 cm below the soil surface. The samples were air-dried at room temperature for 7-10 days and then passed through a 0.8 mm mesh sieve and preserved in sterile polyethylene bags at room temperature before isolation of the bacteria. Portions of 10 g of air-dried soil was then mixed with sterile distilled water (90 ml). The mixtures were shaken for 1 hour on a rotary shaker and allowed to settle for another 1 hour. Portions of 1ml of soil suspensions (diluted 10^{-1}) were transferred to 9 ml of sterile water and subsequently decimal serially diluted up to 10^{-6}. Inoculates consisted of adding 0.1 ml aliquots of 10^{-3} - 10^{-6} soil dilutions to solid sterile starch-casein agar (Kuster and Williams, 1964; Williams and Davies, 1965), supplemented with filter (0.2 µm pore diameter) sterilized antibiotic solution containing cycloheximide, and nystatin (0.005% final concentration), polymixin-β sulphate (0.0005% final concentration) and sodium penicillin (0.0001% final concentration) to inhibit non-actinomycete bacteria and fungi (Baltz, 2006). The samples were spread plated on the test media in triplicates for each dilution. After incubation for 4 - 7 days at 28 ºC, the colonies that had developed on the plates were enumerated and expressed in colony forming units (CFU's) per gram of soil. The isolated colonies of streptomycetes were transferred from the isolation media to a sterile growth media consisting of glucose (10 g), yeast extract (1 g), potassium nitrate (1 g), potassium mono-hydrogen phosphate (0.1 g) and agar (15 g) per liter. The plates were incubated at 28 ºC for 6 days. Individual pure colonies of the cultures were isolated and sub-cultured into freshly prepared yeast malt extract agar at 4 ºC (Demain and Davies, 1999). For ease of identification, isolates were appropriately coded for later use in the antimicrobial activity assay. Thus one isolate coded as WHF2B16 was subjected to antimicrobial bio-assay as described in the following sections. The isolate was obtained from the second sample of soil from the wheat farm and was from a depth of 10-20 cm.

Antifungal bioassay

Two plant pathogenic fungi were used for screening of antimicrobial effect. These fungi are *Fusarium moniliforme* which cause blights and ear rots in maize and *Ascochyta rabiei* which causes *Ascochyta* blight in chick peas. The fungal test pathogens were grown in PDA (potato dextrose agar) for three days; 8 mm disc plug of the fungi was picked using a sterile cork-borer and placed in at 8 mm hole bored in the middle of a PDA plate. A disc of *Streptomyces* colony grown on agar for five days was obtained using 8 mm diameter cork-borer and placed in the PDA media containing fungal test pathogens. Antifungal activity around the *Streptomyces* agar discs was evaluated as described in (Table 1.) below and the ratings used were modified from those of (Lee and Hwang, 2002).

Table 1: Rating scale for inhibition diameter by *Streptomyces* isolates against fungal pathogens (Lee and Hwang, 2002)

Inhibition diameter	Rating
No inhibition	(-) mycelia growth not different from control
5-9 mm (weak inhibition)	(+) partial inhibition of mycelia growth
10-19 mm (moderate inhibition)	(++) almost complete inhibition of mycelia growth
>20 mm (strong inhibition)	(+++) complete inhibition, most mycelia will not grow

Controls were plain agar blocks

Antibacterial bioassay

Antibacterial activity of the isolates was tested against three plant pathogenic bacteria *Xanthomonas campestris* pv, *campestris*, *Pseudomonas savastanoi* pv. *phaseolicola* and *Erwinia carotovora* which causes black rot of cruciferous plants, halo blight disease in beans and soft rot of vegetables respectively. Other standard reference bacterial cultures were also used for the bioassay. These are *Staphylococcus aureus* ATCC 25923; *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853 and *Bacillus subtilis* ATCC 6633. The isolated *Streptomyces* were grown in a broth of the growth media for 72 hours then streaked perpendicularly on plates containing Mueller Hinton agar (Valgas *et al.*, 2007; Balouiri *et al.*, 2016). The plates were incubated for five days at 28°C. A single streak of the test bacteria was done at an angle of 90°C to the

Streptomyces streak (Sanders, 2012). Incubation was done for 24-48 hours and the distance of inhibition was measured.

Submerged cultures and antimicrobial activity of the culture filtrate

Isolates which showed antimicrobial activity were cultivated on casein glycerol agar at 28ºC for 7 days. A 0.6 cm diameter disk of the agar culture was transferred aseptically to 250 ml Erlenmeyer flasks containing 100 ml casein glycerol broth. The inoculated flasks were kept on a rotary shaker at 130 rpm at 28-30ºC for 7 days. Cells were removed by centrifugation of the broth at 5000 rpm for 20 minutes. The cell-free supernatant was separated using 0.2 µm pore size membrane filter (Millipore) and the filtrate collected as the antibiotic sample (Grammer, 1976). The well-diffusion method was used to assay for antifungal and antibacterial activity. For estimation of the antifungal activity, a 10^6 spore suspension of the fungi was prepared and spread plated on PDA plates using a sterile swab. The plates were allowed to dry for 15 minutes before 4 wells per plate were made using an 8 mm sterile cork-borer. Each well was filled with 30 µl of the filtrate (Delahaye *et al.,* 2009). The plates were then incubated at 27°C and observed at day 3, 5 and 7. The test bacteria were grown in Nutrient Broth and compared to a Macfarland standard of 0.5 which is equivalent to an inoculum of 1×10^8 CFU mL^{-1}. Mueller Hinton Agar (MHA) plates were used. The surface of the plates was inoculated using a swab which had been dipped in the inoculum. The swab was streaked three times and each time the plate was rotated at 60°. Using a sterile 8 mm cork-borer, three wells were dug on the seeded agar plates and each well was filled with 30 µl of the filtrate collected as the antibiotic sample (Elleuch *et al.,* 2010). The plates were incubated at 30 °C and the diameter of inhibition was measured round the well.

Extraction of the antimicrobial compound

The culture filtrate was transferred aseptically into conical flasks. Ethyl acetate was added to the culture 72°C for 90 sec and final extension at 72°C for 10 min. The PCR reaction mixture (50 µl) contained PCR beads 1 µl from each primer *8f* and *1492r*, 2.5 µl of CdNTPS, 5 µl of the buffer, 0.2 µl of Taq polymerase, 2 µl of BSA and 1 µl of template DNA up to final volume 50 µl which was reached by adding distilled water. Electrophoresis of the PCR products was carried out on 1% agarose gel in 1xTAE buffer containing ethidium bromide (0.5 µg mL^{-1}), to ensure that a fragment of the correct size was amplified and detected by visualizing under UV light. PCR products were purified using High Pure filtrate in the ratio of 1:1. The mixture was then centrifuged at 5000 rpm for 20 minutes for complete extraction. The ethyl acetate phase that contained the antibiotic was separated from the aqueous phase. It was dried at a water bath at 80-90°C and the obtained residue used for antimicrobial bioassay (Ilic *et al.,* 2005). 6 mm filter paper disks were seeded with the antimicrobial compound and placed on PDA and MHA plates previously inoculated with the test pathogens (CLSI, 2012; Acar and Goldstein, 1996). The zone of inhibition was measured.

Cell morphology, cultural and biochemical characteristics

The cell morphology was done by examining spore hyphae under a microscope. The colour of the colonies was determined by cultivating the isolates on yeast-extract malt extract agar (YME), starch casein agar (SCA), nutrient agar (NA) and inorganic salt and starch agar (ISSA) (Shirling and Gottlieb, 1966). Observation was done after 7, 14 and 21 days. The aerial mass color, presence of soluble pigment and the reverse color was also observed. Microscopic observation was done by cover slip method (Shirling and Gottlieb, 1966). The arrangement of spores on mycelium was observed under high power objective in the light microscope. Biochemical tests for the identification of *Streptomyces* species were done using the method described by (Korn-Wendisch and Kutzner, 1992). These included carbon utilization, catalase test, gelatin liquefaction, Simon citrate and Gram reaction tests.

Identification using molecular procedures

The DNA of the isolate was extracted using the phenol/chloroform/isoamyl method (Kieser *et al.,* 2000).The DNA was semi quantified on a 1% agarose gel in 1 x TAE buffer and visualized under UV by staining with ethidium bromide (Sambrook *et al.,* 1989). Amplification using universal primers of *8f* (5'-AGA GTT TGA TCC TGG CTC AG -3') and *1492r* (5'-TACTTGTTACGACTT-3') under the following condition: 94°C for 5 min, 30 cycles of 94°C for 45 sec, 48°C for 2 min, PCR Product Purification Kiton kit (Roche, Germany) and outsourced for sequencing in Macrogen, South Korea. The 16S rRNA gene sequences were compared to sequences in the public database using Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI), in order to determine the similarity between sequences in the GenBank database (Al-Zahrani, 2007). The phylogenetic tree was drawn using MEGA 6.0 (Tamura *et al.,* 2013).

RESULTS

Cultural and morphological characteristics

Cultural and morphological examination of the isolate WHF2B16 showed that this bacterium was a *Streptomycete*. The aerial mycelium of the isolate on inorganic salt and starch agar (ISSA) was white (Plate 1.), the substrate mycelium on the same media was cream and had a pale yellow soluble pigment. The colonies grew with a white aerial mycelium on YME agar and had a cream substrate mycelium with no soluble pigment. The color of the aerial mycelium on SCA was grey with yellow substrate mycelium and pale yellow soluble pigment as shown in Table 2. The colonies were hard to pick, butryous and formed chalky aerial spores. These are common characteristics of members of the genus *Streptomyces* as listed in the Bergey's Manual of Systematic Bacteriology (Whitman *et al.,* 2012).The spores were observed under a compound microscope at ×250. The spores were flexuous in shape (Figure 1).

Aerial Mycelia on ISSA

Substrate mycelia on

Plate 1: Color of isolate on ISSA media

Table 2: Color of isolate WHF2B16 on different media

Medium	Aerial mycelium	Substrate mycelium	Soluble pigment
SCA	Grey	yellow	pale yellow
YME	white	cream	none
NA	cream	cream	none
ISSA	white	cream	pale yellow

SCA represents starch casein agar; YME is yeast malt extract; NA is nutrient agar and ISSA represents inorganic salts and starch agar.

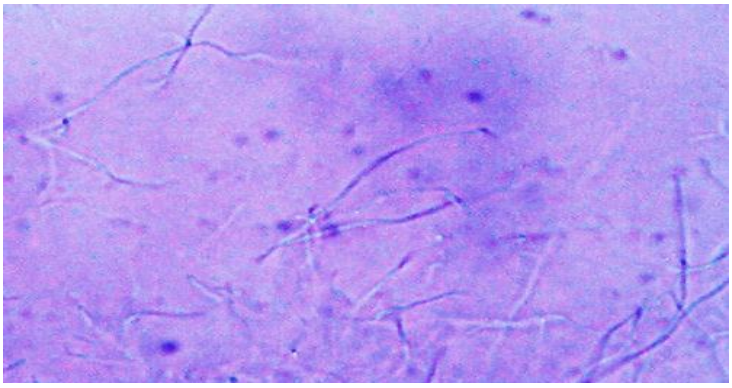

Figure 1: The appearance of the spores of WHF2B16 from a 5 - day old culture (×250).

Biochemical characteristics

The biochemical tests showed that the isolate belonged to the genus *Streptomyces* as compared to other isolates in the Bergey's manual (Whitman *et al.,* 2012). The isolate was able to utilize a wide range of carbon sources and had abundant to fair growth. It had doubtful growth in gelatin after fourteen days after inoculation. The isolate was Gram positive, had a negative catalase test and did not grow on MacConkey and Simon citrate agar (Table 3).

Table 3: Biochemical tests of isolate WHF2B16

Biochemical test	Reaction
D- Glucose	+++
D-Xylose	+
D-Mannitol	+
I-Inositol	+
L-Arabinose	+++
Sucrose	++
L-Rhamnose	++
Starch	+++
Gelatin	±
Catalase	-ve
Simon Citrate	-
Milk Agar	++
MacConkey	-
Casein Hydrolysis	+ve

+++ = Abundant growth, ++ = moderate growth, + = fair growth, ± = Doubtful, - = no growth.

Antifungal and antibacterial activity

Isolate WHF2B16 showed inhibitory activity against the fungal pathogens in the initial screening method. A photograph of the isolates of *Streptomyces* from wheat field showing inhibitory effect on *Ascochyta rabiei* is shown in Plate 2. The isolate inhibited the growth of both fungal isolates with distances that were above 20mm in diameter as shown in Table 4. This shows that the isolate has potential to inhibit control of fungal pathogens of agricultural importance. The results from initial screening showed that isolate WHF2B16 was active against one bacterial phytopathogen i.e. *P. savastonoi* pv. *phaseolicola.* It also had inhibitory activity on reference bacterial cultures such as *B. subtilis, P. aeruginosa, E. coli,* and *S. aureus,* indicating its potential inhibition of bacteria in general.

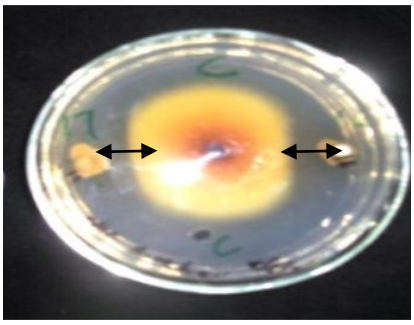

Plate 2. Two isolates with antifungal activity against *Ascochyta rabiei*. Right is WHF2B16 top and bottom is blank agar block which is control and in the center is *Ascochyta rabiei.*

Further tests with culture filtrates and ethyl acetate extract using the disc diffusion method showed higher antimicrobial activities against the test microorganisms *Bacillus subtilis*, *Staphylococcus aureus, Fusarium moniliforme,* and *Ascochyta rabiei* as shown in Table 5. The culture filtrate and the ethyl acetate extract had no effect on the Gram negative bacteria both in the well and disc diffusion methods even for *Pseudomonas aeruginosa* that had shown a positive response in the primary screening test. However, a positive inhibitory effect was found in the Gram positive test organisms.

Molecular analysis

Blast analysis positively identified the test isolate as *Streptomyces* sp. which clustered closely to *Streptomyces* badius, *Streptomyces* sp. E5N135 and *Streptomyces* sp. 514F. It had gene homology of 99% with the *Streptomyces* genus. It was identified as *Streptomyces* Mau 3 ACC. No. KR780774 (Fig. 2).

Table 4: Antimicrobial activity of the isolate from the initial screening method

X. c	P.s.p	E.car	P.a	*E.coli*	S.a	B.s	F. m	A. r
0	15.5±1.2	0	20±0.5	5.3±0.5	19±0.8	20.7±0.9	23±1.2	20±1.2

Values are inhibition zones expressed as mean diameters (mm ± SD). X.c is *Xanthomonas campestris*, P.s.p is *Pseudomonas savastonoi* pv. *phaseolicola*, E. car is *Erwinia carotovora*, P.a represents *Pseudomonas aeruginosa*, *E. coli* is *Escherichia coli*, S.a represents *Staphylococcus aureus*, B. s *Bacillus subtilis*, F. m represents *Fusarium moniliforme* and A.r represents *Ascochyta rabiei*.

Table 5: Antimicrobial activity of the culture filtrate (CF) and the Ethyl acetate extract of isolate WHF2B16

	Isolate	B.s	P.s.p	E. car	E.coli	P.a	S.a	F.m	A.r
CF	WHF2B16	10.3 ± 0.6^{a}	NA	NA	NA	NA	5.7 ± 1.2^{a}	25.7 ± 1.2^{b}	22 ± 0.5^{b}
EAE	WHF2B16	20 ± 2^{b}	NA	NA	NA	NA	18 ± 0.5^{b}	25 ± 2^{b}	23 ± 1^{b}

Values are inhibition zones expressed as mean diameters (mm ±SD). Means followed by the same letters in a row do not differ significantly from one another (P>0.05). B.s represents *Bacillus subtilis*, P.s.p is *Pseudomonas savastanoi* pv. *phaseolicola*, E. car. is *Erwinia carotovora, E. coli* is *Escherichia coli*, P. a is *Pseudomonas aeruginosa*, S.a. is *Staphylococcus aureus*, F.m is *Fusarium moniliforme* and A.r is *Ascochyta rabiei* respectively. NA = no activity.

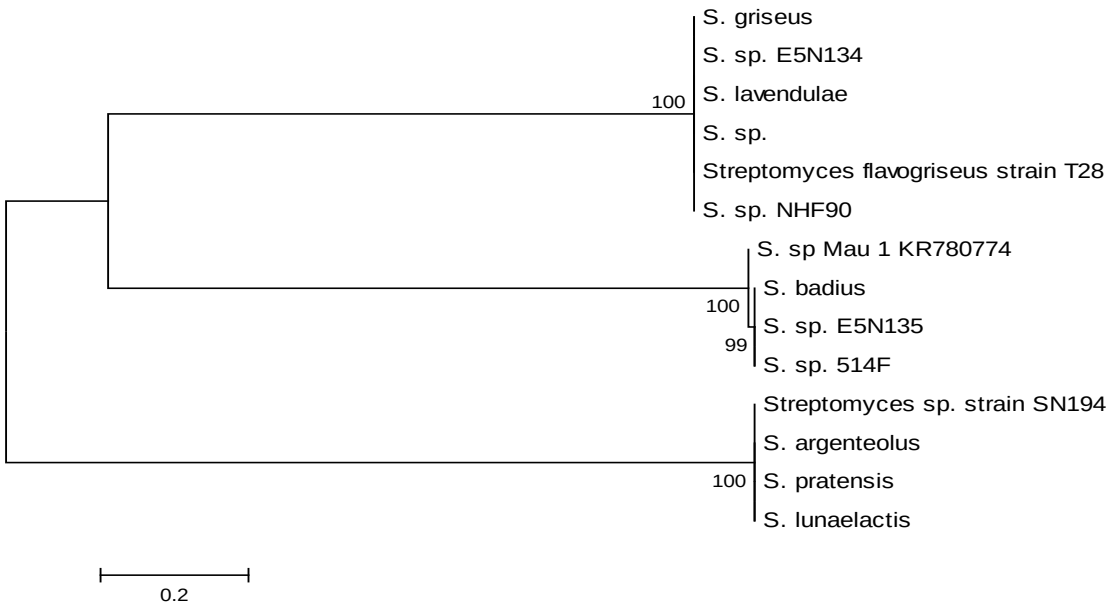

Figure 2: A maximum likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships between WHF2B16 and known *Streptomyces* isolates from the NCBI/BLAST database.

DISCUSSION

This study has reported the first case of *Streptomyces* isolates with antibacterial and antifungal activity from the Mau Forest Complex in Kenya. Isolate WHF2B16 isolated from a wheat farm in the Mau Forest complex showed antifungal and antibacterial activity against selected plant pathogens and standard reference bacteria test pathogens. WHF2B16 significantly inhibited the growth of Gram positive bacteria and fungi as compared to the Gram negative bacteria. These results agree with the findings of Panwar and Saini, 2012 who observed that 10.76% of their isolates had antibacterial activity against *B. subtilis* as compared to 6.45% isolates which showed antibacterial activity against *E. coli.*

Actinomycetes isolates showed more inhibitory activity against Gram positive bacteria than in Gram negative bacteria (Silambarasan *et al.,* 2012). Studies by Laishram *et al.,* 2014 observed that *Streptomyces* isolates inhibited the growth of Gram positive *Staphylococcus aureus* but not Gram negative bacteria, these findings are similar to the current study. This can be attributed to structural differences in cell walls of Gram positive and Gram negative bacteria. The outer membrane layer in the latter group may be an effective permeability barrier due to the presence of structural lipopolysaccharide components which make the cell wall impermeable to lipophillic solutes (Scherrer and Gerhardt, 1971). It may also mean that the antibiotics have an effect on the peptidoglycan development in Gram positive bacteria as is the case with some antibiotics (Kohanski *et al.,* 2012).

The antimicrobial effect of the isolate decreased when the culture filtrate was used as compared to when the isolate was interacting with test pathogens in the solid media. Such findings have been observed by other researchers who noted that antibacterial activity of actinobacteria was higher in solid media than in culture filtrates (Kumar and Kokati, 2012). *Streptomyces* isolates from Jazan showed decreased to negative antimicrobial activity when grown in liquid media as compared in solid media (Al-Zahrani, 2007). These results agree with the findings of the current study. The isolate was characterized using biochemical, microscopical and morphological characteristics and was identified as a member of the genus *Streptomyces*. It was further characterized using 16S rRNA analysis and BLASTN analysis. The isolate was identified as a *Streptomyces* once more and it was placed in the same clade as *Streptomyces badius* which produces hydroxyl marilone C which has potential anti-tumor and anti-viral properties (Osama *et al.,* 2016).

CONCLUSION

The Mau Forest Complex provides a site with unexploited microbial resources in Kenya. *Streptomyces* Mau 1 (Acc. No. KR780774) was isolated from the Complex. This isolate showed antimicrobial activity against Gram positive bacteria and plant pathogenic fungi. 16S rRNA analysis showed

the isolate clustered with *Streptomyces badius* and *Streptomyces griseus* which are antibiotic producing *Streptomyces* that have been exploited by the pharmaceuticals. Hence this isolate can be investigated further for antibiotics production and possibly other beneficial secondary metabolites.

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CONFLICT OF INTEREST

There is no conflict between the authors either financial/commercial or any other form of interest. This paper has not been published elsewhere and is devoid of plagiarism.

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