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Isolation and Characterization of Antibiotic Producing Actinomycetes from Soils of Hawassa, Southern Ethiopia

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ABSTRACT

Antimicrobial resistance has increased drastically in recent years in the developing countries, and it has leading public health concern. With almost all organisms becoming multidrug resistant to the commonly used antibiotics, there is a need to search for more drugs that are novel in order to address this challenge. *Actinomycetes* are considered as one of the most diverse groups of filamentous bacteria capable of thriving into different types of ecological niches due to their bioactive potential. Therefore, this study was aimed at isolation and characterization of *Actinomycetes* from 20 soil samples that were collected from different sites of Hawassa city, Southern Ethiopia. The *Actinomycetes* were isolated using serial dilution followed by spread plate techniques and antimicrobial activity screening done using modified agar disc diffusion method. *Actinomycete* Isolation Agar (AIA) was used to isolate *Actinomycetes*. A total of twenty nine different *Actinomycetes,* identified as AB1-AB29, were isolated.They were differentiated based on the difference in appearance of the colony morphology and mycelial structure. Their metabolites were tested for antibiotic activities through the primary screening using modified agar disk diffusion methods. Test bacteria were; *E.coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Shigella boydii* and *Salmonella typhi*. Actinoycete isolates with broad spectrum activity were further tested against Methicillin resistant *Staphylococcus aureus*(MRSA) using modified agar disk diffusion methods. Out of 29 isolates, 19(65.5%) *Actinomycetes* showed antimicrobial activity against selected bacterial pathogens. Most of the isolates (84.2%) showed good antimicrobial activity against *Salmonella typhi* though significantly lower than the control drug Ciprofloxacin. Maximum zone of inhibition was 29.2mm observed against *S.typhi*. As the result indicates the *Actinomycetes* isolates showed higher inhibition zone against Gram negative bacteria than Gram positive bacteria. The study indicated that soils of Hawassa may have potential group of *Actinomycetes* with broad spectrum antimicrobial activity. It is therefore suggested that a combination of several molecular analysis methods such as DNA re-association and PCR-based fingerprinting techniques may extremely help to provide broader information about the total genetic diversity of soil *Actinomycetes* obtained in this study.

INTRODUCTION

Antibiotics are substances normally of low molecular weight capable of inhibiting or slowing the growth of pathogenic microorganisms. They are often secondary metabolite produced by microorganisms and seem to have no definite role in the growth of the cell source. Microorganisms produce antibiotics normally during their late log phase of growth until their stationary phase. One of their key benefits to the source organism is said to be their ability to inhibit the growth of other microorganisms growing in the same

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environment in nature hence providing the source with a competitive advantage. Antibiotic producing microorganisms can then compete with others and survive in nature for a long time (Onlamoon, 2008).

Antimicrobial compounds are produced by various living organisms such as bacteria, fungi, and plants. Among the various groups of organisms that have the capacity to produce antimicrobial agents, the *Actinomycetes* are the most capable candidate (Gebreselema *et al*., 2013). *Actinomycetes* are slow growing, Gram-positive bacteria, having high G+C content from 55-75 % (Ningthoujam *et al*., 2009). They resemble fungi because of their filamentous appearance and spore production property and bacteria because of the presence of peptidoglycan in their cell wall and possession of flagella (Mythili and Das, 2011). *Actinomycetes* are inexhaustible producers of antimicrobial agents (Atta *et al*., 2011). Around 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by *Actinomycetes*, representing 45% of all bioactive microbial metabolites discovered (Berdy, 2005). The secondary metabolites obtained from the class *Actinomycetes* are of special interest because of their diverse biological activities such as antibacterial, antifungal, antioxidant, antitumor and antiviral. Among *Actinomycetes*, *Streptomyces* species produce around 7,600 compounds. Many of these secondary metabolites are potent antibiotics, which has made *Streptomycetes* the primary antibiotic-producing organisms exploited by pharmaceutical industry and responsible for the formation of more than 60 % of known antibiotics. Further 15 % are made from a number of related *Actinomycetes, Micromonospora, Actinomadura, Streptoverticillium,* and *Thermo Actinomycetes* (Jensen *et al*., 2007; Ramesh *et al*., 2009).

The emergence of resistance to the commercially available antibiotics and multidrug-resistant pathogenic bacteria are issues of extreme concern in present time for the whole human community. Due to these issues, there is rapid spread of infectious diseases leading to morbidity and mortality especially among the elderly and immune-compromised patients (Hong *et al*., 2009). To overcome this situation the discovery of novel drugs with lesser side effects is need of present time.

Choice of natural materials like soils in researches is based on the assumption that samples from widely diverse locations are more likely to yield novel microorganisms and therefore hopefully, novel metabolites are exploited as a result of the geographical variation. Besides, the important approaches helpful in discovering new microbial species or unknown bioactive substances include isolation and characterization of microorganisms from relatively unknown or unstudied areas (Moncheva *et al*., 2002).

In Ethiopia, a few studies showed the existence of antibiotic producing microorganisms from different ecosystems. Biniam (2008) isolated antimicrobial producing *Actinomycetes* from southern part of Ethiopian Rift Valley alkaline lakes like Hora and Chitu. The potential of a mushroom compost as a good source for antibiotic producing thermophlic *Actinomycete* was also reported by Moges (2009). Atsede and Fassil (2018) also isolated and screened antibiotic producing *Actinomycetes* from soil

collected from the rhizosphere of plants and agricultural soils of Ethiopia. However, there is no such scientific report on antibiotic producing microorganisms from soil samples collected in Hawassa city. Therefore, the present study aimed to isolate and characterize antibiotic-producing *Actinomycetes* from soil samples of Hawassa, Southern Ethiopia.

MATERIALS AND METHODS

Description of the study area

The study was conducted in Hawassa City. It is located 275 km away from Addis Ababa, the capital city of Ethiopia. It is located at 70' 03'' latitude and 80'29" east and lies at an altitude of 1708 m above sea level. The city has a total area of 15,720 hectares and the city Administration of Hawassa consists of 8 sub cities and 32 kebelles. The city experiences a sub humid type of climate having an average annual temperature of about 20.3^{o}Cand mean annual precipitation of about 933.4 mm (Hawassa city Administration, 2007)

Collection of Soil Samples

Twenty (20) soil samples were collected from four different sites of Hawassa city namely: Main campus (5), Monopol (5), Mount Tabor (5) and Mount Alamura (5). Two hundred grams of soil samples were taken from a depth of 11-16 cm from the soil surface using sterile spoon (Chaudhary *et al*., 2013). The soil samples were collected and placed in dry, clean, sterile polyethylene bags and transported aseptically to Microbiology Laboratory of the Department of Biology, Hawassa University where the entire research work was carried out. The collected samples were labeled with details such as: name of collection site, date of collection and pH of soil. The pH of soil was measured before collecting the soil samples. The collected soil samples were air dried at room temperature for a week to reduce gram negative bacteria (Oskay *et al*., 2004). The soil samples from sterile plastic bag were grinded using sterile mortar and pestle and sieved aseptically using 250 µm pore size mesh to remove small pieces of stones and organic matter. The samples were then placed in polyethylene bags to avoid external contamination and kept in refrigerator at 4°C until used.

Isolation and Cultivation of *Actinomycetes*

From each sample, 1g of soil sample was added in the test tube containing 10 ml distilled sterile water and shaken well using vortex mixer for 3 minutes and serially diluted by using serial dilution method up to 10^{-7}. These test tubes were considered as stock cultures for different soil sample sites. From the stock culture, 1 ml was used to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} by serial dilution method. Thereafter 0.1 ml of the suspension from 10^{-3}, 10^{-5}, and 10^{-7} was taken and aseptically spread onto *Actinomycete* Isolation Agar (AIA) medium (Millipore, and Sigma, Germany) by applying spread plate technique and incubated at 30°C for 7 d. The colonies were picked and sub cultured for purity by streaking on nutrient agar. The pure colonies were isolated and identified by the color of hyphae, colony morphology and the presence or absence of aerial and substrate mycelium (Reddy *et al*., 2011). After incubation, the slants containing pure *Actinomycetes* isolates were preserved at 4°C for the further studies.

Screening of *Actinomycetes* for Antimicrobial Activity

Test Microorganisms

Antimicrobial properties against selected microorganisms acquired from the Ethiopian Health and Nutrition Research Institute (EHNRI) and Hawassa University Referral Comprehensive Specialized Hospital were investigated *in vitro*. The test bacteria used for primary screening were *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia, Shigella boydii* and *Salmonella typhi*. Methicillin-resistant *Staphylococcus aureus* (MRSA) (clinical isolate) which was provided by Hawassa University Referral Comprehensive Specialized Hospital was also used for secondary screening.

Primary Screening of the Isolates by Disc Diffusion Method

Antimicrobial screening was done using disc diffusion method as described by Kirby Bauer (1979). The stocked *Actinomycetes* isolates were revived by sub-culturing on Nutrient Agar plates. The colony was then picked and inoculated into 5ml nutrient broth and incubated at 30°C for 10 days. Thereafter, the prepared culture was standardized to 0.5 McFarland turgidity standard using the spectrophotometer (optical density of 1.0 at 625 nm) by adding sterile distilled water to obtain the desired cell density of 1.5 X 10^{8} (cell/ml) (CLSI, 2012).

Paper discs (6 mm in diameter) were prepared from Whatman No 1 filter papers and sterilized by autoclaving at 121°C, 15psi for 15 minutes (Ngeny *et al*., 2013). The disc (6 mm in diameter) was impregnated with 15µl of the 7d old culture broth and placed on Mueller Hinton Agar inoculated with the test isolates. Standard antibiotic (Ciprofloxacin) was used as a positive control and filter paper disc soaked with sterile distilled water was used as a negative control. They were then incubated at 37°C for 24 hours. The isolates with antimicrobial activities were identified by measuring the inhibition zone in millimeters (mm) using a ruler. The absence of growth or a less dense growth of test bacteria near the disc was considered as positive for production and secretion of antibacterial metabolite by the isolates (Kekuda *et al*., 2010).

Secondary Screening of the Isolates

Based on the zone of inhibition in primary screening, *Actinomycete* isolates that have broad spectrum antimicrobial activity were further assessed against Methicillin-resistant *Staphylococcus aureus* (clinical isolate). Antimicrobial activity of the secondary metabolites that had broad spectrum activity in primary screening was determined by use of Kirby Bauer disk diffusion method (Brown, 2009). One week old broth cultures of the *Actinomycetes* were used with sterile paper discs soaked in the cultures for 30 minutes were used to inoculate *Actinomycetes* on Muller Hinton Agar (MHA) media seeded with methicillin resistant *Staphylococcus aureus* (MRSA) (clinical isolate). The petri dishes were incubated at 37°C for 24 hours. Standard antibiotic (Ciprofloxacin) disc was used as a positive control and filter paper disc soaked with sterile distilled water was used as a negative control. The antibacterial activity was determined by measuring the diameter of the inhibitory zones with a ruler (CLSI, 2012).

Morphological Characterization

A loop full of the isolates were streaked on each medium and incubated at 30°C for 7 to 10 days. The color of aerial mycelium, reverse color, and nature of the colony was observed and recorded.

Microscopic Characterization

The arrangement of spores and sporulating properties of the selected isolates were examined microscopically by using cover slip culture method by inserting sterile cover slip at an angle of 45°C in the *Actinomycete* isolation agar medium. A loop full of each isolate was taken separately from 7 d old culture, inoculated at the insertion of cover slip and incubated at 30°C for 7 d. Then, the cover slip was removed by using sterile forceps and placed upward on a clean glass slide. Finally, the cover slip was observed for the morphology of isolates under the microscope at 100x magnifications (Cappuccino and Sherman, 2002).

Gram Stain

A thin smear of the 7 d old *Actinomycetes* cultures were inoculated into grease free slides. Thereafter, they were heat fixed and placed in a staining rack. The slides then flooded with crystal violet for one minute and then rinsed with distilled water gently. Gram's iodine was then gently flooded on the smears and allowed to stand for one minute before gently rinsing with distilled water. This was then decolorized using 95% ethyl alcohol for 20 seconds and immediately rinsed with water to avoid over decolonization. Finally Safranin was gently flooded on the smears and let to stand for one minute before rinsing with distilled water. The slides were then blot dried using absorbent paper and then viewed using a light-microscope under oil-immersion (100x) (Cappuccino and Sherman, 2002).

Physiological Characterization

Temperature on Growth

The identified isolates were streaked on *Actinomycetes* isolation agar and incubated at 25°C, 30°C, 37°C and 40°Cat pH of 7 and after 7 d their growth was observed. The optimum temperature for maximum growth was determined by visual examination of the growth.

Biochemical Characterization

Urea hydrolysis

For this, isolates were inoculated into sterile urea agar slants and incubated at 30°C for 7 d and a change in color was observed (Betson., 1994; Collee *et al.,* 1996).

Catalase test

Catalase enzyme present in some microorganisms breaks down hydrogen peroxide to water and oxygen and this helps them in survival since hydrogen peroxide is lethal to cells. A modified version of the method described by Cappuccino and Sherman, (2002) was used in which isolates were grown on starch casein agar plates at 30°C for 7 d and thereafter a colony was picked with a sterile stick and placed on a sterile glass slide containing a drop of hydrogen peroxide. Production of bubbles was indicative of positive results hence the production of free oxygen (Collee *et al.,* 1996).

Starch Hydrolysis

The isolates were streaked on starch agar plates and incubated at 30°C for 7 d. After incubation, iodine solution was poured on the agar and examined for hydrolysis of starch by the production of clear zone around the microbial growth and representing a positive result. Starch in the presence of iodine imparts a blue-black color to the medium indicating the absence of starch-splitting enzymes and representing a negative result (Benson, 1994;Collee *et al*., 1996).

Citrate test

Simmons citrate agar slant were prepared and a single colony of the isolates were streaked on the surface of the slant culture. Then the slants were incubated at 30ºC for 7d. After 7d of incubation the citrate utilizing bacteria were produced a blue color in slant surface of the media as a result of alkaline end products. This indicates the tested bacteria were citrate positive (Collee *et al*., 1996)

Oxidase test

A small amount of *Actinomycete* isolates were obtained from 7 day old culture and put on sterile filter paper. Then 1-2 drops of tetramethyl phenylenediamine dihydrochloride was added to the culture and the reaction was observed. A positive reaction was indicated by a color change to dark blue or purple and a negative test will result in the absence of color (Betson*.,* 1994; Collee *et al.,* 1996)**.**

Statistical Analysis

The collected and recorded data was analyzed using SPSS (version 20.0)software. The different inhibition zone measurements in triplicate were compared by performing One-way ANOVA ranked with Duncan's multiple range tests with descriptive analysis type on different isolates against different test pathogens. All statistical results with $P<0.05$ were considered to be statistically significant.

RESULTS

Isolation of *Actinomycetes*

Twenty nine (29) isolates of *Actinomycetes* were isolated from 20 different soil samples of which 12(41.38%) from Mount Tabor, 11(37.93%) from Main campus, 4(13.79%) from Mount Alamura and 2(6.89%) from Monopol (Table 1). All the 29 isolates grown on *Actinomycetes* isolation agar showed morphology typical of *Actinomycetes*. The colonies were slow growing, aerobic, folded and with aerial and substrate mycelia of different colors.

Table 1: *Actinomycetes* isolates from soil samples collected from different sites of Hawassa city

Collection sites	No. of soil samples	No. of isolates	Codes
Mount Tabor	5	12	AB1-AB12
Main campus	5	11	AB13-AB23
Mount Alamura	5	4	AB24-AB27
Monopol	5	2	AB28-AB29

Antimicrobial activity screening of the isolated *Actinomycetes*

From the 29 isolated *Actinomycetes*, 19 (65.5%) showed antimicrobial activity against at least one of five test bacteria isolates. Sixteen (16) *Actinomycetes* isolates showed antimicrobial activity against *S. typhi*, thirteen (13) showed antimicrobial activity against *S. aureus*, nine (9) showed antimicrobial activity against *K. pneumonia*, seven (7) *Actinomycetes* isolates showed antimicrobial activity against *S. boydii* and two (2) showed activity against *E. coli* (Fig.1).

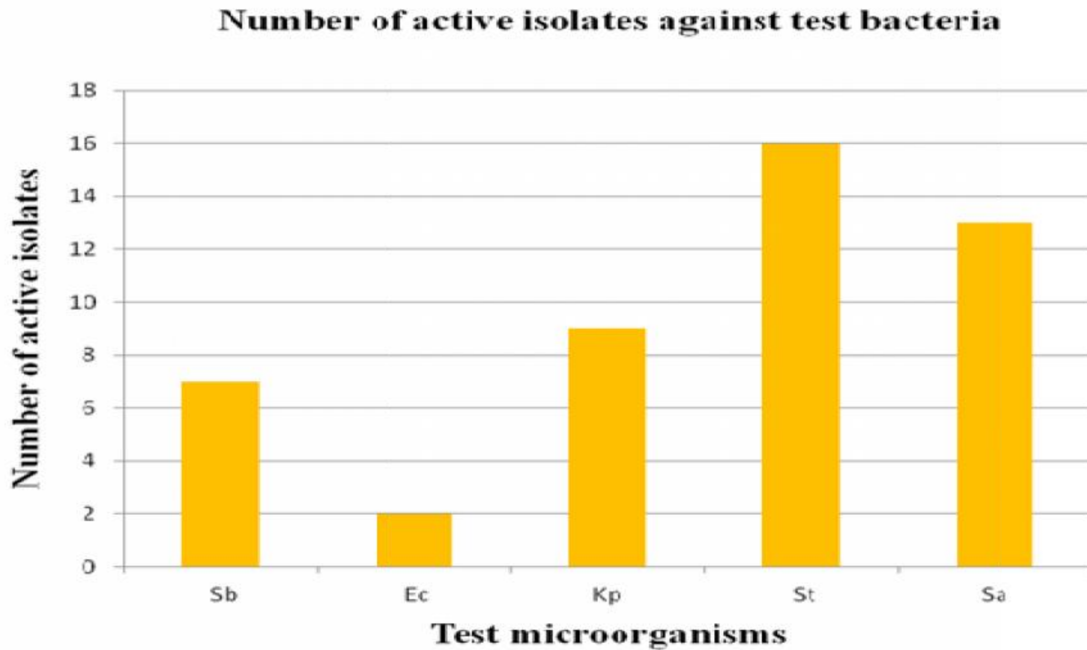

Figure 1: Number of active *Actinomycetes*that inhibited tested microorganisms

(**Sb-***Shigella boydii*, **Ec-***Escherichia coli*, **Kp-***Klebsiella pneumonia*, **St-***Salmonella typhi*, **Sa-** *Staphylococcus aureus*)

Antimicrobial Screening

Total number of isolates which showed positive result in antibacterial activity (at least against one test bacteria) was 19 (65.5%). *Salmonella typhi* was susceptible to all the isolates apart from isolates AB2, AB10 and AB14. On the other hand, *E. coli* was resistant to most of the isolates apart from isolates AB9 and AB25. *Shigella boydii* was also resistant to most of the isolates apart from isolate AB1, AB2, AB6, AB13, AB19, AB22 and AB25. Among the tested isolates AB1, AB4, AB5, AB6, AB9, AB13, AB22, AB25 and AB27 proved to be a broader antibiotic spectrum as the acted against most of the test isolates. Among broader antibiotic spectrum isolates AB13 and AB25 were proven to inhibit four of five test pathogens. Isolates AB16 and AB20 showed poor activity. They were only active for *Salmonella typhi*. Isolates AB2, AB3, AB7, AB10, AB14, AB19, AB24 and AB28 showed dual inhibition. Among active isolates tested against human pathogens, six (6) isolates were active against Gram negative bacteria and only

thirteen (13) isolates were active against both Gram positive and Gram negative bacteria, and no isolate was found active against only Gram positive bacteria (Table 2).

Table 2: Sensitivity of selected test microorganisms for *Actinomycetes* isolated from different sampling sites

Actinomycetes Isolates	Test microorganisms						Spectrum activity
	Sampling site	*E.coli*	*K.pneumonia*	*S.boydii*	*S.typhi*	*S.aureus*	
AB1*	Tabor	-	-	+	+	+	3
AB2	Tabor	-	-	+	-	+	2
AB3	Tabor	-	-	-	+	+	2
AB4*	Tabor	-	+	-	+	+	3
AB5*	Tabor	-	+	-	+	+	3
AB6*	Tabor	-	-	+	+	+	3
AB7	Tabor	-	+	-	+	-	2
AB8	Tabor	-	-	-	-	-	0
AB9*	Tabor	+	+	-	+	-	3
AB10	Tabor	-	+	-	-	+	2
AB11	Tabor	-	-	-	-	-	0
AB12	M.C	-	-	-	-	-	0
AB13*	M.C	-	+	+	+	+	4
AB14	M.C	-	+	-	-	+	2
AB15	M.C	-	-	-	-	-	0
AB16	M.C	-	-	-	+	-	1
AB17	M.C	-	-	-	-	-	0
AB18	M.C	-	-	-	-	-	0
AB19	M.C	-	-	+	+	-	2
AB20	M.C	-	-	-	+	-	1
AB21	M.C	-	-	-	-	-	0
AB22*	M.C	-	+	+	+	-	3
AB23	M.C	-	-	-	-	-	0
AB24	Alamura	-	-	-	+	-	1
AB25*	Alamura	+	-	+	+	+	4
AB26	Alamura	-	-	-	-	-	0
AB27*	Alamura	-	+	-	+	+	3
AB28	Monopol	-	-	-	+	+	2
AB29	Monopol	-	-	-	-	-	0

Legend: + = active against test organism; - = inactive against test organism, *= Show broad spectrum activity, M.C=Main campus

From a total of 29 isolates of *Actinomycetes* tested for antimicrobial activity against human pathogenic bacteria: *Escherichia coli*, *Klebsiella pneumonia*, *S. boydii, Salmonella typhi* and *Staphylococcus aureus*, 19 (65.5%) isolates showed antimicrobial activity against at least one test microorganism. All of the inhibition zones produced by isolates showed significant differences when compared with control ciprofloxacin tested against test organisms (P<0.05). The antimicrobial activity of all the isolates tested against *K. pneumonia* were statistically significant (P<0.05) from ciprofloxacin (25.16 mm) which was the control drug (Table 3). *Escherichia coli* was resistant to all of the isolates except isolates AB9 and AB25. The two isolates that showed antimicrobial activity against *E. coli* were

significant as compared with control drug ciprofloxacin (18.33 mm). When compared with control drug ciprofloxacin (18.33 mm), isolate AB25 showed good antimicrobial activity (15.8mm) as opposed to isolate AB9 (9.45mm). The zones of inhibition of active isolates against *S. boydii* were significant (P<0.05). Isolate AB6 showed good activity (21.90mm) when compared with control ciprofloxacin (26.25mm) as opposed to the rest which were active against *S. boydii*.

Table 3: Primary screening of antimicrobial activity (mm) of Actinomycetes isolates

'-': refers inactive, '-ve control ':filter paper disc soaked with sterile distilled water, '+ve' control: Ciprofloxacin

Isolates	Test microorganisms				
	Gram negative			Gram positive	
	E.coli	*K.pneumonia*	*S.boydii*	*S. typhi*	*S.aureus*
AB1	-	-	9.25±0.97	13.75±0.91	11.88±0.41
AB2	-	-	15.79±0.82	-	11.20±0.75
AB3	-	-	-	17.62±0.42	12.50±0.68
AB4	-	10.88±0.41	-	26.63±0.33	10.74±0.44
AB5	-	11.92±1.10	-	18.55±0.62	13.57±0.72
AB6	-	-	21.90±0.40	26.21±0.55	11.04±0.37
AB7	-	17.95±0.44	-	13.30±0.69	-
AB9	9.45±0.50	14.48±0.48	-	11.24±0.67	-
AB10	-	10.51±0.78	-	-	11.35±0.44
AB13	-	12.43±0.60	7.93±0.68	17.30±0.27	12.00±0.50
AB14	-	10.54±0.77	-	-	15.08±0.54
AB16	-	-	-	26.10±0.38	-
AB19	-	-	11.68±0.85	18.99±0.58	-
AB20	-	-	-	8.32±0.17	-
AB22	-	13.58±0.66	8.00±1.00	10.80±0.38	-
AB24	-	-	-	15.00±1.00	16.86±0.34
AB25	15.80±0.18	-	10.65±0.75	21.80±0.34	6.57±0.25
AB27	-	10.25±0.86	-	20.00±0.00	14.33±0.76
AB28	-	-	-	29.11±0.57	18.69±0.32
+ve control	18.33±0.57	25.16±0.76	26.25±0.66	30.33±0.57	25.00±1.00
-ve control	-	-	-	-	-

Salmonella typhi was sensitive to most of the *Actinomycete* isolates (84.2%). The highest zone of inhibition was also shown for isolate AB28 against *Salmonella typhi* (29.11mm). Inhibition zones produced by AB4, AB6, AB16 and AB28 against *Salmonella typhi* were 26.63mm, 26.21mm, 26.10mm and 29.11mm, respectively, which were strong active when compared with control ciprofloxacin (30.33mm) but all of them were statistically significant (P<0.05). *Salmonella typhi* showed resistant against isolates AB2, AB10 and AB14. Thirteen isolates (68.42%) exhibited antimicrobial activity against *Staphylococcus aureus* which was the highest next to *Salmonella typhi*. The isolate AB28 (18.69 mm) showed the highest inhibitory activity against *Staphylococcus aureus* when compared to others. Isolates that showed the

second, third and fourth antimicrobial activities against *Staphylococcus aureus* were AB24 (16.86mm), AB14 (15.08mm) and AB27 (14.33mm), respectively. All the active isolates tested against *Staphylococcus aureus* also showed statistically significant result (P<0.05) as shown in Table 3.

Among active isolates tested against pathogens, six (6) isolates were active against Gram negative bacteria only, thirteen (13) isolates were active against both Gram positive and Gram negative bacteria and none of the isolate was found active against Gram positive bacteria.

Among the 19 active isolates from soil, the highest number was from mount Tabor (47.37%) followed by Main campus (16.58%), Mount Alamura (15.79%) and Monopol site (5.26%). According to the spectrum of 19 active *Actinomycetes*, it was found that most *Actinomycetes* inhibited two tested microorganisms (8 isolates) followed by 7, 2, and 2 isolates that inhibited 3, 4 and 1 tested microorganisms, respectively (Table 4).

Table 4: Antimicrobial activity (mm) of selected isolates against Methicillin resistant *Staphylococcus aureus* (MRSA) in secondary screening

	Isolates									Control
	AB1	AB4	AB5	AB6	AB9	AB13	AB22	AB25	AB27	Cipro
MRSA	-	11±0	14±1	8±1	16±1	-	-	13±1	-	24±1

Cipro: ciprofloxacin, MRSA: methicillin resistant Staphylococcus aureus -: no inhibition zone

Secondary Screening

Based on primary screening, 9 isolates with wider spectrum activity were further tested against Methicillin resistant *Staphylococcus aureus* (MRSA) (clinical isolate) using modified disc diffusion method. Secondary screening of 9 isolates selected from primary screening revealed that 5 isolates showed inhibition against clinical isolate of Methicillin resistant *Staphylococcus aureus* (MRSA), with inhibition zone size above 8mm to 16mm diameter. Isolates AB1, AB13, AB22 and AB27 did not show any antimicrobial activity during secondary screening against MRSA. The antimicrobial activities of isolates were statistically significant (P<0.05) when compared to the standard antibiotics of ciprofloxacin. Among those five isolates, 4 of them were from mount Tabor site and 1 from Mount Alamura site. Three isolates (AB5, AB9 and AB25) showed maximum inhibition zone size of diameter against Methicillin resistant *Staphylococcus aureus* (MRSA) (clinical isolate). Two of them were isolated from Mount Tabor site and one was from Mount Alamura (Table 4).

Characterization of selected *Actinomycete* isolates

After taking the pH of all soil samples, it was found that almost all soil samples were neutral to alkaline except three samples from Monopol site which were acidic (less than pH value of 6). Based on secondary screening against Methicillin resistant *Staphylococcus aureus*

(MRSA) (clinical isolate), five (5) active broad spectrum *Actinomycete* isolates namely AB4, AB5, AB6, AB9, and AB25 were used in characterization.

Morphological Characterization

Macroscopic characteristics of the selected isolates were studied by growing the isolates on *Actinomycetes* Isolation Agar (AIA), Nutrient Agar and yeast extract malt agar. The isolates showed different growth patterns on each of the medium. The growth of the *Actinomycete* isolates were highest in *Actinomycetes* Isolation Agar (AIA), moderate growth was observed at yeast extract malt agar, and low growth was seen at Nutrient Agar comparatively.

The nature of colonies was found rough, smooth, chalky, and powdery and it was noted that colonies had different colors ranging from white, whitish, yellow, brown, and pink colonies on *Actinomycetes* Isolation Agar (AIA) plates. Some colonies were very hard to pick from agar surface, which is also a characteristic of *Actinomycetes*. The microscopic observations showed that all the isolates were Gram positive as they retained the primary color (crystal violet) hence appeared blue and this is a characteristic of *Actinomycetes* (Table 5).

Table 5: The morphological characteristics of the isolates on Actinomycetes isolation agar

Isolates	Appearance of colonies	Gram stain
AB4	White powdery	Gram positive
AB5	Brown rough	Gram positive
AB6	Pink	Gram positive
AB9	Yellow smooth	Gram positive
AB25	Whitish yellow	Gram positive

Physiological and Biochemical Characterization

In biochemical tests, all the isolates were able to hydrolyze both starch and urea. Citrate was positive for all the isolates, all the isolates showed positive result for catalase test and oxidase was also positive for all the isolates. Isolates AB4 was grown at a temperature range of 25-30°C, AB9 on a temperature range of 30-37°C while none of the isolates were able to grow at the temperature of 40°C. Optimum temperature for most of the isolates was found at 30°C (Table 6).

Table 6: Physiological and biochemical characteristics of selected isolates

	Characteristics of isolates				
Types of test	AB4	AB5	AB6	AB9	AB25
Starch	+	+	+	+	+
Citrate	+	+	+	+	+
Catalase	+	+	+	+	+
Urea	+	+	+	+	+
Oxidase	+	+	+	+	+
Opt. T^o	25-30°C	30°C	30°C	30-37°C	30°C

Opt. T^o: optimum temperature; +: positive; -: negative

DISCUSSION

Antibiotic resistance is one of the most pressing public health issues worldwide. Presently, antibiotic-resistant organisms are extensively emerging and causing great challenge for a number of infectious diseases and current clinical care. As a result, there has been growing interest in searching valuable antibiotics from soil *Actinomycetes* in diversified ecological niches (Abo-Shadi *et al*., 2010). *Actinomycetes* are the richest sources of bioactive compounds (Suthindhiran and Kannabiran, 2009). Almost 70% of all recognized antibiotics have been isolated from *Actinomycetes* of which 75% and 60% are used in medicine and agriculture respectively (Kumar *et al*., 2012). Isolation of *Actinomycetes* has always been facing difficulties while comparing with other bacteria and fungi (Williams and Cross, 1971). This may be due to their long incubation period. However, *Actinomycetes* isolation ratio has increased by pretreatment of the samples by air drying for a week (Oskay *et al*., 2004).

In this study, out of total 29 *Actinomycete* isolates, 19 (65.5%) showed antimicrobial activity against the test pathogens. Primary screening using the disc diffusion methods revealed 65.5% (n=19/29) of the isolates were effective inhibitors against the test pathogens. This finding is higher than the finding (26.7%) of the previous study done by Abebe*et al*. (2013) from soil samples of Gondar town, North West Ethiopia. However, this result is in agreement with the report of Atsede and Fassil (2018) that 60% of *Actinomycete* isolates showed antimicrobial activity against at least one test pathogen. Sawasdee *et al*. (2011) reported that 80% of the isolates showed antimicrobial activity against at least one test microorganism and this value was higher than the present findings. Khasabuli and Kibera (2014) also reported that all the isolates IS1-IS15 showed positive results against at least one tested pathogen.

Test bacteria showed varied responses to metabolites of *Actinomycete i.e.,* being susceptible to one isolate and resistant to the other isolate. *Salmonella typhi* was susceptible to most of the isolates other than AB2, AB10 and AB14. This was consistent with the report of Khasabuli and Kibera (2014). *Escherichia coli* were resistant to all of the isolates except from AB9 and AB25 isolates. *Shigella boydii* was susceptible to metabolites from isolates such as AB1, AB2, AB6, AB13, AB19, AB22 and AB25 while it was resistant to the other metabolites from the remaining isolates.

The results of this study showed that the inhibition zones were maximum against Gram negative bacteria when compared to Gram positive bacteria. This finding differs from the previous reports of Abebe *et al*. (2013), Gebreselema *et al*. (2013) and Atsede and Fassil (2018) who reported higher inhibitory effect in Gram positive pathogens than in Gram negative. Several other studies conducted elsewhere also showed Gram positive isolates were more susceptible to the antibiotics produced by *Actinomycetes* than Gram negative bacteria (Sawasdee *et al*., 2011; Sheik *et al*., 2017). However, a study done in Chennai, India reported higher inhibitory effect on Gram negative bacteria than Gram positive bacteria (Fatima *et al*., 2017) which is in agreement with the present study. Kamal *et al*. (2018) also reported highest inhibitory effect in Gram negative pathogens than Gram positive bacteria. The reason for higher antibacterial activity of

Actinomycetes towards Gram negative bacteria in comparison to Gram positive bacteria tested might be due to the nature of the cell wall of the Gram negative bacteria which is easier to break than those of the Gram positive bacteria. However, this hypothesis did not hold good with the findings of some researchers, who observed much higher inhibitory reaction against the Gram positive bacteria than the Gram negative bacteria (Basilio *et al*., 2003; Oskay *et al*., 2004; Sacramento *et al*., 2004).

On secondary screening using the disk diffusion methods against Methicillin resistant *Staphylococcus aureus* (MRSA) five out of nine *Actinomycete* isolates showed antimicrobial activity with different inhibition zones. The previous study indicated that, the inhibition zone of isolates against MRSA ranged from 0-15 mm (Yucel and Yemac, 2010). In this study, the inhibition zone of nine isolates against MRSA ranged from 0-16 mm which was found to be good when compared to Yucel and Yemac's results (Yucel and Yemac, 2010). However, the present result is less when compared to the activity reported by Abebe *et al*. (2013) who found zone of inhibition ranged from 0-20mm. Such differences in the results might be due the variation in the strains and biotypes of Actinomycetes from the different habitats. According to the present result, ciprofloxacin had 24±1 mm inhibition zone against MRSA, which had greater inhibition zone when compared to the isolates tested.

The results demonstrate that, the type of culture medium and incubation temperature has a significant effect on the production of antibiotics by the antibiotic producing organisms. The aerial mycelium, substrate mycelium growth and pigmentation showed distinct variation based on the culture media in which the isolates were grown. Among the three culture media used, the preferred medium in this experiment was *Actinomycete* Isolation Agar where the maximum numbers of colonies were isolated and this may be due to the inclusion of sufficient amount of nutrient in this media under 30°C. This is in agreement with the results of Atsede and Fassils (2018) who reported 30°C was optimum temperature for most of the potential *Actinomycetes* isolates. Kumar *et al.* (2012) also reported AIA as the best media for the isolation of *Actinomycetes*. All the potential isolates in this study have the ability to hydrolyze starch and urea. Oxidase, citrate and catalase tests were also positive for all potential isolates. Therefore, after observation of cultural, morphological, physiological and biochemical characteristics it was confirmed that these isolates obtained from soil of Hawassa belong to the species of the genus Streptomyces. Previous studies conducted by Kalyani *et al*. (2012); Sudha and Hemalatha (2015); Midhun and Girijasankar (2016); Ramendra *et al*. (2016); Sreejetha *et al*. (2016); Sujatha and Swethalatha (2016) showed that Streptomyces sp. being producers of useful bioactive metabolite have an antibacterial effect with a broad spectrum of activities. Another study showed that Streptomyces species produced about 7,600 compounds which have antimicrobial properties which are highest among *Actinomycetes* producing antibiotics in the soil (Das *et al*., 2010).

CONCLUSION & RECOMMENDATIONS

Antimicrobial resistance is a global problem which demands for novel antimicrobial structure against pathogenic microbes. Actinomycetes are famous for antibiotic production and continued

to be explored in hope of getting novel antibiotics. The isolates from soil of Hawassa city might be a promising candidate for discovering novel. *Actinomycetes* isolates recovered from mount Tabor samples account largest number of antimicrobial bioactive compounds. It is therefore suggested that a combination of several molecular analysis methods such as DNA re-association and PCR-based fingerprinting techniques may extremely help to provide broader information about the total genetic diversity of soil Actinomycetes community. Perhaps, such methods may lead to the improvement in isolating antibiotic producing strains of soil Actinomycetes obtained in this study.

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