ISOLATION AND CHARACTERIZATION OF ANTIBACTERIAL COMPOUNDS ISOLATED FROM ENDOPHYTIC ACTINOMYCETES (TG-16)

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Abstract

The research was concerned with isolation, purification and partial characterization of antibacterial compounds of isolated endophytic Actinomycetes TG-16 from the stem of *Tadehagi triquetrum* (L.)H.Ohashi. These experiments were conducted by solvent extraction, thin layer chromatography, silica gel column chromatography, UV and FT IR spectroscopic analyses. The plant sample was collected from Pathein University Campus. The fermented broth of isolated strain TG-16 was carried out by paper chromatography using with four solvents for the extraction of antibacterial metabolites. the Actinomycetes culture filtrate was studied by the different ratio of two solvents. According to this result, the active metabolite, 15 liter was extracted by using *n*-butanol to fermented broth (1:1v/v). The analysis of separation, purification, and partial characterization of isolated compounds indicated that compound A (aromatic derivatives, 15 mg, colorless crystal) and compound B (steroid derivatives, 14 mg, white amorphous solid). Further evaluation of minimum inhibitory concentration of compounds showed the value of 3.125 µg/mL in compound B while compound A did not exhibit antibacterial activity against *E. coli*. This study revealed the presence of bioactive compounds in isolated strain TG-16, which may be a promising resource for the discovery of bioactive metabolites against multidrug resistance of *E. coli.*

Keywords: paper chromatography, silica gel column chromatography, minimum inhibitory cocentration

Introduction

Endophytic Actinomycetes can produce novel antibiotic compounds and other secondary metabolites and this has resulted in the isolation of metabolites from Actinomycetes. Only a few resent studies have heighted the bioactive importance of endophytic actinomycetes, including biocontrol of fungal plant pathogens, production of antimalarial and antimicrobial agents, production of anticancer compounds and production of enzymes (EI- Shatoury *et al*., 2006). Dilution methods are used to determine the minimum inhibitory concentration (MIC) of antimicrobial agents and are reference methods for antimicrobial susceptibility testing (Owuama, 2017). Among microbes producing bioactive secondary metabolites, actinomycetes produced highest number of bioactive metabolites (Prashith Kekuda, 2016). The aim and objectives of this study was to be extraction, isolation and purification of two compounds from endophytic streptomyces, TG-16 and to observe partial characterization and minimum inhibitory concentractions (MIC) of these compounds.

Materials and Methods

Paper Chromatography (Tomita, 1988)

The extraction of antibacterial metabolites of selected strain TG-16 was done by using paper chromatography. The filter paper and four solvents; 20% NH_4Cl, *n*-butanol saturated with water, *n*-butanol-acetic acid -water (3:1:1) and ethyl acetate saturated with water, were used for preliminary characterization of antibacterial metabolites. The obtained fermented broth samples (100 µL) were applied on the paper and allowed to dry. The papers were chromatographed in each

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solvent. Each paper was placed on assay agar plate. Then, bioautography was done to check the antibacterial activity of each.

Preparation of *n*-BuOH extract fromTG-16 fermented broth (Natara *et al*., 2010

The actively growing culture of TG-16 was inoculated with broth of ISP-2 medium and incubated at room temperature for 5 days. It was them centrifuged at 100 rpm for 15 min. After incubation period and the supernatant was passed through a filter paper to get a spore free filtrate. For extraction of antibacterial compounds containing in fermented broth, the filtrate was then treated with an equal volume ratio of *n*-BuOH to fermented broth. Then, the mixture was shaken in a separating funnel. The organic layer was separated and collected. The solvent was removed in vaccum using a rotary vaccum evaporator.

Thin layer chromatographic analysis (Verma *et al*., 2014)

Thin layer chromatography (TLC) was performed on *n*-BuOH crude extract from the culture broth of the isolated strain TG-16. For this, bioactive culture extracts were applied to the TLC plate (Merck silica gel plate 60 GF 254, 0.2 mm) using chloroform : methanol (30:1 v/v), chloroform : ethyl acetate (5:1, 1:1 v/v), chloroform only, hexane : chloroform (1:1 v/v), hexane : ethyl acetate (10:1,5:1, 3:1, 1:1 v/v) and hexane : *n*-BuOH (60:1, 50:1, 40:1, 30:1,20:1, 10:1,5:1, 3:1, 2:1, 1:1 v/v). The spots on the plates were developed and observed under UV light at 254 nm and 365 nm and also observed by color developing chemical reagents such as I_2 vapour, 5 %H_2SO_4 and 5% $FeCl_3$ and their R_f values were recorded.

Isolation of organic metabolites by silica gel column chromatography (Simon and Gray, 1998)

According to thin layer chromatographic analysis, the *n*-butanol extract residue of selected Actinomycetes metabolite was developed to isolate the active compound by silica gel column chromatography with *n*-hexane:*n*-BuOH as eluting solvent. Silica gel (60-120 mesh) (ca.50g) was dissolved in *n*-hexane:*n*-BuOH 60:1 v/v and the column was packed by wet method. Gradient elution was performed successively with increasing polarity (*n*-hexane:*n*-BuOH, 60:1,50:1, 40:1, 30:1, 20:1 10:1, 5:1, 2:1,and 1:1 v/v). Fractions of 2 mL each were collected individually and the compounds present were checked on TLC. The fractions with same spots were mixed and the solvent was evaporated on rotary evaporator.

Characterization of Isolated Antibacterial Compounds

In an attempted to characterize the isolated antibacterial compounds, the following tests were performed;

Determination of solubility of isolated compounds

A 0.5 mg each of isolated compounds was subjected to 0.5 ml of polar and non-polar solvents such as H_2O, MeOH, EtOH, EtOAc, $CHCl_3$, *n*-BuOH and Hexane in order to known their solubility.

Determination of some chemical properties of isolated compounds

The isolated compounds were subjected to TLC analysis and then treated with some coloured reagents such as Liebermann-Burchard reagent, 5%H_2SO_4, I_2 vapour, 5% $FeCl_3$ and 5 % Anisalehyde sulphuric acid and noted their behavior on TLC.

Study on UV-visible spectroscopy

For the identification of isolated compounds, ultra violet absorption spectra were also recorded and examined. A Shimadzu UV-18000 UV-visible spectrophotometer at Customer Support & Laboratory (AMTT).

Study on FT IR spectrometry

The FT IR spectra of the isolated compounds A and B were recorded by using Spectrum II (Perkin elmer) spectrophotometer at Chemistry Department, Pathein University.

Antimicrobial activity of isolated compounds

Isolated compounds A and B were checked for their activity against *E.coli* by agar well diffusion method. The isolated compounds A and B (100 µg each) were separately dissolved in 1 mL of methanol and their antibacterial activity were performed by agar well diffusion method.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration (MIC) was determined by tow fold serial dilution method (Andrew, 2001). The using concentrations were ranging from 50µg/mL to 0.195µg/mL with control solution. The minimum inhibitory concentration of compound A and B were determined by streak culture method and agar well diffusion method. The selected test organism was *Escherichia coli.* After incubation for 24 hours, checked the MIC values of isolated compounds.

Results

Paper Chromatography

In this study, according to R_f values, *n*-butanol was the most extractable solvent for the antibacterial metabolites (R_f : 0.79) followed by ethyl acetate solvent (R_f : 0.76), ethyl acetate-acetic acid-water 3:1:1 (R_f : 0.74) and finally NH_4Cl the lowest R_f :0.21). The chromatography bioautographic assay was shown in Figure 1

1. 20% NH_4Cl
2. *n*-butanol saturated with water
3. ethyl acetate- acetic acid-water (3:1:1v/v)
4. ethyl acetate saturated with water

Figure 1 Paper chromatography bioautographic assay

Comparison of antibacterial activity of TG-16 extracted with different ratios of *n*-BuOH and EtOAc to fermented broth against *E. coli*

In this study, the *n*-BuOH esxtracts obtained by *n*- BuOH to (fermented broth) FB in the ratios of (1:1, 2:1, 3:1 v/v) showed antibacterial activety with inhibition zone diameter of 27.01 mm, 26.02 mm and 25.00 mm, respectively. Similarly, EtOAc extracts obtained by EtOAc to FB in their ratio of 1:1, 2:1, 3:1 v/v showed antibacterial activity against *E.coli* with inhibition zone diameter 22.47 mm, 20.80 mm, 20.72 mm, respectively. According to the results, the *n*-BuOH extract (1:1v/v) of TG-16 displayed the highest antibacterial activity against *E.coli*. with

inhibition zone diameter of 27.01 mm. Therefore, antibacterial metabolites were extracted with equal volume of *n*-BuOH to fermented broth.

Table 1 Comparison of Antibacterial Activity Aginst *E.coli* of TG- 16 Extracted with Different Ratios of Solvent to Fermented Broth

Different ratio of solvent to fermented broth (v/v)	Inhibition zone diameter (mm)	
	***n*-BuOH extract**	**EtOAc extract**
1:1	27.01	22.47
2:1	26.02	20.80
3:1	25.00	20.72

Agar well size = 8mm

Extraction of antibacterial metabolite

15 liters of selected strain TG-16 were fermented in suitable synthetic fermentation medium (120 h, 20 % size of inoculums, at room temperature, pH 8, 6day fermentation period, under shaking culture) and extracted with equal ratio of *n*-butanol to fermented broth (1:1 v/v) to yield 4.0g was obtained from the culture filtrate.

Thin layer chromatographic analysis

According to the thin layer chromatography (TLC) was performed on *n*-BuOH crude extract by employing various solvent systems, the extract showed well- separated spots on TLC by using *n*-hexane : *n*-BuOH solvent system under UV 365nm and some color reagent tests. So, the solvent system (*n*-hexane: *n*-BuOH) was selected to isolate pure compounds by silica gel column chromatography.

Isolation of organic metabolites from *n*-BuOH extract of fermented broth of TG-16 by silica gel column chromatography

In this study, successive fractions obtained were combined on the basic of their behavior on TLC. Finally, fifteen main fractions (F1 to F15) were collected. Antibacterial activity of each fraction was examined in bioassays to determine the fraction containing the active compound by agar well diffusion method. Fractions F-1-2, F-4 and F-15were found to be inactive against *E.coli*, F-5, F-6 and F-9 were significantly showed antibacterial activity against *E.coli* while remaining fractions have a few effect on it. From the resulting fractions, compounds A and B were obtained from the respective fractions F-6 and F-9 and the remaining fractions were Found as mixture (Figure 3).

Figure 2 The antibacterial activity of collected fractions F-1 toF-15 from *n*-BuOH extract

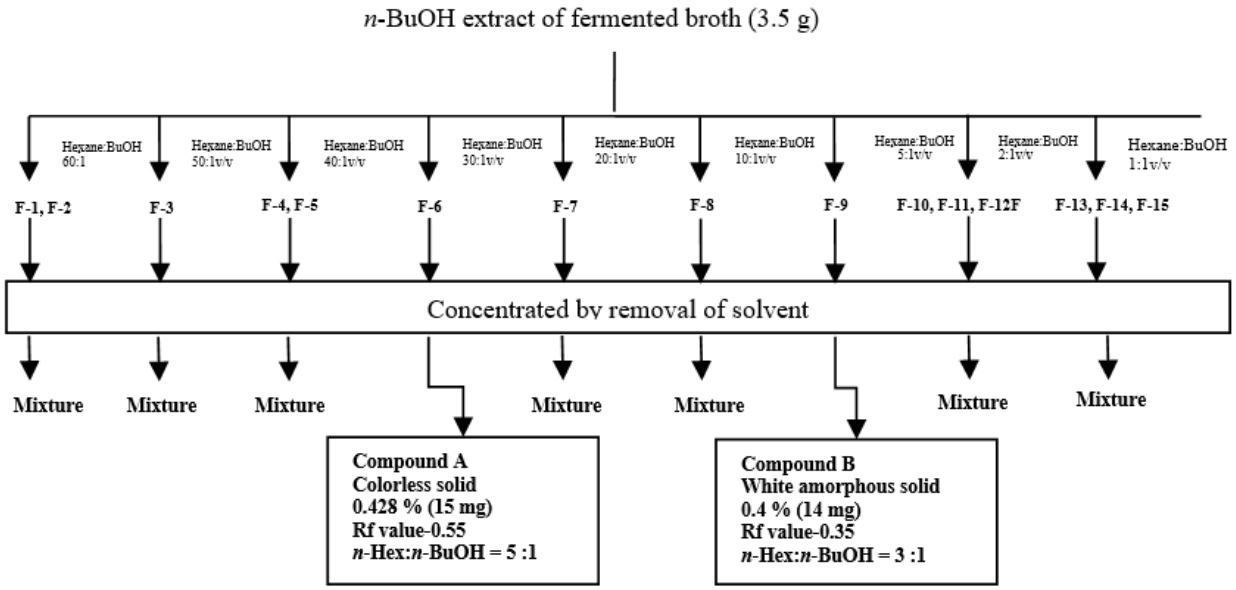


Figure 3 Isolation of organic metabolites from n-BuOH crude extract, culture broth of isolated strain TG-16 by column chromatography with *n*-Hexane : *n*-BuOH solvent system

Compound A
R_f value : 0.55
Solvent system : *n*-Hexane : *n*BuOH (5:1 v/v)
UV 365 nm : Fluorescence

Compound B
R_f value :0.35
Solvent system : *n*-Hexane:*n*-BuOH (3:1 v/v)
Spraying agent : 5 % Anisaldehy sulphuric acid

Figure 4 Isolated compound A and B and their thin layer chromatogram

Characterization and classification of isolated compounds

The isolated compounds A and B were characterized by physical properties such as R_f value, solubilities and by some chemical reagent tests and modern spectroscopic techniques such as UV and FT IR.

Compound A

From the above result, fraction F-6 was evaporated and washed with *n*-Hexane and then with *n*-Hexane : *n*-BuOH (60:1 v/v) and then purified by recrystallization from MeOH, to give 0.428 % (15 mg) of compound A as colorless solid. It was soluble in EtOH, Hexane, BuOH, $CHCl_3$, MeOH but insoluble in H_2O.The R_f value of compound A was found to be 0.55, solvent system of *n*-Hexane:*n*-BuOH (5:1v/v) and it fluorescence under UV 365 nm, it gave yellow spot on TLC with iodine vapor but not responds in spraying with 5% H_2SO_4, anisaldehyde sulphuric acid, Libermann Burchard and $FeCl_3$ (Table 2). In the UV spectrum, maximum absorption bands at λ_{max} of 204 nm and 283 nm indicating the present of conjugated double bond system (Figure 5 and Table 3).

The IR spectrum of the compound included a diagnostic peak at 3339 cm^{-1} which is indicative of the -OH group. However, the peak at 3068 cm^{-1} was assigned to aromatic -CH stretching. The peak appearing at 2921cm^{-1}, 2855 cm^{-1} was assigned to C-H stretching vibration of $-CH_3$– and $-CH_2$. The peak absorption band appearing at 1713 cm^{-1} was assigned to C-O stretching vibration of ketone, the absorption bands at 1640 cm^{-1} was assigned to C=C stretching vibration of olefinic group, the absorption bands at 1534cm^{-1} and 1513cm^{-1} were due to C= C stretching

vibration of aromatic ring. The absorption bands at 1458cm^{-1} and 1376 cm^{-1} were due to bending -CH_3 and -CH_2. The peak appearing at 1040cm^{-1}was assigned to C-O group of alcohol and the peak at 910cm^{-1}wasdue to C-H bending vibration of olefinic group. The absorption bands at 749cm^{-1}and 719cm^{-1} were due to C-H bending vibration of monosubstituted aromatic compound (Figure 6 and Table 4). According to the results of UV, FT IR and some chemical coloration tests, compound A may be classified as an aromatic derivative.

Table 2 Some Chemical Properties of Isolated Compound A and B

Experiment	Observation (compounds)	
	A	B
UV (MeOH /nm)	Active	Inactive
5% H_2SO_4	Colorless	Pink
I_2 vapor	Yellow	Yellow
5% $FeCl_3$	Colorless	Colorless
Anisaldehyde Sulphuric acid	Colorless	Violet
Libermann Burchard	Colorless	Greenish blue

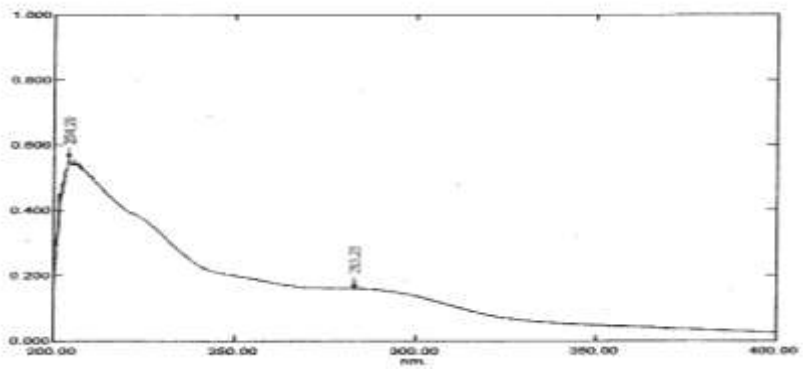


Figure 5 Ultraviolet absorbance of the compound A

Table 3 UV Spectral Data of Isolated Compound A

Solvent used	Observed (nm)	Remark
MeOH	204,283	Conjugated double bond

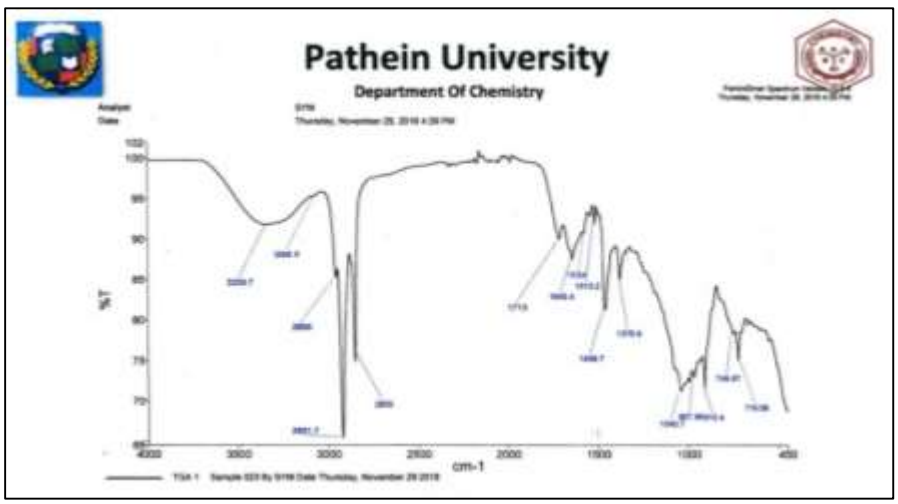


Figure 6 FTIR spectrum of the compound A

Table 4 FTIR Spectral Data of Isolated Compound A

Wave number (cm^{-1})	Literature value*(cm^{-1})	Band assignment
3339	3400-3200	υ_{O-H} of alcohol
3068	3100-3000	$\upsilon_{=C-H}$ of aromatic ring
2921, 2855	3000-2850	υ_{C-H} (asym & sym) of CH_3 and CH_2
1713	1730-1715	$\upsilon_{C=O}$ of ketone
1640,1534,1513	1600-1400	$\upsilon_{C=C}$ of aromatic ring
1458,1376	1470-1450	δ_{C-H} of (asym & sym) of $CH_3$and CH_2
	1390-1370	
1040	1300-1000	υ_{C-O} of alcohol
910	900-680	δ_{C-H} of olifinic group
749, 719	900- 675	$\delta_{(oop)C-C}$ of aromatic ring

*Silverstein *et al*., 2005

Compound B

Compound B isolated from fraction F-9, it washed with *n*-Hexane and then with *n*-Hexane : *n*-BuOH (30:1 v/v) and then purified by recrystallization from MeOH, to give 0.4 % (14 mg) of compound B as white amorphous solid. It was soluble in *n*-Hexane, EtOH, *n*-BuOH, $CHCl_3$, MeOH but insoluble in H_2O and it was inactive under UV (365 & 254 nm).The R_f value of compound B was 0.35 in the solvent system of *n*-Hex to *n*-BuOH (3:1v/v). It gave a yellow with iodine vapor, pink coloration while spraying 5% H_2SO_4 followed by heating and developed into violet coloring while spraying anisaldehyde sulphuric acid followed by heating (Table 2). It gave greenish blue coloration with Liebermann Bruchard reagent. The IR spectrum of the compound included a diagnostic peak at 3315 cm^{-1} which is indicative of the -OH group of alcohol. The peak appearing at 2921cm^{-1}, and 2873 cm^{-1}were assigned to asymmetric and symmetric -CH stretching vibration of -CH_3–and–-CH_2 of groups. The peak at 1640 cm^{-1} was assigned to C=C stretching vibration of olefinic group, and the absorption bands at 1413cm^{-1}, 1376 cm^{-1}were due to -CH bending vibration of -CH_3 and -CH_2 groups, the C=O stretching vibration of alcohol was observed in 1007cm^{-1} (Figure 7 and Table 5). From the results of FTIR and some chemical coloration tests, compound B may be classified as a steroid derivative.

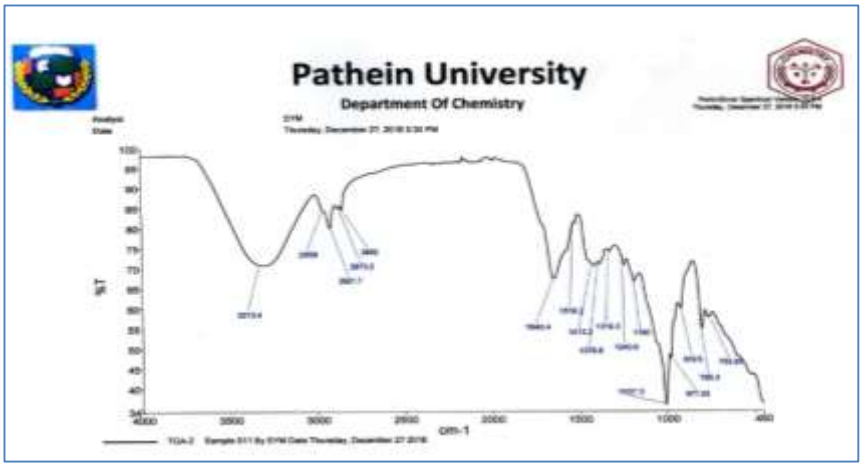


Figure 7 FTIR spectrum of the compound B

Table 5 FT IR Spectral Data of Isolated Compound B

Wave number (cm^{-1})	Literature value* (cm^{-1})	Band assignment
3315	3400-3200	υ_{O-H} of alcohol
2921,2873	3100-2850	υ_{C-H} asym & sym of CH_3 and CH_2
1640	1680-1600	$\upsilon_{C=C}$ of olefinic group
1413-1376	1465 -1375	δ_{C-H} of CH_3 and CH_2
1007	1300-1000	υ_{C-O} of alcohol

*Silverstein *et al*., 2005

Antibacterial activity of isolated compounds

Bioassay for determination of isolated compounds were undertaken by agar well diffusion method. From the results, isolated compound A exhibited antibacterial activity against *E.coli* with inhibition zone diameter :16.82 mm and compound B showed antibacterial activity with inhibition zone diameter: 22.98 mm. The photograph illustrating the inhibition zones provited by the isolated compounds against *E.coli* are presented in Figure 8.

Compound A **Compound B**

Figure 8 Antibacterial activity of isolated compound A and B

Concentration (MIC) of isolated compounds

In the study of Minimum Inhibitory Concentration (MIC) of isolated compounds, it was observed that MIC value of compound B was 3.125 µg/mL but isolated compound A did not exhibit antibacterial activity against *E.coli* in this experimental concntration range from 50 µg/mL to 0.195 µg/mL.

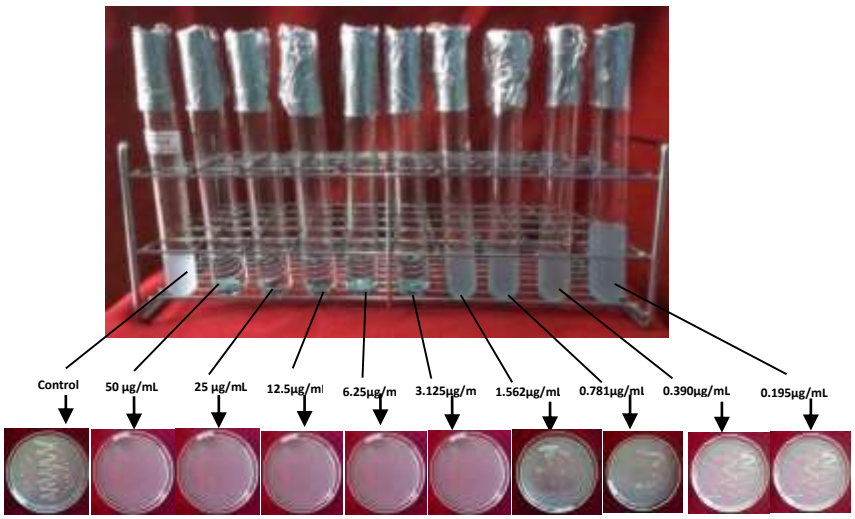
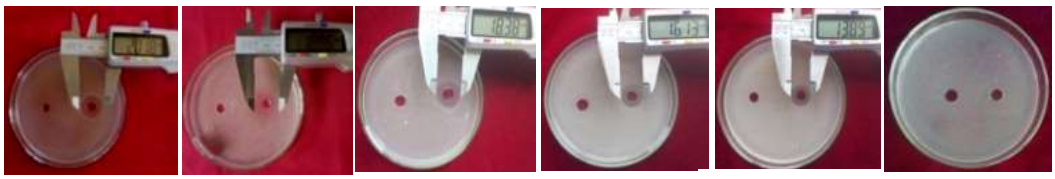


Figure 9 Minimum inhibitory concentration of secondary metabolites from compound B against *E. coli* (Streak culture method)



50 µg/mL 25 µg/mL 12.5µg/mL 6.25µg/mL 3.125 µg/mI 1.562µg/mL

Figure 10 Minimum inhibitory concentration of secondary metabolites from compound B against *E. coli* (agar well diffusion method)

Discussion and Conclusion

Investigation on the extraction of antibacterial metabolites of TG-16, the active metabolites were the best extracted with *n*-BuOH to fermented broth, 1:1 v/v. The present result was described by Sekiguchi, *et al*., 2007. In the study of separation of active compounds by thin layer chromatography, the crude extract showed well separated spots on TLC by using *n*-Hexane: *n*-BuOH solvent system. According to the result of silica gel column chromatographic analysis, all the collected fractions was analyzed by TLC and the fractions with similar behavior on TLC were combined, which ultimately resulted in major fifteen fractions. In the study of bioassay for measuring the antibacterial activities of collected fractions 1-15, fraction F_1, F_2, F_4, and F_{15} were found to be inactive on *E.coli* and F_5, F_6 and F_9 were to have the highest activity.

From column chromatographic separation of fermented broth TG-16, compound A and B were obtained from the respective fractions F6 and F9 and remaining fractions were found as mixture. In the UV spectrum of compound A, it was seen that a maximum absorption bands at λ_{max} 204 nm and 283 nm were due to conjugated double bond system. From the results of FT IR spectral data of compounds A, the absorption band at 3339.7, which indicates OH- groups, peaks at 1640 cm^{-1} (C=C groups), it shows the presence of aromatic ring. In addition, absorption band at 2921cm^{-1}, it exhibits the presence of CH- groups and peak at 1040cm^{-1} was assigns to C-O groups of alcohol. The UV and FT IR spectra data were agreement with Oskay, 2011 Augustine *et al*., 2005 and Dhanasekaran *et al*., 2008. From the results of UV, FT IR and some chemical coloration tests, compound A may be characterized as an aromatic derivative.

The compound B was inactive under (365 nm and 254 nm). The result of FT IR spectral data of compound B indicated that bands at 3420 cm^{-1} indicated the presence of OH groups of alcohol, the peak appearing 1640 cm^{-1} was assigned to C=C of olefinic group. These characters were aggrement with Jenifer *et al*., 2013 from the results of some chemical reagent tests on TLC and FT IR spectral data, compound B may be classified as a steroid derivative. The results were expressed as the minimum inhibitory concentration of the compound B against *E coli* were 3.125 µg/mL. Isolated compound A did not exhibit antibacterial activity against *E.coli* in this experimental concentration range of 50µg/mL to 0.195µg/mL. Antimicrobial resistance in *E.coli* has been reported worldwide and increasing rates of resistance among *E.coli* is a growing concern in both developed and developing contries (Kibret and Abera, 2011). This research indicated that the selected strainTG-16 produced antibacterial compounds, which are found effective against multi drug resistant bacteria, *E.coli.*

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References

Andrews, J.M., (2001). **Determination of minimum inhibitory concentrations**. J. Antimicrobe. Chemother. 48:5-16

Augustine, S.K., Bhavsar S.P. and Kapadris, B.P., (2005). **Production of a growth dependent metabolite active against dermatophytes by *Streptomyces* rochei AK39,** Ind. J Med.Res.121, 164-170

Dhanasekaran, D., N.Thajuddin, A. Panneerselvam, (2008). **An Antifungal Compoun:4' Phenyl-1-Napthyl- Phenyl Acetamide from *Streptomyces* sp. DPTB16.** Facta Universitatis, Medicine and Biology, Vol.15, No.1, pp.7-12

EI- Shatoury, S., H. Abdulla, O. EI- Karaaly, W. EI- Kazzaz and A. Dewedar, (2006). **Actinomycetes from selected Medicinal Plants in the World Heritage Site of Saint Katherine, Egypt In J. of Botany: Volume 2(3): 307-312**

Jenifer J.A., M.B.S. Donio, V.Thangaviji, S. Velmurugan, S. Albindhas, and T.Citarasu, (2013). **Halo-alkaliphilic Actinomycetes from solar salt works in Inmdia: diversity and antimicrobial activity**. Blue Biotechnol J. 2(1): 137-151.

Kibret M., and B. Abera, (2011). **Antimicrobial susceptibility patterns of *E.coli* from clinical Sources in northeast Ethiopia**. Afr. Health Sci. (Suppl 1): 540-545.

Natara, K.. S.S. Rabiya, and R. Satish, (2010). **Screening for antibacterial compound in broth extracts of Aspergillus nigra MTCC2208.** Drug invention Today. 2(8). 385-386

Oskay M., (2011). **Isolation and Purification of Two Metabolites (KGG32-A and KGG32-B) from a Soil Bacterium, *Streptomyces* sp., KGG32.** International Journal of Agriculture and Biology. Issn Print: 1560–8530

Owuama, C.I., (2017). **Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentraction (MBC) using a novel dilution tube method.** Academic Journal Vol. 1. Pp.977-980.

Prashith Kekuda, (2016). **Isolation, Characterization and Antimicrobial Potential of Endophytic Actinomycetes.** J. Curr. Microbiol. App. Sci. 5(7): 100-116

Sekiguchi, M., N. Shiraish, K. Kobinata, T. Kudo, I. Yamaguchi, (2007). **RS-22A B and C: new macrolid antibiotics from *Streptomyces violaceusniger I*. Taxonomy, fermentation, Isolation and biological activities.** J.Antibiot., 48(4), pp.289-292

Silverstein, R.M., F.X. Webster, D.T. Kiemle, (2005). **Spectroscopic Identification of Organic Compounds**, New Tork, 7th ED., John witely & Sons Inc.

Simon, G. and A.I Gray, (1998). **Isolation by planar Chromatography.** 209-246

Tomita, F., (1988). **Laboratory Method, Hokkadio University Japan**. Riddhi , Valli, S., O.S. Aysha, P.Nirmala, VK.Pinoth, A.Reena, 2012.**Antimicrobial potential of Actinomycetes species isolated from marine environment.** Asian Pac.J. Trop.Biomed.2(6):469-147

Verma, A., N.Singh, and A. Kumart, (2014). **Antagonistic evaluation of bioactive metabolite from endophytic fungi, *Aspergilllus flavipes* KF671231**. J Mycol, 2014;5 Article ID 371218.