ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

In-vitro studies on biocontrol of *Alternaria solani* and *Botrytis fabae.*

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ABSTRACT

Thirty-two isolates of actinomycetes were isolated from tomato field infected with early blight disease at Giza Governorate, Egypt. The isolates were screened for the production of antifungal compounds against *Alternaria solani* and *Botrytis fabae* using Agar plug diffusion method. Three *Streptomyces* strains were the most potent for active metabolites production on starch nitrate medium. They were identified to be *Streptomyces recifensis*, *Streptomyces gelaticus* and *Streptomyces nodosus*. *Streptomyces nodosus* was the most potent antimicrobial producer strain. Highly active metabolites were extracted from *Streptomyces nodosus*. The active metabolite has been identified according to physicochemical characteristics. Elemental analysis (C, H, N, O & S), spectroscopic characteristics (UV absorbance, HPLC, FT-IR, ^{1}H NMR and Mass spectrum), melting point, solubility and color have been investigated. These analyses indicate a suggested empirical formula of $C_{47}H_{73}NO_{17}$. The purified antifungal agent shows high level of identity to the known antibiotic amphotericin B. The minimum inhibition concentrations of the purified antifungal agent were also determined.

Keywords: Antifungal, Actinomycetes, *Streptomyces nodosus*, *Alternaria solani*, *Botrytis fabae*.

https://doi.org/10.33887/rjpbcs/2020.11.2.12

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ISSN: 0975-8585

INTRODUCTION

One of the world's most popular cultivated crops is tomatoes (*Solanum lycopersicum* L., syn. Lycopersicon esculentum Mill.) [1]. Tomato production in Egypt has been ranked fifth with global tomato production, Recently, Tomato production in Egypt increased to 8.3 million tons [2].

The early blight disease of tomato is caused by the *Alternaria solani* fungus which is one of the most common foliar diseases of tomatoes that damage leaves, stems and fruits causing severe damage of the aerial part and reduction of fruit size and number, resulting in severe losses of yield up to 80 % [3].

Faba bean infected by numerous diseases. One of the most serious diseases is chocolate spot disease caused by *Botrytis fabae*. It cause large yield loss due to the damage of leaves , reduced amount of seeds and reduced crop production [4].

Plant diseases control is based mainly on the use of chemical pesticides. Several efficient pesticides have been suggested but, due to cost issues, exposure risks, fungicide residuals and other health and environmental threats, theses pesticides are not considered to be long-term solution. In an effort to alter this condition, certain alternative control methods have been introduced. So, Mycologists focus on the development of biocontrol techniques for managing plant diseases that are environmentally secure, durable and efficient [5].

Actinomycetes can synthesis various secondary biologically active metabolites such as antibiotics, pesticides, herbicides and enzymes inhibitors. Antibiotics are important therapeutically compounds, and about 70 % of known antibiotics were isolated from actinomycetes. *Streptomyces* strains can produce large numbers of bioactive metabolites more than other actinomycetes strains [6]. Actinomycetes bioactive metabolites play an important application role in agriculture due to their role in phytopathogens control [7]. Amphotericin B is a polyene macrolide antibiotic Figures 1, of formula $C_{47}H_{73}NO_{17}$ produced in submerged cultures of certain *Streptomyces nodosus* [8]. The importance of this antibiotic lies in its broad antifungal spectrum activity, fungicidal activity, high activity against resistant strains of fungi [9].

This study aimed to isolate and identify the causal agent of early blight disease and chocolate spot disease and evaluate the efficacy of isolated actinomycetes species in controlling of causal agent of these diseases in vitro.

MATERIALS AND METHODS

Isolation and identification of early blight and chocolate spot pathogens:

During growing season of the year 2016/2017, tomato plants with a typical early blight symptoms were collected from fields in Giza Governorate (30°10'53.8"N 31°02'47.3"E) and infected faba bean plants showing chocolate spot symptoms were collected from field at Menoufia Governorate (30°40'38.9"N 30°55'36.0"E). Isolation took place in the same collection day. The infected leaves were sterilized for 2-3 minutes with sodium hypochlorite (0.5 percent) then washed several times by sterilized distilled water. These sterilized parts have been dried between sterilized two filter papers and transferred on to PDA plates and incubated for 5-7 days at 25 °C [10]. Pure cultures were kept on PDA slants and stored at 5 °C in a refrigerator. The disease symptoms appeared on tomato and faba bean plants showed in Figures 2 and 3.

Purification and identification of the fungal isolates:

The developed fungal colonies were purified by using a technique of hyphal tip as reported by [11,12] which based on their morphological characteristics as described by [13–15]. The identification was confirmed by Assiut University Mycological Center (AUMC), Assiut, Egypt.

Biological control:

Soil sampling and isolation of actinomycetes:

Tomato fields in Giza Governorate and faba bean fields at Menoufia Governorate were used for soil samples collection. Twenty-five samples of soil were collected. Each sample consists of three samples taken from different locations in the same site. Actinomycetes isolates were isolated from soil samples using a dilute plating method and starch nitrate agar as growth medium. It was supplemented by nystatin (50 µg / ml) then incubated for a seven days at 30 °C. All isolates have been purified by streaking on starch agar nitrate medium. Actinomycetes colonies were selected and stored on slants of starch nitrate agar medium which stored in 5 °C [16–18].

Antimicrobial activity of actinomycetes isolates against *A. solani* and *B. fabae* on PDA plates by using agar plug diffusion method:

Actinomycetes isolates were grown on starch nitrate agar medium and incubated for 7 days at 30°C, 5 mm discs were prepared from the developed colonies and transferred to the surface of PDA medium seeded with *A. solani* and *B. fabae* spores. Plates were then incubated for 7 days at 25 °C. Diameters of inhibition zones were examined daily for 7 days [19].

The antimicrobial activity of the three actinomycetes isolates showed the highest bioactivity against *A. solani* and *B. fabae* were tested by using agar disc method. Diameters of inhibition zones were examined daily for 7 days.

Identification of the most potent isolates:

Three isolates showed the highest bioactivity were identified as *Streptomyces* spp. in accordance to the standard method and media mentioned in the International *Streptomyces* Project (ISP) introduced by [20–24], the key mentioned by [25]; [26] and [27] was also used. The spore surface was examined using Scanned Electron Microscope at Applied Center for Entomonematodes (ACE), Faculty of Agriculture, Cairo University, Cairo, Egypt.

Production and purfication of the active compounds:

Flasks containing 50 ml of starch nitrate broth medium were inoculated with 5 mm disc of *S. nodosus* and incubated in shaking incubator at 180 rpm for 7 days at 30 ºC. Then, filtrates were separated from biomass and collected together. The filtrate was extracted twice with n-butanol (1:1) by shaking vigorously in separation funnel for 15 min and kept stationary for another 15 min to separate the organic phase from the aqueous phase. Then, the yellowish crude extract was obtained at the end of evaporation process using rotary evaporator [28].

TLC of crude extract and bio-autography:

The crude yellowish powder was dissolved in a small volume of n-butanol and was further purified by thin layer chromatography using different solvent systems. The best solvent system was n-butanol: ethanol: Acetic acid: H_2O (50:15:15:20) (v/v). The activity of metabolite(s) was checked using bio-autography method in which the developed chromatogram strip was placed on the surface of *A. solani* and *B. fabae* seeded in PDA plates. The plates were then, incubated at 25°C for 72 h. the active spots was determined by the presence of inhibition zones around the strips [29].

Column chromatography and bio-autography of pure fraction:

Column chromatography was carried out for the separation of active metabolite(s) of *S. nodosus* crude extract. The crude extract is adsorbed on to stationary phase of silica gel which was packed in a glass column 2 cm in diameter, resulting in 31 cm bed height. Mobile phase was n-butanol: ethanol: Acetic acid: H_2O (50:15:15:20) (v/v). Eleven fractions were collected and checked with TLC for purity. The fractions with same R_f were mixed together and the solvent was evaporated on rotary evaporator. The activity of eleven fractions

and combined fractions were checked using the bio-autography method in which the developed chromatogram strip was placed on the surface of PDA plates seeded with *A. solani* and *B. fabae*. The plates were then incubated at 25 °C for 72 h. The presence of inhibition zones around the active spot was determined.

Identification of the produced active metabolite:

Identification of the active metabolite was carried out by determining its R_f values and spectroscopic analysis with UV spectrophotometer (Unicam 1300), UV-visible absorption spectra were recorded with spectrophotometer in the UV-visible region (200-800 nm), HPLC assay using by using YL-9100 HPLC apparatus [30], FTIR spectral analysis using FT-IR spectrophotometer 4100 Jasco apparatus, ^{1}H NMR (400 MHz) spectrum recorded by Bruker Biospin AVANCEII 300 spectrometer, Mass spectra using GCMS-QP2010 SE apparatus, testing solubility and melting point with digital Stuart SMP3 electric melting point apparatus. The elemental analysis of C, H, N, O, and S was carried out using Vario EL III CHNOS Elemental Analyzer apparatus. Standard amphotericin B obtained from (Sigma–Aldrich).

The minimum inhibitory concentration assay:

Minimum inhibitory concentrations (MICs) of bioactive metabolite were performed using agar dilution technique. *A. solani* and *B. fabae* were seeded in onto PDA medium containing different concentrations of active compound [31].

Statistical analysis:

The obtained data were subjected to analysis of variance and means were compared by Tukey's test ($P \leq 0.05$) to determinate statistical significance. All experiments were performed three times.

RESULTS AND DISCUSSION

Isolation and identification of early blight and chocolate spot pathogens:

As showed in Figure 4; isolates were isolated and identified as *Alternaria solani* and *Botrytis fabae* according to their morphological characteristics of conidia and mycelial appearance and the identification was confirmed by Assiut University Mycological Center (AUMC), Assiut, Egypt.

Isolation of actinomycetes:

Thirty two actinomycetes isolates were isolated. These isolates belong to different groups according to their colors; grey (40%), white (19%), pale yellow (15 %), green (13 %) and pale red (13 %).

Antimicrobial activity of actinomycetes isolates against *A. solani* and *B. fabae* on PDA plates by using agar disc method:

The results obtained upon using the cut disc method which represented in Table (1) indicate that among the thirty two actinomycetal isolates, only sixteen isolates could antagonize *A. solani* and *B. fabae*. The antagonistic action varies according to the organism as indicated by the difference in diameter of inhibition zones. Isolates number 1, 5 and 15 were considered as the most potent antagonistic organisms. They produce the highest inhibition zones (19.4 - 28.6 mm).

From the results above, isolates number 1, 5 and 15 could be considered of the most promising producer of active metabolites. So, their antimicrobial activity against *A. solani* and *B. fabae* was tested by using agar disc method for conformation. The results obtained showed in Table (2).

As a result of the experiment, isolate number 15 was selected for further investigations throughout the subsequent experiments.This results are agreed with Cuppels (2013) and El Hussein (2014) who confirmed the abilities of *Streptomyces* isolates in inhibiting the mycelial growth of *A. solani* and they are significant also in reducing the disease severity of early blight when compared with control in tomato [32,33] and Mahmoud

(2004) who found that *Streptomyces exfoliates* was effective in controlling *Botrytis fabae* which causes chocolate spot disease in faba bean [34].

Identification of experimental isolates:

From the results above, isolates number 1, 5 and 15 could be considered of the most promising producer of antibiotics on the starch nitrate agar medium. According to morphological and biochemical studies showed in Table (3) and Figures 5, 6 and 7, the isolates were identified as *Streptomyces recifensis* (isolate 1), *Streptomyces gelaticus* (isolate 5) and *Streptomyces nodosus* (isolate 15). *S. nodosus* is found to be the most potent antimicrobial producer.

TLC of crude extract and bio-autography:

Separation of active metabolites using different solvent systems is represented in Table (4). Two spots were detected on TLC plate R_f (A) is 0.68 and R_f (B) is 0.56 by using n-butanol: ethanol: Acetic acid: H_2O (50:15:15:20). The result of bio-autography showed that out of two spots detected on TLC plate. Only one spot with R_f 0.56 showed antifungal activity against *A. solani* and *B. fabae*.

Column chromatography and bio-autography of pure fraction:

Based on TLC profile, Fractions 1–6 detected at R_f (0.56) and fractions 7–11 detected at R_f (0.68). The antifungal activities of the 11 fractions were tested by bio-autography assay against *A. solani* and *B. faba.* Fractions 1–6 were highly active and produced inhibition zones when investigated on *A. solani* and *B. fabae* Table (5).

Fractions 1–6 and 7–11 were then combined, respectively, into two fractions (F1–F2), each one giving one spot in TLC with R_f (0.56) and R_f (0.68) respectively. The antifungal activities of the two fractions were tested by bio-autography assay against *A. solani* and *B. faba,*. F1 fraction was highly active and produced inhibition zones when investigated on *A. solani* and *B. fabae* Table (6).

Identification of the Produced Antibiotic:

The active metabolites fraction has been investigated using UV spectrophotometer at range (280-500 nm). The active metabolite showed three specific UV absorption peaks at 408, 383 and 364 nm Figure 8; where the maximum peak was at 408 nm. By using standard amphotericin B obtained from (Sigma–Aldrich), the active metabolite and standard amphotericin B gave the same R_f (0.56) after using TLC and both showed three absorption UV peaks at 408, 383 and 364 nm.

From HPLC assay, active metabolite shows peak at retention time 9.023 min which is more or less as the same showed by standard amphotericin B with retention time 9.017 min as showed in Figures 9 and 10.

FTIR Spectra of standard Amb recorded presence of hydroxyl group (OH) and hydroxyl group (OH) of the acidic carboxylic function and amino group (NH) which stretching absorption peak at 3362 cm^{-1}, aliphatic hydrocarbon group (CH) showed bands around 3009-2877 cm^{-1}, carbonyl acidic group (COOH) showed absorption at 1690 cm^{-1} and (C=C) functional group which showed stretching absorption at 1631-1553 cm^{-1} as showed in Figure 11.

FTIR Spectra of active metabolite recorded presence of hydroxyl group (OH) and hydroxyl group (OH) of the acidic carboxylic function and amino group (NH) which stretching absorption peak at 3400 cm^{-1}, aliphatic hydrocarbon group (CH) showed bands around 3012-2934 cm^{-1}, carbonyl acidic group (COOH) showed absorption at 1711 cm^{-1} and (C=C) functional group which showed stretching absorption at 1636-1563 cm^{-1} as showed in Figure 12.

^1H NMR (400 MHz) spectrum of the active metabolite in DMSO has peaks in the region 0.9-1.2 ppm which probably indicates methyl (CH_3) protons Figure 13.

ISSN: 0975-8585

The mass spectrum of the active metabolite is shown in Figure 14. The molecular weight of the metabolite is 924.

Elementary analysis (%) of the active metabolite shows that: C=56.70%, H=7.72% N=1.87%, O=33.7l% and S= 0.00%.These analyses indicate a suggested empirical formula of $C_{47}H_{73}NO_{17}$.

The antibiotic was obtained as yellow powder which melted at 170 ºC. It was soluble in Dimethylsulfoxide (DMSO), slightly soluble in methanol and methyl alcohol; insoluble in water, benzene and toluene. So, from chemical characterization of active metabolite, it is expected to be related to amphotericin B according to properties described in [35–39].

The Minimum Inhibitory Concentration Assay:

Antifungal activity of active metabolite against *A. solani* and *B. fabae* fungi and corresponding MIC values are indicated in Table (7). The active compound showed antifungal activity against the two tested fungi where it showed MIC value against *A. solani* (12.5 µg.ml^{-1}) and against *B. fabae* (3.125 µg.ml^{-1}). This result is agreed with Ellis (2002) who reported that amphotericin B is a polyene macrolide antibiotic produced by *S. nodosus* and it has broad spectrum of action in treating fungal diseases [40]. It alters the permeability of cell membrane by binding with ergosterol on fungal cell membrane which causes ion leakage of intracellular sodium and potassium, thus killing fungal cells. Also, Tevyashova (2013) reported that hydroxyl groups exhibit antifungal activity against numerous pathogenic fungi, Li (2008) reported that amine group characterizing with antifungal activities [41,42]. Meanwhile, Hadi and Irawan (2008) reported that carboxylic acids exhibit greater antifungal activity against various pathogenic fungi [43].

Table 1: Potentiality of actinomycetes isolates against *A. solani* and *B. fabae*

Isolate No.	Inhibition zone (mm)	
	A. solani	*B. fabae*
1	21.8±0.4 b	19.4±0.2 c
5	22.5±0.3 b	21.5±0.3 b
7	2.8±0.3 g	2.6±0.2 m
8	1.8±0.2 h	4.1±0.2 j
9	3.8±0.3 f	3.7±0.2 l
12	5±0.2 e	11.7±0.1 d
15	26.2±0.3 a	28.6±0.2 a
18	3±0.1 g	0.9±0.1 n
20	6.1±0.3 d	3.4±0.2 l
22	3.2±0.2 f g	6.4±0.3 h
23	0.9±0.1 i	5.7±0.2 i
24	5.4±0.3 de	8.4±0.3 g
26	6.2±0.4 d	10.2±0.3 e
27	7.1±0.3 c	9.4±0.2 f
30	3.2±0.2 f g	3.4±0.4 l
32	1.6±0.3 hi	6.2±0.4 h

Means and Standard deviation followed by the same letters per column are not significantly different according to Tukey's test (P ≤ 0.05).

Table 2: Potentiality of actinomycetes isolates against *A. solani* and *B. fabae*

Isolate No.	Inhibition zone (mm)	
	A. solani	*B. fabae*
1	21.3±0.8 c	19.4±0.2 c
5	22.6±0.15 b	21.4±0.2 b
15	26.5±0.20 a	28.6±0.1 a

Means and Standard deviation followed by the same letters per column are not significantly different according to Tukey's test (P ≤ 0.05).

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Table 3: Cultural, morphological and physiological characteristics of the most potent isolates

characteristics			isolate no. 1	isolate no. 5	isolate no. 15
Culture and morphological	Growth of vegetative mycelium		+++	+++	+++
	Aerial mycelium		grey	gray	gray
	Substrate mycelium		brown	brown	brown
	Soluble Pigment		-ve	-ve	-ve
	Shape of spore chain		spiral	hock	spiral
	Spore surface		smooth	smooth	smooth
physiology	Melanin formation		-ve	-ve	-ve
	Streptomycin sensitivity		sensitive	sensitive	sensitive
	Growth on Czapek's agar media		Slight(+)	Slight(+)	Slight(+)
	Carbon utilization	No carbon (control)	-	-	-
		D-Glucose	+++	++	++
		D-Xylose	++	++	++
		L-Arabinose	-	-	-
		D-Fructose	++	++	++
		D-Galatose	+++	+++	+++
		Raffinose	+++	-	-
		D-Mannitol	++	+++	++
		Sucrose	+++	-	-

+++: Excellent growth, ++: moderate growth, +: weak growth, -: no growth

Table 4: Solvent Systems for TLC

Solvent system	R_f (A)	R_f (B)
n-butanol: ethanol: Acetic acid: H_2O (50:15:15:20)	0.68	0.56
n-butanol: Acetic acid: H_2O (3:1:1)	0.66	0.51
Methanol: Acetone: Acetic acid (8:1:1)	0.61	0.46
Chloroform: CH_3OH: 20% NaOH (2:2:1)	0.53	0.41
n-butanol	0.38	0.21

Table 5: Diameter of inhibition zones of the fractions (1-11)

Fraction	Inhibition zone (mm)	
	A. solani	*B. fabae*
1	26.12	27.13
2	27.23	27.56
3	27.91	28.52
4	28.19	29.32
5	26.81	28.43
6	25.54	26.95
7-11	00.00	00.00

ISSN: 0975-8585

Table 6: Diameter of inhibition zones of the fractions (F1&F2)

Fraction	Inhibition zone (mm)	
	A. solani	*B. fabae*
F1	28.72	30.41
F2	00.00	00.00

Table 7: Antimicrobial activity of active metabolite against *A. solani* and *B. fabae*

Concentration ($\mu g.mL^{-1}$)	Average linear growth (mm)	
	A. solani	*B. fabae*
0.0	90.00	76.00
1.5	61.4	12.5
3.125	45.5	0.00
6 .25	28.7	0.00
12.5	0.00	0.00

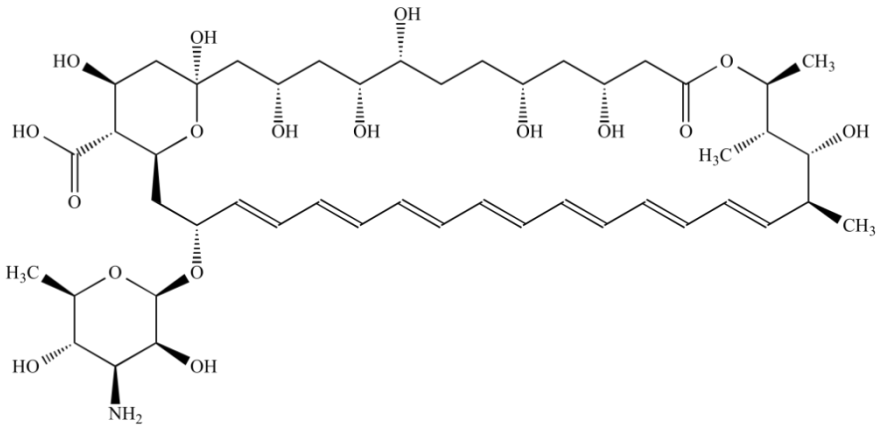

Fig. 1: chemical structure of Amphotericin B

Fig. 2: Infected tomato plant showing early blight symptoms

ISSN: 0975-8585

Fig. 3: Infected faba bean plant showing chocolate spot symptoms

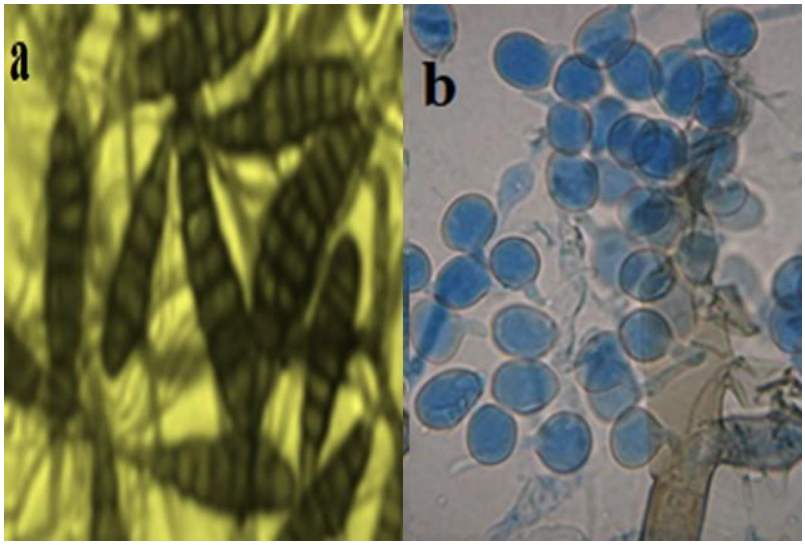

Fig. 4: a) conidia of *A. solani* b) conidia of *B. fabae*

ISSN: 0975-8585

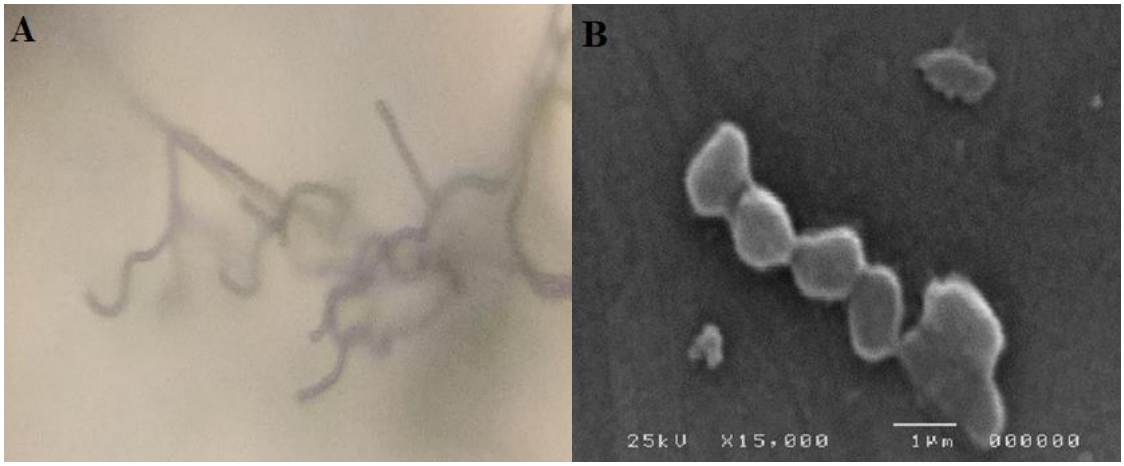

Fig. 5: *Streptomyces recifensis* (1)
A. Microphotograph of spore chain
B. Scanned Electron Microscope of spore surface

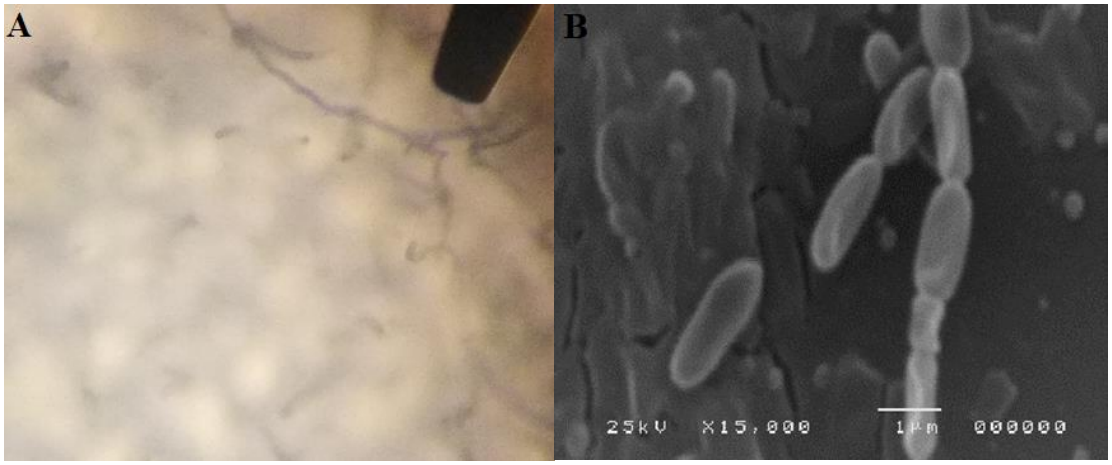

Fig. 6: *Streptomyces gelaticus* (5)
A. Microphotograph of spore chain
B. Scanned Electron Microscope of spore surface

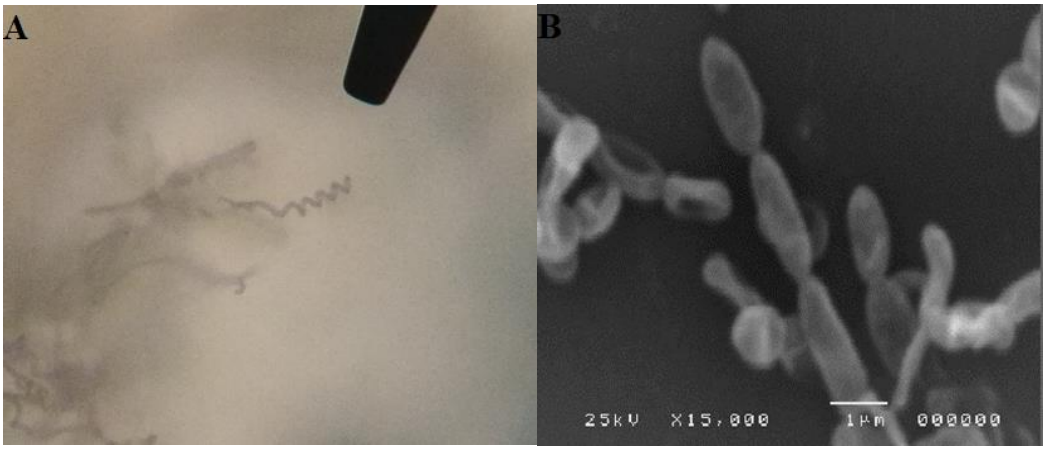

Fig. 7: *Streptomyces nodosus* (15)
A. Microphotograph of spore chain
B. Scanned Electron Microscope of spore surface

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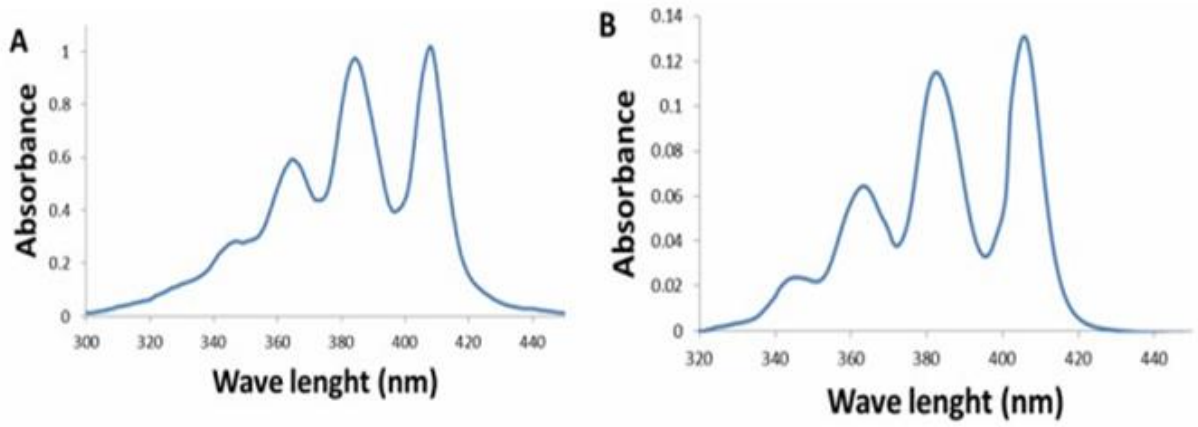

Fig. 8: A) UV spectra of standard AmB B) UV spectra of active metabolites

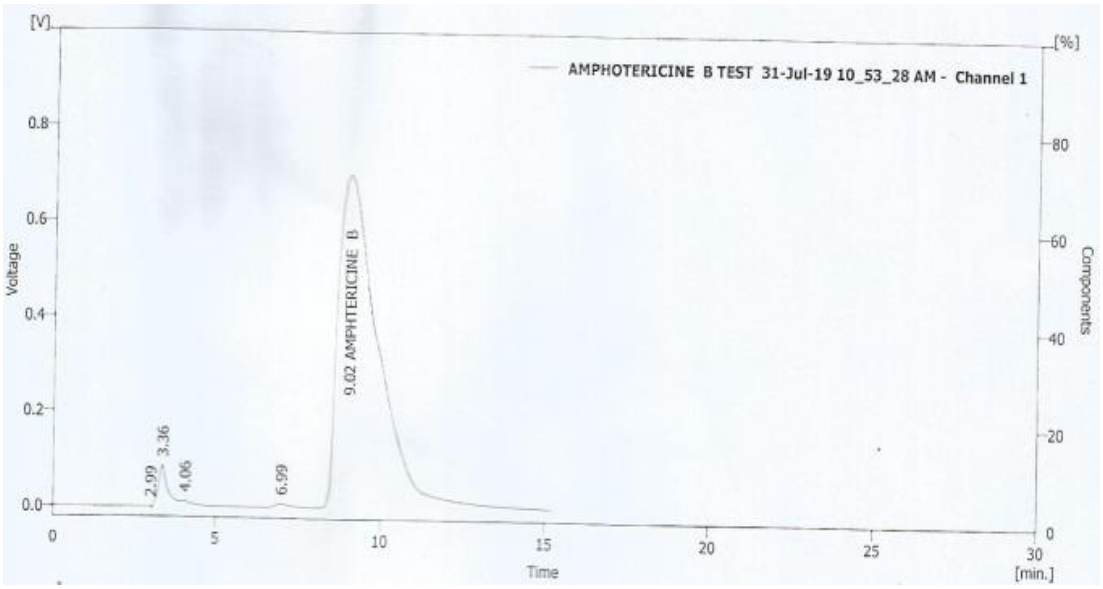

Fig. 9: HPLC for the active metabolite

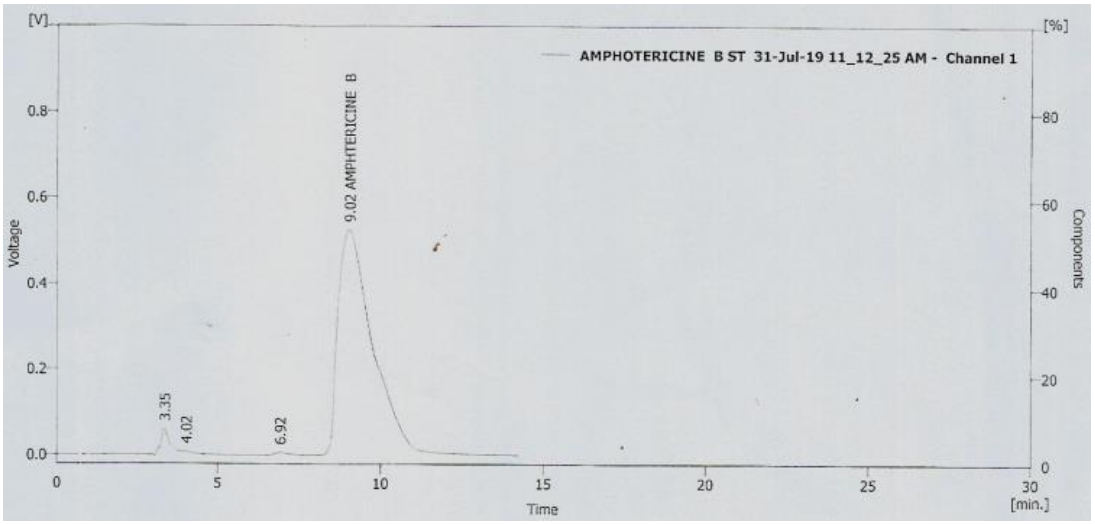

Fig. 10: HPLC for the active metabolite standard AmB

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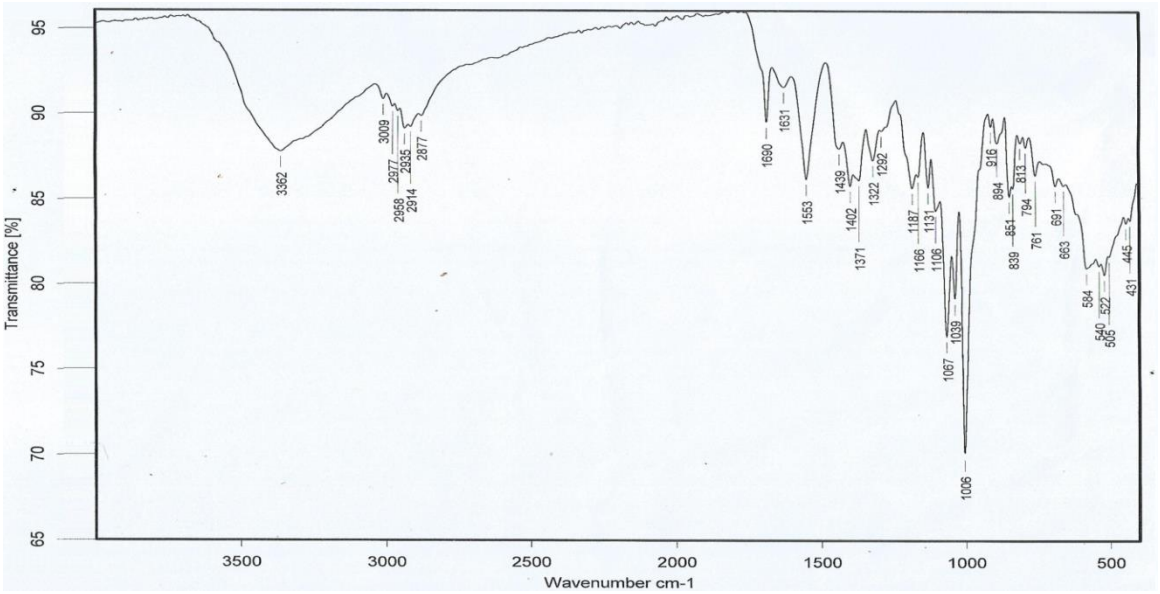

Fig. 11: FTIR Spectra of standard Amb

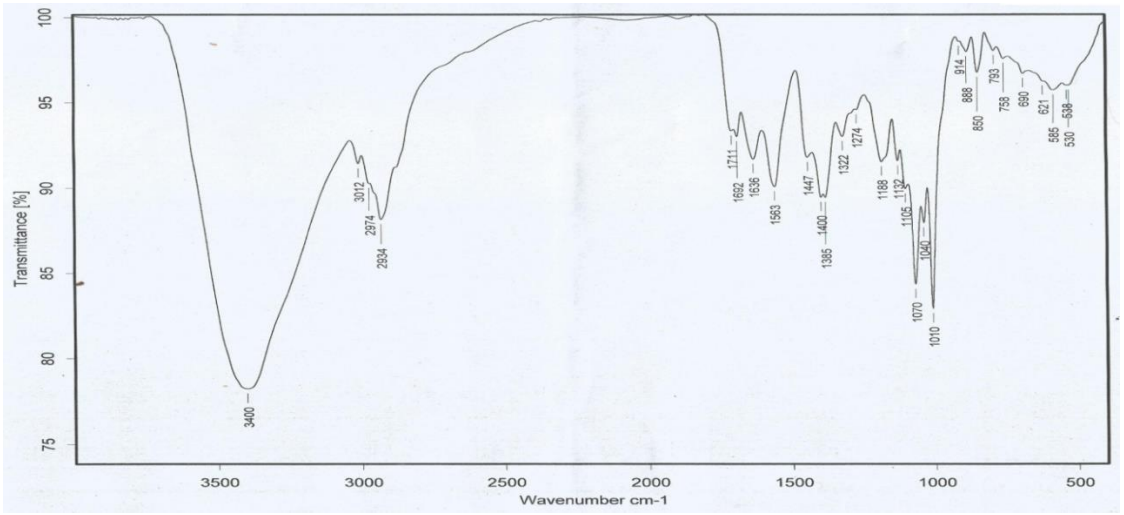

Fig. 12: FTIR Spectra of the active metabolite

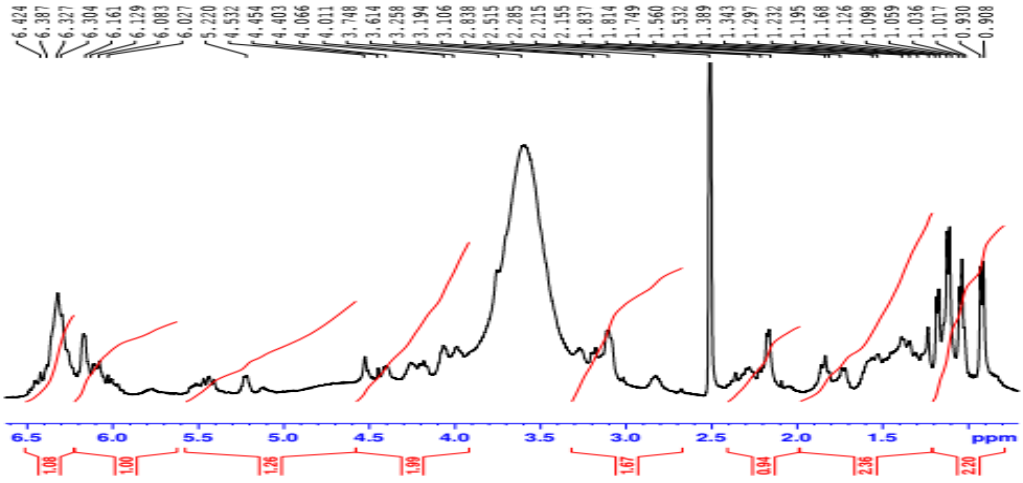

Fig. 13: ^{1}H NMR (400 MHz) spectrum of the active metabolite

ISSN: 0975-8585

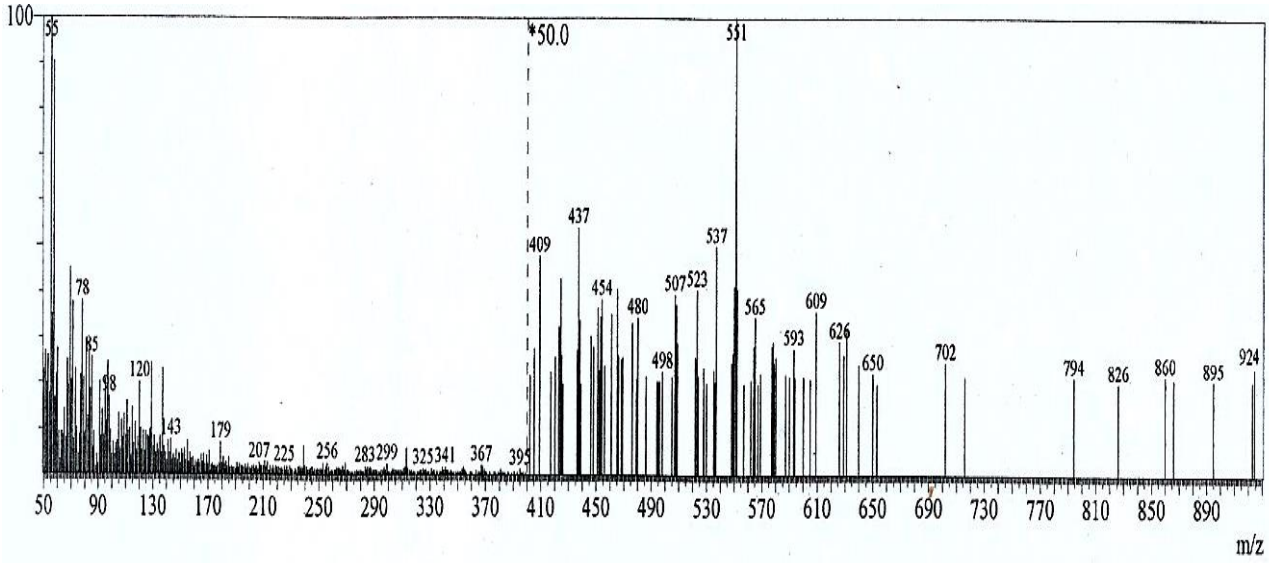

Fig. 14: The mass spectra of the active metabolite

CONCLUSION

Actinomycetes are important producers of potent metabolic that can be used as antibiotics and novel drugs. The present study focusing on obtaining isolates of actinomycetes which are capable of producing an antimicrobial agent. An interesting scope for further research would be to improve the antimicrobial agent production by *S. nodosus* against *A. solani* and *B. fabae*. The amphotericin B ‘‘polyene’’ antibiotic produced by *S. nodosus* (isolate 15) demonstrated inhibitory effects against pathogenic fungi.

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