

A new antifungal compound from *Streptomyces exfoliatus*El-Sabbagh S.M.¹, Emara H.A.², Metwally A.M.³, Saba H.A.⁴¹ Botany Department, Faculty of Science, Menoufia University, Menoufia, Egypt.² Microbiology Department, Soil, Water and Environment Research Institute, Agriculture Research Center, Giza, Egypt.³ Botany Department, Faculty of Science, Tanta University, Gharbya, Egyptsabhaahmed63@hotmail.com

Abstract: This study investigated the biological control of *Streptomyces exfoliatus* against some fungi (*Aspergillus flavus*, *A. niger*, *Fusarium oxysporum* and *Candida albicans*). The optimum conditions for the best growth and antifungal activity of *S. exfoliatus* were on starch nitrate media after 8 days of incubation at 30 °C, pH 7.0 in the presence of starch and potassium nitrate as carbon and nitrogen sources respectively. The active metabolite was extracted using a mixture of chloroform: methanol (9: 1, v/v). The separation of the active ingredient and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The identification of antifungal compound was made by determining the melting point, by means of elemental analysis and spectral analysis such as UV, IR and proton NMR, mass spectra. Based on the spectral characteristics, the antifungal compound was identified as diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate. It showed antifungal activity against *Aspergillus flavus*, *A. niger*, *Fusarium oxysporum* and *Candida albicans* with minimal inhibitory concentrations ranging between 0.25-16 µg/ml.

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1. Introduction:

Fungal phytopathogens cause serious problems worldwide in agriculture and food industry by destroying crops and economically important plants in the field and during storage (Pohanka, 2006). In addition, many fungal species produce mycotoxins, which are harmful to humans and livestock.

There is a growing interest in the use of secondary metabolites, such as toxins, proteins, hormones, vitamins, amino acids and antibiotics from microorganisms, particularly from actinomycetes, for the control of plant pathogens as these are readily degradable, highly specific and less toxic to nature (Dombou et al., 2001).

Many species of actinomycetes, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several plant pathogenic fungi (Xiao et al., 2002; Joo, 2005; Errakhi et al., 2007, Li et al., 2010). The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds (Ouhdouch et al., 2001; Getha and Vikineswary, 2002; Fguira et al., 2005; Taechowisan et al., 2005).

Niphithricins A, B, elaiophylin and nigericin produced by *Streptomyces violaceuniger* TU 905 were biologically active against Gram-positive bacteria and fungi. The mode of action is attributed to an alteration of the membrane permeability (Fiedler et al., 1981). 2-methyl-heptyl isonicotinate isolated

from the culture filtrate of *Streptomyces* sp. 201 exhibited marked antimicrobial activity against *Bacillus subtilis*, *Shigella* sp., *Klebsiella* sp., *E. coli*, *Proteus mirabilis*, and the pathogenic fungi, *Fusarium moniliforme*, *F. semitectum*, *F. oxysporum*, *F. solani* and *Rhizoctonia solani* (Bordoloi., et al., 2001). A non-polyene antifungal antibiotic, A121, produced by an unidentified strain of *Streptomyces* species was active against a number of filamentous fungi including some plant and human pathogens (Singh and Samanta, 1992).

(Leptomycins A and B) produced by a strain of *Streptomyces* exhibited strong inhibitory activity against *Schizosaccharomyces* and *Mucor* (Hamamoto et al., 1983). Thraustomycin, was an antifungal nucleoside antibiotic produced from *Streptomyces exfoliates* (Kneifel et al., 1974) and AZ-SH514 (4' phenyl-1-naphthyl-phenyl acetamide) was an antifungal produced by *Streptomyces olivaceiscleroticus*, (Atta., 2009). The antifungal compounds Bafilomycin B1 and C1 are produced by *Streptomyces halstedii* (Frändberg et al., 2000).

Rhizovit R produced from *Streptomyces rimosus* is used in the control of a wide range of fungi such as *Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani*, *Alternaria brassicola*, and *Botrytis* sp. Marten et al. (2001). Also *S. rimosus* showed a high antagonistic activity against *Fusarium solani*, *F. oxysporium* f sp. cucumarinum, *Verticillium dahliae*, *R. solani*, *Fulvia fulva*, *Botrytis*

cinearia, *A. alternat*, *Sclerotinia sclerotiorum* and *Bipolaris maydis* (Liu *et al.*, 2004).

Streptomyces sp. strain 5406 has been used in China for the last 35 years to protect cotton crops against soilborne pathogens (Valois *et al.*, 1996). Blasticidin-S and Kasugamycine from *S. griseochromogenes* and *S. kasugaensis* were used in the control of rice blast pathogen, *Piricularia oryzae* (Agrios, 1988). Also, (Prabavathy *et al.*, 2006) discovered two antifungal aliphatic compounds, SPM5C-1 and SPM5C-2 with a lactone and ketone carbonyl unit, respectively obtained from *Streptomyces* sp. Antifungal agents are used in several fields: human and animal therapy, agriculture for protection of plant and crops, food industries and treatment of wood. The toxicity of present antifungal therapy due to the biochemical similarity between fungal pathogens and infected host (all eukaryotes) and the increase of mycosis caused by opportunistic fungi encourage the research for novel antifungal antibiotics (Ouhdouch, 1989).

In the present study, we report the optimization of culture conditions for production, purification and characterization of the antifungal compound from *Streptomyces exfoliatus*.

2. Materials and Methods

2.1. Source of bacteria and fungi

Aspergillus flavus, *A. niger*, *Fusarium oxysporum* and *Candida albicans* were provided by Prof. Dr. Hamdy Ali Emar, Professor of Microbiology (Mycology), Microbiology Department, Soil, Water and Environment Research Institute, Agriculture Research center, Giza, Egypt.

The actinomycetes strain was obtained from the culture collection of Microbial Resource Center (MIRCEN), Faculty of Agriculture, Ain-Shams University, Cairo, Egypt. The Actinomycetes strain was maintained on starch nitrate agar, fungi were maintained on Czapek's Dox agar.

2.2. The antagonistic activity of the actinomycetes strain against (*Aspergillus flavus*, *A. niger*, *Fusarium oxysporum* and *Candida albicans*) by Hallow well technique

This method was used as described by Holmalahti *et al.* (1994). The actinomycetes strain was grown in starch-nitrate in 250 ml Erlenmeyer flasks on rotary shaker (200 rpm) for 8 days at 30 °C. The contents of each flask were centrifuged at 6000 xg for 20 minutes and the supernatant was sterilized through a 0.45 µm bacterial filter. Aliquots (50 µl) of each sterilized cell-free supernatant was applied into wells (5 mm diameters) which were made in the previously prepared plates seeded with 200 µl of 10⁶ spores/ml of each fungus (*A. flavus*, *A. niger*, *F. oxysporum* and *C. albicans*) using sterilized cork borer. After incubation at 30 °C for 3 days, the

appearance of inhibition zone was considered as positive results.

2.3. Effect of cultural conditions on growth and production of the antifungal compound by *Streptomyces exfoliatus*

2.3.1. Effect of different culture media

To find out the best medium for the growth and antifungal activity of *Streptomyces exfoliatus*, Erlenmeyer flasks (250 ml) each containing 48 ml of one of the tested media (starch nitrate, starch peptone, Emerson, G.B.A, sucrose nitrate, Bennet, glucose asparagines, inorganic salt starch, glycerol asparagines, yeast extract malt extract, nutrient agar and Czapek's Dox media) were inoculated with 2 ml (2 x10⁶ cfu / ml) the selected organism. The flasks were agitated at 200 rpm on a rotary shaker at 30°C for 7 days. At the end of incubation period, the antifungal activity was determined by the diameter of inhibition zones of growth of *Aspergillus flavus* using well assay method. The biomass dry weight of actinomycetes was determined as g/L.

2.3.2 Effect of incubation period

Erlenmeyer flask (250 ml) containing 48 ml of starch nitrate media were inoculated with 2 ml of the culture of *Streptomyces exfoliatus* (2x10⁶ cfu/ml). The flasks were exposed to the same conditions as mentioned above for different incubation periods (2 to 14 days). The growth and antifungal production were determined for each flask as mentioned before.

2.3.3. Effect of different pH values

The effect of pH on growth and antifungal production by the tested strain was studied using buffered starch nitrate medium. The following buffer systems were utilized: citrate phosphate buffer pH 4.0, pH 5.0, phosphate buffer pH 6.0, pH 7.0, Tris-HCl buffer pH 8.0 and pH 9.0, after inoculation and incubation at 30°C on rotary shaker at 200 rpm for 8 days, the antifungal production and growth were measured.

2.3.4. Effect of temperature

To determine the optimal temperature for growth and antifungal production by the tested strain, the organism was grown in starch nitrate medium. The media were adjusted to pH 7.0; the growth was carried out at different temperatures (15, 20, 25, 30, 35 and 40°C). After incubation for 8 days on a rotary shaker, the antifungal production and growth were measured.

2.3.5. Effect of agitation

The inoculated flasks were maintained at the optimum conditions for growth and antifungal production (starch nitrate media for 8 days at pH 7.0 and 30 °C). The flasks were incubated either at static conditions or shake conditions at different speeds (50, 100, 150, 200 and 250 rpm). The growth and the

antifungal production were determined at the end of the experiment.

2.3.6. Effect of different carbon sources

Starch in starch nitrate medium was substituted with an equivalent amount of one of the different carbon sources (D-fructose, glycerol, sucrose, maltose and glucose). The media were adjusted to pH 7.0 before autoclaving, after inoculation and incubation at 30 °C for 8 days on rotary shaker (200 rpm), the antifungal production and growth were measured.

2.3.7. Effect of different nitrogen sources

The effect of different nitrogen sources was investigated by substituting potassium nitrate (KNO₃) in the starch nitrate medium with equimolecular weights of various nitrogenous compounds. The following nitrogen sources (asparagine, NaNO₃, NH₄Cl, (NH₄)₂SO₄ and peptone) have been tested. The pH was adjusted to 7.0 before autoclaving. After inoculation and incubation at 30°C for 8 days on a rotary shaker (200 rpm), antifungal production and growth were measured.

2.4. Purification of the antifungal substance produced by the experimental actinomycete

After cultivation of the tested strain in starch nitrate media (pH 7.0) for 8 days at 30 °C on a rotary shaker (200 rpm), the culture broth (5 litres) were centrifuged at 6000 xg for 20 min to separate the supernatant from mycelial cake. The resultant supernatant was extracted with equal amount of ethanol, ethyl acetate, diethyl ether, n-hexan, dichloromethane and chloroform by shaking using separating funnel for three times according to (Hussein *et al.*, 1998). The organic layer was separated and dried at room temperature. The resulting material was dissolved in dimethylsulfoxide (DMSO) and stored at 4 °C until used. The antifungal activity was determined in each extract using well agar diffusion method as described by Holmalahti *et al.* (1994) against *Aspergillus flavus*.

The antifungal substances obtained from extraction with dichloromethane, were analyzed by thin layer chromatography (Balagurunathan and Subramanian, 2001) on silica gel plates. About 30 µl of dichloromethane extract was spotted on the start (2 cm away from the edge). The choice of the developing solvent is determined by the principle of chromatography to be employed. With reference to silica gel the following series of solvents in order of increasing elution power (petroleum ether, cyclohexane, carbon tetrachloride, benzene, dichloromethane, chloroform, ether, ethyl acetate, acetone, n-propanol, ethanol, methanol, water and pyridine). The solvent used for running was chloroform: methanol (9: 1 v/v) were being used with great success in this work which showed good

separation. After convenient development, the plates were dried and examined under UV lamp. Chromatographic plates were usually developed once by the ascending technique at room temperature to a high of 15-18 cm.

The bioautography method described by Rahalison *et al.* (1993) was followed for the detection of active compound separated in TLC. Chromatogram developed as described above was placed in a sterile bioassay Petri dish and overlaid with 10 ml molten Czapek's- Dox agar medium inoculated with 0.1 ml of *Aspergillus flavus* and incubated for 48 hrs at 30°C. An inhibition zone resulted in the area in which biologically active compound diffused. This inhibition zone was used to define the area to scrape out of a parallel TLC carried out in the same conditions.

The active compound was eluted with dichloromethane, concentrated to produce a creamy powder (120 mg). This powder was loaded on column (380 ×100 mm) containing Silica gel (60-120 mesh) and eluted with solvent system of chloroform-methanol (100: 0, 95: 5, 90: 10, 85: 15, 80: 20, 70: 30, 60: 40, 50: 50 and 0: 100). Twenty seven fractions were collected and each fractions (20 ml) was collected as described by Mostafa *et al.* (2006). TLC of each fraction was performed using pre-coated TLC plates to detect the antifungal compound. TLC plates were developed and each band was visualized with UV light. The fractions having same R_f value were mixed and the solvent was evaporated at room temperature. The fractions were scrapped and tested for their antifungal activity by well agar diffusion method. The fraction was purified by using column chromatography system as mentioned above and confirmed purity by using TLC plates. Thus, the obtained white powder was stored at 4°C until subjected to the following analysis in order to reveal its structure.

2.5. Characterization of diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate.

The antifungal compound from *Streptomyces exfoliatus* was characterized by the Harindran *et al.* (1999). Quantitative analysis of the diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate

2.5.1. Solubility test.

The solubility of the substance was tested using various solvents like ethyl acetate, chloroform, ethanol, diethyl ether, dichloromethane, petroleum ether, benzene, DMSO and hexan.

2.5.2. Melting point. The melting point was determined using an apparatus consisting of a round bottom flask filled with conc. H₂SO₄. It was fitted with a thermometer. One side opened capillary tube with powdered antifungal compound along with the thermometer in a test tube was introduced into the

flask. The flask was heated and the temperature was noted when the antifungal compound was first get melted to a clear liquid.

2.5.3. Ultra Violet spectrum. The ultra violet spectral measurement of the pure diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate was made 200-400 nm by using Shimadzu UV-Vis 2100s double beam recording spectrophotometer using quartz cuvette containing the antifungal substance, chloroform was used as a solvent.

2.5.4. FT-Infra Red spectrum. The FT-Infra Red spectrum of diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate was analysed by Fukuda *et al.* (1990).

The infra red spectra (IR) of the pure compound of *Streptomyces exfoliatus* was determined using Jasco FT / IR 260 plus (made in Japan) spectrophotometer.

2.5.5. NMR. ¹H NMR spectra were analysed by Ivanova and Schlegel (1997). The antifungal substance was dissolved in DMSO (dimethyl sulfoxide). The different functional groups were identified using (NMR) Nuclear magnetic resonance (Varian Gemini 200 MHZ).

2.5.6. Mass spectrum. Mass spectrum of the antifungal substance was recorded using mass spectrophotometer (Chimadzu QP1000 EX GCMS). The pure product was subjected to steam of high energy of electrons at elevated temperature up to 100°C. Cleavage fragments were yielded which can be characterized by mass / charge from mass spectra data.

2.6. Determination of minimum inhibitory concentration (MIC) by tube dilution method

Standard antifungal agent voriconazole and the antifungal agent under study were prepared. Serial dilutions of antifungal agent (64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.0313 µg/ml) were prepared. *A. flavus*, *A. niger*, *Fusarium oxysporum* and *Candida albicans* were incubated in 5 ml broth tubes containing different concentrations of the tested antifungal agents. Incubations were allowed for 4 days at 30 °C. The rate of growth was determined by measuring the optical density at 660 nm. MIC was the lowest concentration of antifungal agent which inhibited the growth of *A. flavus*. One tube without antifungal agent with spore inoculum was used as positive control, and a sterile broth medium was used as negative control NCCLS (2000).

3. Results

3.1. Antifungal activity of actinomycetes strain against *Aspergillus flavus* by Hallow well technique

Streptomyces exfoliatus produced antifungal activity against the four tested fungi and the diameter of inhibition zones were 30.6, 25, 35, 31 mm against

Aspergillus flavus, *A. niger*, *Fusarium oxysporum* and *Candida albicans* shown in Table 1.

Table (1): The antagonistic activity of *Streptomyces exfoliatus* against the tested fungi using hallow well method

Fungi	Diameter of inhibition zone (mm)
<i>Aspergillus flavus</i>	30.67 ± 0.58
<i>A. niger</i>	25.00 ± 0.00
<i>Fusarium oxysporum</i>	15. ± 0.58
<i>Candida albicans</i>	31.67 ± 0.58

3.2. Physical and nutritional factors influencing the growth and antifungal activity.

3.2.1. Effect of different culture media

The effect of 12 different media on the growth and antifungal substance produced by the tested strain were studied. The results were recorded in Figure (1). The antifungal activity was determined by recording the diameter of the inhibition zone using hallow well method. Glycerol asparagine medium was the most favorable for growth, whereas, starch nitrate medium was the most suitable for antifungal activity while Emerson medium gave the minimal growth and antifungal activity.

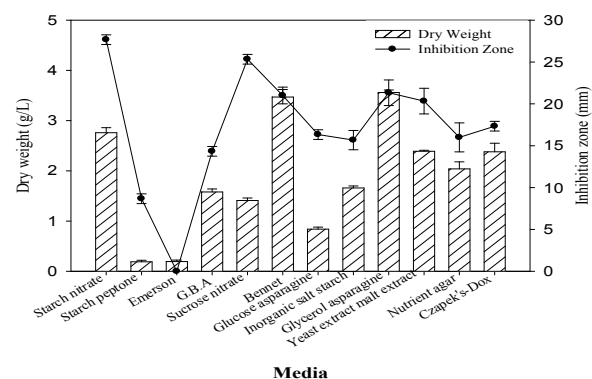


Figure (1): Effect of different culture media on the growth and antifungal activity.

3.2.2. Effect of incubation period

This experiment was designed to determine the best incubation period required for the best growth and antifungal production by the tested strain. According to Figure 2, the maximum growth and antifungal activity were obtained after 8 days of incubation.

3.2.3. Effect of temperature

Temperature is an important factor as it influences metabolic activities and microbial growth. From the data shown in Figure (3), it can be noticed that the growth and antifungal production by the tested strain increased with elevating temperature. Elevation of temperature increased the growth and the antifungal activity until the optimum temperature

and the rate began to decrease at higher temperatures. The optimum temperature for antifungal activity and growth was 30 °C. It could be noticed that there's no noticeable activity at 40 °C. The minimum growth and the antifungal activity of the isolate were at 15 °C.

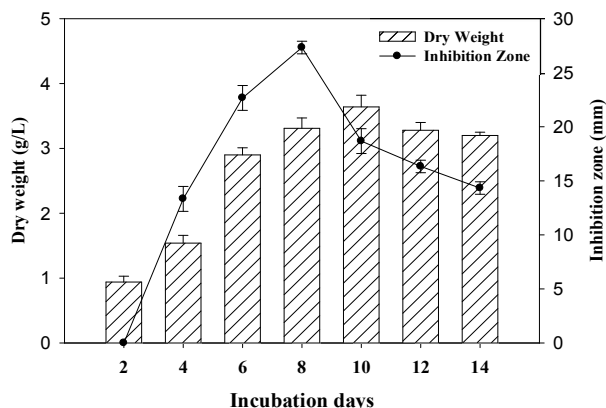


Figure (2): Effect of incubation periods on the growth and antifungal activity.

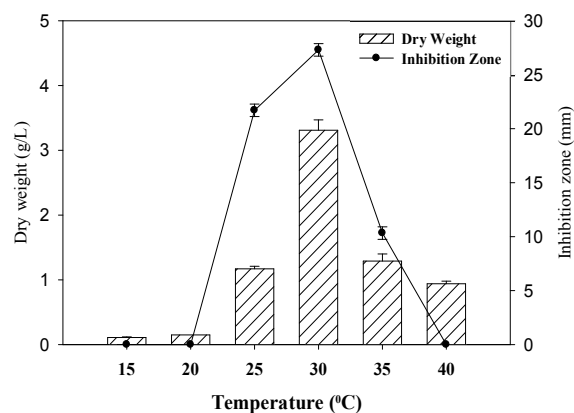


Figure (3): Effect of temperature on the growth and antifungal activity.

3.2.4. Effect of agitation

In this experiment, the culture media of the strain were incubated either at static conditions or shake conditions at different speeds (50, 100, 150, 200 and 250 rpm). The microbial growth and the antifungal potency against *Aspergillus flavus* were estimated. The antifungal activity against agitation speed is represented in Figure 4.

It was found that growth and antifungal activity depend on shaking which allowed more aeration of the media. The strain showed the highest growth value and antifungal activity at 200 rpm. The minimum growth and the antifungal activity of the isolate were at static culture.

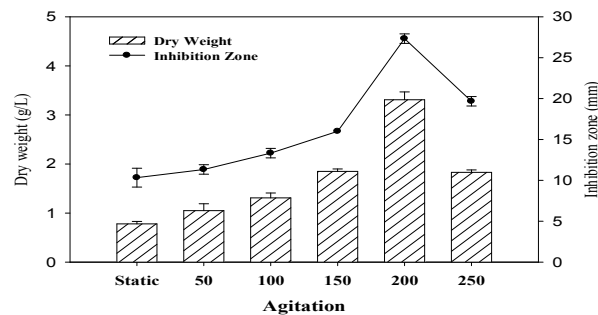


Figure (4): Effect of agitation on the growth and antifungal activity.

3.2.5. Effect of different pH values

The results obtained in Figure (5) showed that the growth and antifungal production by the tested strain increased with increasing the pH value up to pH 7.0, which represented the optimum pH value after which antifungal activity and growth decreased. The maximum growth and diameter of inhibition of the selected isolate were at pH 7.0. The minimum growth was at pH 5.0 and the minimum antifungal activity was observed at pH 8.0.

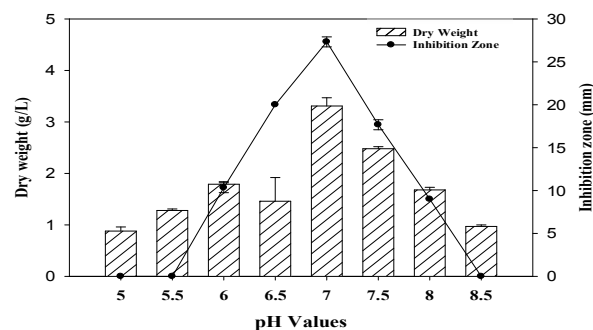


Figure (5): Effect of different pH values on the dry weight and antifungal activity.

3.2.6. Effect of different carbon sources

In this experiment, the effect of various carbon sources on growth and antifungal production by the tested strain was studied. The results presented in Figure (6) showed that the starch was the most suitable carbon source for growth and antifungal activity. Glycerol increased the antifungal activity but less than starch and was followed by glucose. However, growth and antifungal activity on maltose were the lowest of the tested carbon sources.

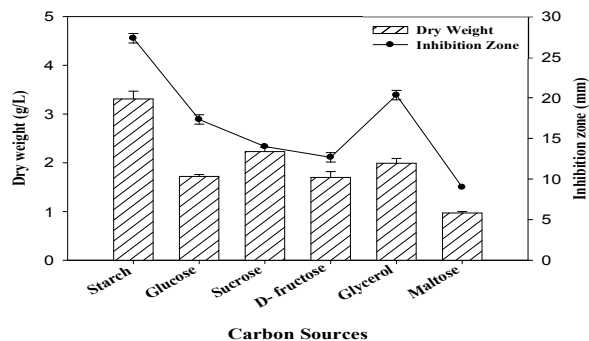


Figure (6): Effect of different carbon sources on the dry weight and antifungal activity.

3.2.7. Effect of different nitrogen sources

The effect of different nitrogen sources on the growth and antifungal production by the tested strain revealed that it differed according to the nitrogen source. The results presented in Figure (7) showed that the highest values of growth and antifungal activity were attained in the presence of potassium nitrate whereas peptone yielded the lowest value for antifungal activity and growth.

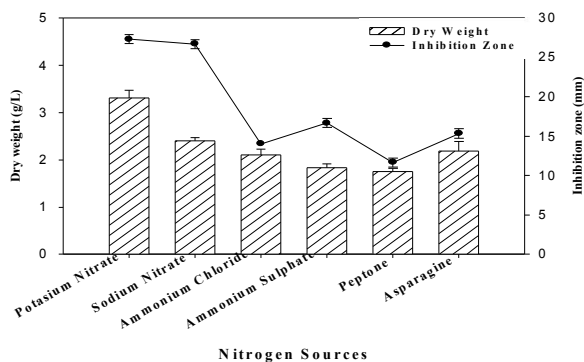


Figure (7): Effect of different nitrogen sources on the growth and antifungal activity.

3.3. Purification and characterization of the antifungal substance produced by the *Streptomyces exfoliatus*

The crude antifungal compound showed good separation on TLC with one spot using chloroform: methanol (9: 1) as a solvent. The activity of purified compound was detected in TLC sheet itself by agar overlay bioautography. Based on the results of bioautography, the crude compound was purified by column chromatography which gave single separated band in TLC.

The purified compound was off-white in color, the melting point was 165°C and is freely soluble in chloroform, dichloromethane and methanol but couldn't be dissolved in ethylacetate, diethyl ether, petroleum ether, hexane and benzene as shown in Table (2).

Table (2): Physical properties of purified antifungal compound

Physical properties	Results
Color	Off-white
Consistency	White crystalline powder
Melting point	165 °C
Solubility	Soluble in chloroform, dichloromethane and methanol. Sparingly soluble in DMSO. Insoluble in ethylacetate, diethyl ether, petroleum ether, hexane and benzene.

The UV spectrum of the purified antifungal substance isolated from *Streptomyces exfoliatus* was carried out in chloroform. This spectrum showed absorption maxima at 246, 266 nm which can assigned to an aromatic compound (Figure 8).

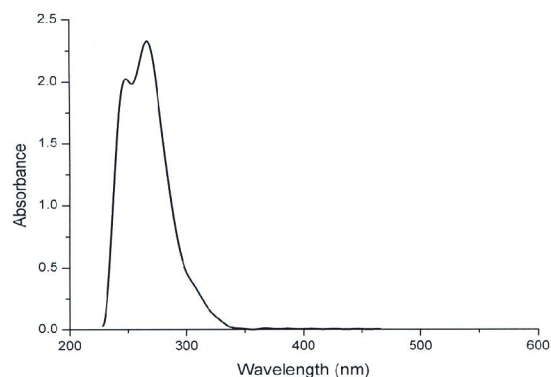


Figure (8): UV spectrum of diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate.

The infra red (IR) spectrum of the antifungal compound (Figure 9) showed absorption bands at 3459, 3059, 2927, 1696, 1510, 1454 and 1141 These bands revealed the presence of the following functional groups: -NH stretch group, aromatic =C-H stretch group, aliphatic alkanes -C-H stretch group, C=O stretch group, aromatic C=C stretch group, C=N stretch group and C-O stretch group respectively.

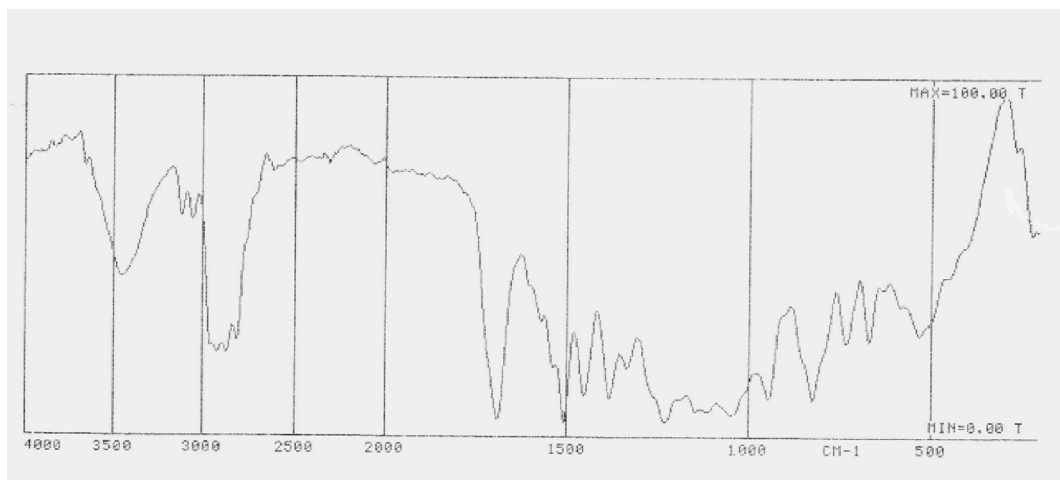


Figure (9): FT- IR-spectrum of diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate.

Proton nuclear magnetic resonance spectra (^1H NMR) of the antifungal compound (Figure 10) showed that the peaks of value at 8.4 to 7.08 (C=CH

due to aromatic protons), 6.8 to 7.1 (aromatic protons and NH), 3.8 – 4.6 (heterocyclic ring) and 1.3 – 2.5 aliphatic protons (-CH).

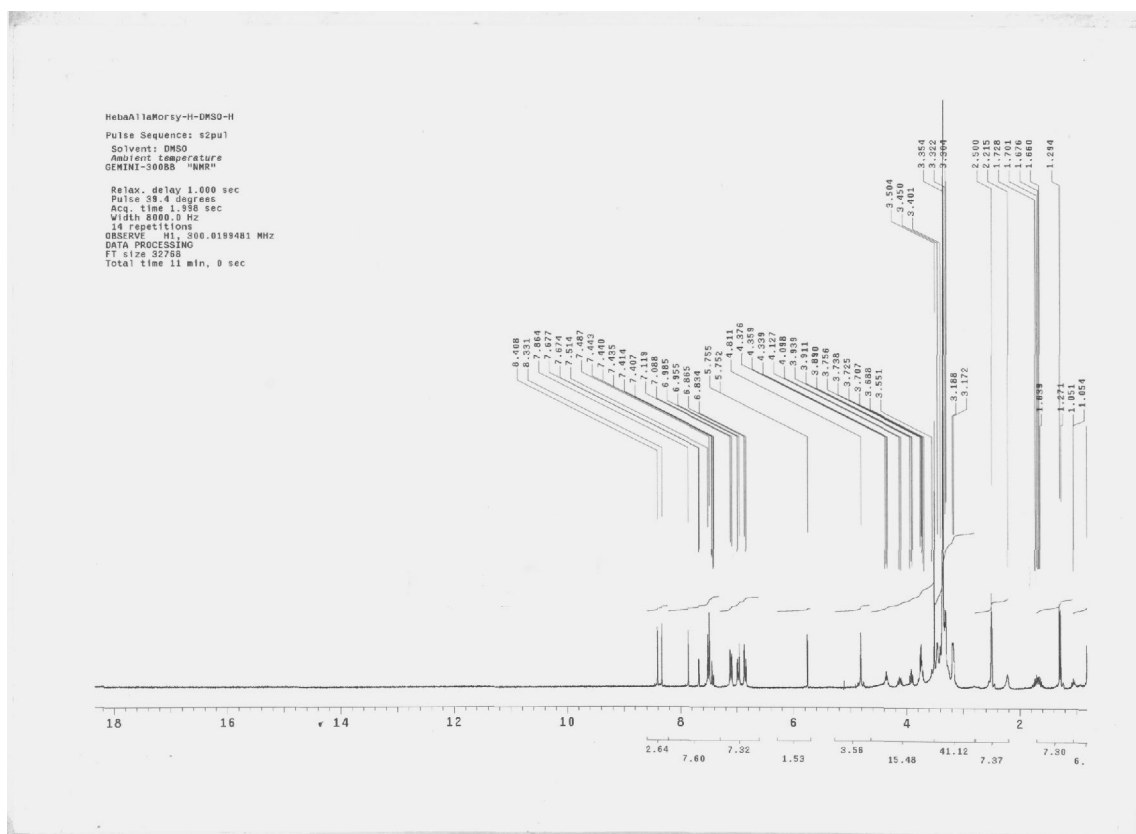


Figure (10): ^1H NMR spectrum of diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate.

The mass spectrum analysis of antifungal compound (Figure 11) showed that molecular weight

of the antifungal compound at m/e 490.28 (The molecular ion peak).

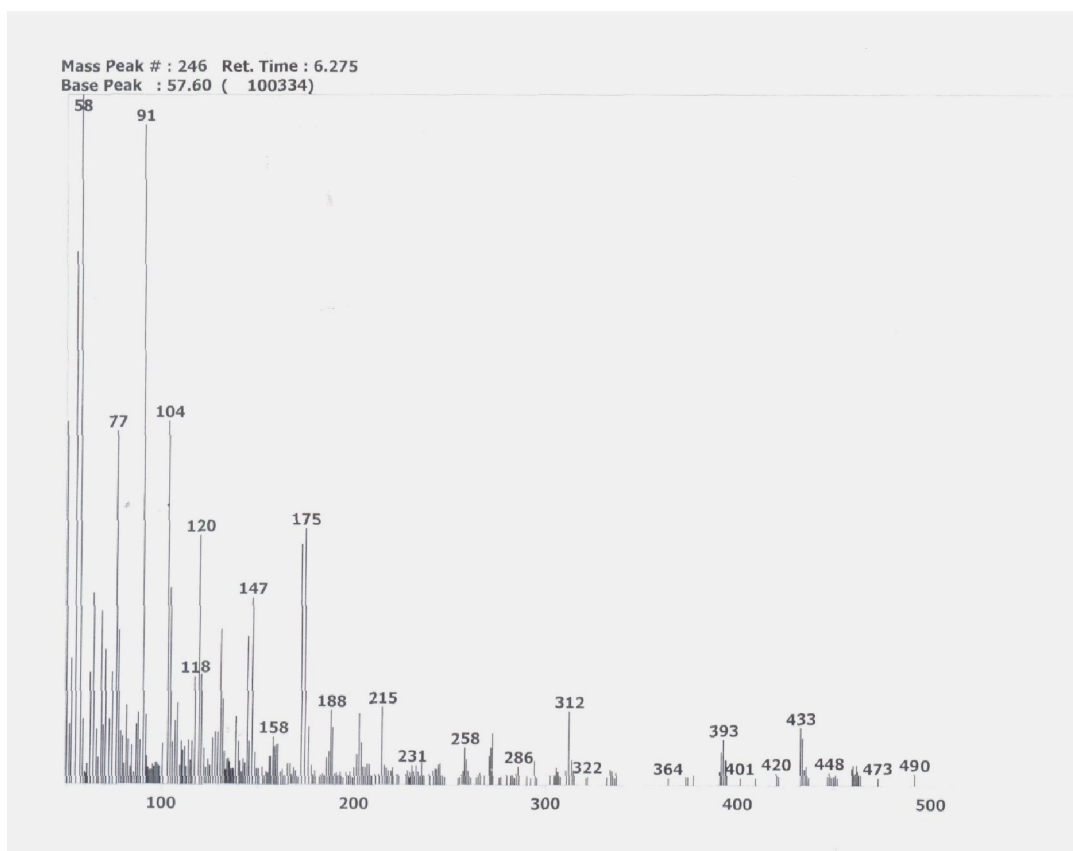
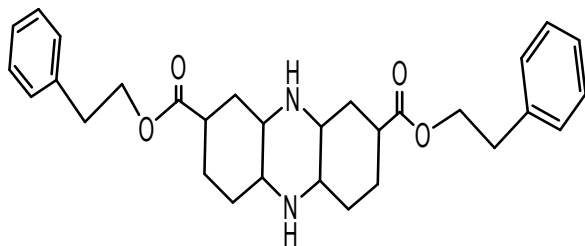


Figure (11): Mass spectrum of diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate.

Mass spectra and ^1H NMR indicates, suggested molecular formula $\text{C}_{30}\text{H}_{38}\text{N}_2\text{O}_4$. The suggested chemical structure of the purified antifungal compound produced by *Streptomyces exfoliatus* is indicated in Figure 12.



Diphenethyl tetradecahydrophenazine-2,8-dicarboxylate

Figure (12): Structure of the purified antifungal compound produced by *Streptomyces exfoliatus*.

3.2. Determination of minimum inhibition concentration (MIC)

The antifungal compound had a minimal inhibitory concentrations ranging between 0.25-16 $\mu\text{g/ml}$ against the tested fungi as compared with the control (voriconazole) which had a minimal inhibitory concentration ranging between 1.0-4.0 $\mu\text{g/ml}$ (Table. 3).

Table (3): Minimum inhibitory concentrations (MIC, $\mu\text{g/ml}$) of the antifungal compound produced by *Streptomyces exfoliatus*.

Fungal sp. tested	Minimum inhibitory concentration ($\mu\text{g/ml}$)	
	Antifungal compound	Voriconazole (standard antifungal)
<i>A. flavus</i>	0.25	1.0
<i>A. niger</i>	0.5	1.0
<i>Fusarium</i>	16	4.0
<i>Candida albicans</i>	0.25	0.5

4. Discussion

In the present study, the biological control of *Aspergillus flavus*, *A. niger*, *Fusarium oxysporum* and *Candida albicans* by *Streptomyces exfoliatus* was the main target. In order to achieve this control, the strain screened for its potentiality to inhibit the growth of fungi by hallow well technique. The results showed that the strain produced high antifungal activity. Similar results obtained by Dhanasekaran *et al.* (2008) who found that *Streptomyces sp.* DPTB16 produced detectable quantities of antifungal compounds in starch casein broth but contrast results obtained by Pandey *et al.* (2004) who showed that the isolates of actinomycetes during the screening of the

novel secondary metabolite often encountered which show antibiotic activity on agar but not in liquid culture.

The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds (El-Tarabily *et al.*, 2000; Lee and Hwang, 2002; Augustine *et al.*, 2005; Pohanka, 2006).

Many reports refer to the role of actinomycetes, mostly *Streptomyces* strains, in plant protection and are antagonists against most of the phytopathogenic fungi. Bressan (2003) reported the effectiveness of *Streptomyces* sp. strains previously isolated from maize rhizosphere and found that two *Streptomyces* sp. strains inhibited seed pathogenic fungi, *Aspergillus* sp. and *Fusarium subglutinans* whereas they did not suppress the development of *Penicillium* sp.

It has been reported that nutritional requirements of *Streptomyces* play an important role during metabolite synthesis process. Amongst various nutritional requirements, antifungal substance production has been known to be influenced by the media components and cultural conditions, such as aeration, agitation, pH, temperature, carbon, nitrogen source and incubation time, which vary from organism to organism (Yu *et al.*, 2008).

The effect of different growth media (starch nitrate, starch peptone, Emerson, G.B.A, sucrose nitrate, Bennet, glucose asparagine, inorganic salt starch, glycerol asparagine, yeast malt extract, nutrient agar and Czapek's-Dox) on the growth of *S. exfoliatus* and its antifungal activity was studied. The study of influence of different nutritional media on antifungal compound production indicated that the highest antifungal activities were obtained in the case of starch nitrate medium was used as a base. This result was in agreement with Rathna and Chandrika (1993).

In our study the maximum biomass production was reached during 8th day of incubation after which a decline phase was observed. The antifungal activity also increased with increase in biomass production and reached maximum of 27.3 mm at 8th day of incubation and on further incubation there was decrease in the antifungal activity production. This could be because the organism might have reached death phase. Usually synthesis of the secondary metabolites occurs after the growth ceases.

Contrast results were obtained by (Singh and Vibhuti., 2012) who found that the antibiotic production by *Streptomyces rimosus* subsp. *Rimosus* against gram positive and gram negative bacteria as well as filamentous fungi including human pathogens was detected in culture broth after 24 h of incubation and reached maximum at stationary phase after 96 h

of incubation. Mycelium growth gradually increased up to 96 h of incubation.

One of the factors which seemed to play an important role in bacterial growth and its antifungal activity is the incubation temperature. The effect of temperature on *S. exfoliatus* growth and its antifungal activity was estimated and showed that the optimum temperature for *S. exfoliatus* was 30°C. These results are in agreement with several workers, such as Rathna and Chandrika (1993) and Rizk *et al.* (2007), who revealed that the optimum temperature for *Streptomyces* growth and antifungal production was 30°C. Deviation from optimum temperature for antifungal metabolite production severely affects the yield of the antifungal metabolite (Yoshida *et al.*, 1972).

pH is a significant factor that influences the physiology of a microorganism by affecting nutrient solubility and uptake, enzyme activity, cell membrane morphology, by product formation and oxidative reduction reactions (Bajaj *et al.*, 2009). Our results revealed that as pH value increased, there was a corresponding increase in bacterial growth and its antifungal activity up to pH 7.0 which seemed to be the optimal pH, above which a gradual decrease was observed in the growth and antifungal production. The present results are running parallel with those reported by Rathna and Chandrika (1993).

Agitation of the culture medium is another factor that influences the growth and the antifungal production. Agitation affects aeration and mixing of the nutrients in the fermentation medium. The culture media of the strain were incubated either at static conditions or shaking conditions at different speeds (50, 100, 150, 200 and 250 rpm). Similar results were represented by Augustine *et al.* (2005) who reported that the potency of *Streptomyces rochei* AK 39 against the dermatophytes increased with increasing agitation to 200 rpm.

The effect of different carbon sources on growth of *S. exfoliatus* and their antifungal activity was studied and the results indicate that starch was the best carbon source for bacterial growth and antifungal productivity. This results are in agreement with those obtained by Jia *et al.* (2008) and Syed *et al.* (2009) who showed that *Streptomyces pristinaespiralis* CGMCC 0957 preferred starch as a carbon source for the production of secondary metabolites (pristinamycin).

The growth and antifungal activity of our experimental organism was affected by different nitrogen source in the culture media. The results indicated that potassium nitrate was the best nitrogen source for bacterial growth and antifungal productivity. Another studies found that soy meal was the best nitrogen source for the optimal

rapamycin production by *Streptomyces hygroscopicus* ATCC 29253. (Sallama *et al.*, 2010).

Generally, the optimum conditions for the best growth and antifungal activity of *S. exfoliatus* were on starch nitrate media at 30 °C, pH 7.0 after 8 days in the presence of starch and potassium nitrate as carbon and nitrogen sources. The inhibitory effect of antifungal compound differs among the fungi. According to Strzelczyk *et al.* (1990) and Marwick *et al.* (1999), the production of bioactive secondary metabolites varies with species and growth conditions of the actinomycetes.

The antifungal metabolites from *Streptomyces exfoliatus* were eluted by the organic solvent tested namely, n-butanol, ethyl acetate, diethyl ether, n-hexane, dichloromethane and chloroform. This is an indication that, the antifungal substance in this culture filtrate was more soluble in dichloromethane and chloroform. Organic solvents with different polarities have been used by many researchers for the extraction of antimicrobial compounds from actinomycetes (Selvameenal *et al.*, 2009). Febles *et al.* (1995) reported that methanol extraction yielded higher antimicrobial activity than n-hexane and ethyl acetate. On the contrary, Sastry and Rao (1994) found that chloroform was better than methanol.

The purified compound was off-white in color, the melting point was 165°C and is freely soluble in chloroform, dichloromethane and methanol but couldn't be dissolved in ethylacetate, diethyl ether, petroleum ether, hexane and benzene. In the present research, the identification of antifungal compounds was made by determining the melting point, elemental analysis and spectral analysis such as UV, IR, NMR and Mass spectra. In order to elucidate the nature and structure of the compounds, several parameters such as, solubility, melting point, pH and temperature stability UV, IR, Mass spectrum and ¹H NMR elemental analysis were carried out for the isolate of *Streptomyces exfoliatus*. Based on the spectral characteristics, the compound was identified as diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate with molecular formula C₃₀H₃₈N₂O₄.

Minimal inhibitory concentrations of the purified compound were 0.25-16 µg/ml as compared with voriconazole concentration. Voriconazole has good *in vitro* activity against a range of *Aspergillus* species, including *A. flavus* (Diekema *et al.*, 2003). The MIC is not a constant for a given agent, because it is affected by the nature of the test organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration. Frändberg *et al.* (2000) reported that Bafilomycins B1 and C1 produced by *Streptomyces halstedii* K122 were equally active against the fungal species tested (*Aspergillus fumigatus*, *Mucor hiemalis*, *Penicillium*

roqueforti, and *Paecilomyces variotii*., with MIC values in the range of 0.5-64 µg/ml. Arasu *et al.* (2013) found that the antifungal activities of polyketide metabolite from marine *Streptomyces* sp. AP-123 it exhibited MIC values of 12.5 and 25 µg/ml, against *Candida albicans* and *Aspergillus niger* respectively. Otani *et al.* (1988) and Sakai *et al.* (2004) isolated and characterized the antifungal compounds from *Streptomyces* sp. and showed the narrow spectrum activities, while (Dhanasekaran *et al.*, 2008) showed the broad spectrum activity of 4' phenyl 1-naphthyl phenyl acetamide produced by *Streptomyces* sp. DPTB16 against *Candida albicans*, *Aspergillus niger*, *A. fumigatus* *A. flavus*, *Mucor*.

Many antibiotic substances were reported to be isolated from *S. exfoliatus* such as exfoliamycin (antibacterial) with molecular formula C₂₂H₂₆O₉ (Potterat *et al.*, 1993). A nucleoside antibiotic inhibiting zygospore formation of *Mucor hiemalis*, the molecular formula of the antibiotic is C₁₁H₁₄O₉ with properties similar to a carbohydrate (Kneifel *et al.*, 1974). Exfoliazone antibiotic against *Valsa ceratosperma* the causative fungus of the apple canker disease, the antifungal antibiotic had molecular formula C₁₅H₁₂O₄N₂ (Imai *et al.*, 1990). Antibiotic MYC8005 extracted from mycelium of *S. exfoliatus* had strong activity against bacteria and fungi, the antibiotic had maximum UV absorption in methanol at 259 nm (Den Admirant *et al.*, 1972).

Phenazines are heterocyclic compounds that are produced naturally and substituted at different points around their rings by different bacterial species. The antagonistic effects of almost all of phenazine derivatives are usually attributed to one general characteristic redox activity Price-Whelan *et al.* (2006).

Our study is the first in which the purified antifungal compound had molecular formula C₃₀H₃₈N₂O₄ isolated from cell free culture filtrate of *S. exfoliatus*. This compound identified as diphenethyl tetradecahydrophenazine - 2, 8-dicarboxylate produced by *Streptomyces exfoliatus* could be exploited for its future use as a biofungicide as reported by (Thomashow and Weller, 1988; Delaney *et al.*, 2001), they discovered a phenazine derivative like the phenazine compound (phenazine-1-carboxylic acid) produced by *Pseudomonas* spp., such as *Pseudomonas fluorescens*, *Pseudomonas aureofaciens*, and *Pseudomonas chloraphis*, was active against *Gauemannomyces graminis* var. *tritici* Walker Ggt, was the causal agent of take-all disease, the most damaging root disease of wheat worldwide. The same compound was effective against *Seiridium cardinale* and *Seiridium unicorne*, were the causal agent of cypress canker of some plants (Raio, 2011).

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