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Biological Control of Rice Blast Disease by *Streptomyces flavotricini*.

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ABSTRACT

Fifty isolates of actinomycetes were isolated from Egyptian soil, rice field. All these isolates were subjected for antagonistic test against rice blast fungus *Pyricularia grisea*. The highest antifungal activity was obtained, identified according to (ISP) and based on molecular identification of 16S sequences as *Streptomyces flavotricini*. The best conditions for the production of antifungal compounds were at 28^{o}C for 9 days, pH 7.2. Ethyl acetate extract was separated in to several fractions using chromatographic techniques and the purified fractions were tested against *Pyricularia grisea* using agar well diffusion method. The highest antifungal activity compound was identified using Infra Red spectroscopy (IR), Nuclear Magnetic Resonance (NMR) and Mass Spectra (MS). This compound was identified as Dihydroxy viridiofungin $C_{37}H_{58}N_2O_{10}$.

Keywords: Biological control, *Streptomyces flavotricini*, *Pyricularia grisea*, Rice blast disease, Dihydroxy viridiofungin.

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INTRODUCTION

The fungus of rice blast disease is *Magnaporthe grisea* (Hebert) Barr, [anamorph*, Pyricularia grisea* Sacc.] [1] highly specific to rice, although certain strains that don't attack rice can harm weeds in the rice field. Once on a rice plant, the fungus rapidly produces thousands of spores, which are carried readily through the air, by wind or rain, onto neighboring plants [2]. Selection of antagonistic microorganisms for biological control is considered an alternative practice to chemical fungicides [3]. Although a variety of strategies have been developed for the prevention and control of rice blast, only limited success is achieved because of the rapid genetic variations of *Magnaporthe oryzae* [4].

Actinomycetes were able to produce bioactive substances effective against phytopathogenic fungi [5] and they have been used to control rice blast disease [6].

Streptomyces species are abundant in soils and able to produce a group of secondary metabolites, such as antibiotics and extracellular enzymes, which have a role in degradation of complex molecules especially, cellulose, xylan and lignin, that play an important role in decomposition of organic matter [7, 8]. The antifungal aliphatic compound SPM5C-1 obtained from *Streptomyces* sp. PM5 was evaluated under *in vitro* and *in vivo* conditions for rice blast disease fungus which indicated inhibition of mycelial growth of this fungus [9]. In additional, *Staphylococcus* sp. strain LZ16 was isolated from seawater collected in the East China Sea. Both culture filtrate and cell lysate of LZ16 possessed strong growth inhibition activities against *Magnaporthe oryzae* [10]. The bioactive compound radicinin, isolated from *Curvularia* sp. FH01, actively killed rice blast fungal (*Magnaporthe oryzae*). Many fungicides have been developed to control blast, which represents the second fungicide market worldwide. Systemic fungicides are often used to control blast in many rice-growing areas [11]. The use of fungicides with similar modes of action over extensive periods is not recommended because it has resulted in the emergence of resistant populations of the pathogen [12].

The aim of this work is to show biological control of fungus rice blast disease (*Pyricularia grisea*) using local *Streptomyces* species isolated from Egyptian soil and to study the potentiality of the secondary metabolites that are produce by these species for control of *Pyricularia grisea* in their culture medium.

MATERIAL AND METHODS

Fifty actinomycetes isolates were isolated from various locations of rice fields in Al-Sharqiya Governorate. The samples were air-dried, sieved and mixed thoroughly with $CaCO_3$ (10% w/w) then incubated at 30°C for 10 days to increase the number of *Streptomyces* [13], serial soil dilution technique was used [14].

The antagonistic activity of all fifty isolates of actinomycetes were tested against *Pyricularia grisea* [*Magnaporthe grisea* (Hebert) Barr, teleomorph. EMCC Number 781 (Mousa 2001)] using dual culture technique [15], eight mm diameter mycelia disc of each test antagonist *Streptomyces* isolates taken from 5-7 day old culture on starch nitrate agar was paired against eight mm diameter mycelia disc of *Pyricularia grisea* at opposite end on PDA. The PDA plates were inoculated only with phytopathogen served as control. The plates were incubated at 28±2^{o}C. The zone of inhibition was recorded as the distance in mm between the fungal pathogen and the area of antagonist growth.

Morphological (macroscopic and microscopic) and physiological characterization of the *Streptomyces* species were tested by following the standard protocol of the International *Streptomyces* Project (ISP) [16, 17] and on molecular identification of 16S. The macroscopic characters, the color of aerial mycelium was noted using cultures developed on solid starch nitrate medium (ISP 1) incubated for 7-14 days at 28^{o}C, Glycerol-Asparagine agar (ISP 5), Malt extract-yeast extract agar (ISP 2) and glucose-nitrate agar medium. The color of substrate mycelium noted using cultures grown at 28^{o}C for 7, 14 and 21 days on (ISP 1) and the presence of soluble pigments were investigated on (ISP 2, 5) medium. The microscopic characters of spore chain morphology was assessed as described [18] and spore ornamentation examined by Transmission Electronic Microscope (TEM).

The physiological activities used with other characters to identify the species of actinomycetes isolates by using many tests as gelatin liquefaction for (production of proteolytic enzymes), carbon sources utilization by using 9 carbon source (D-glucose, D-fructose, sucrose, D-mannitol, xylose, arabinose, raffinose,

inositol and rhamnose), coagulation and peptonization of milk (production of proteolytic enzymes), cellulose decomposition, starch-hydrolysis and production of melanin pigments.

Molecular identification was performed using DNA extraction from the harvested mycelia, which was frozen in liquid nitrogen and ground in a mortar according to the protocol recommended for the DNA purification of Gene JET™ genomic DNA purification kit (Thermo). PCR were carried out using Maxima Hot Start PCR master mix (Thermo) in 50 µl of reaction system containing (25µl) Maxima Hot Start PCR master mix (2X), (1 µl) 20µM 16S rRNA forward primer (5'-AGA GTT TGA TCC TGG CTC AG-3'), (1 µl) 20µM 16S rRNA reverse primer (5'-GGT TAC CTT GTT ACG ACT T-3') [19], (5 µl) DNA template, (18µl) water, nuclease-free. The Initial denaturing was at 95 ^{o}C, 3 min; and then 35 cycle 95 ^{o}C 30 s; 65 ^{o}C 60 s; 72 ^{o}C 90 s; and final extension at 72 ^{o}C 10 min then purification of PCR product was using Gene JET™ PCR Purification kit (Thermo). Finally, the sequencing of the PCR product was done by GATC Company, Germany was using ABI 3730xl DNA sequencer by using forward and reverse primers.

The method of production, extraction and purification was described [20]. The production of antifungal compounds of *Streptomyces flavotricini* was cultivated in 4 L of potato dextrose broth media (Sigma-Aldrich) using 1000 ml Erlenmeyer flask. The pH was adjusted to 7.2. The medium was sterilized by keeping it in autoclave for 15 min at 1.5 psi, 121^{o}C, then inculcated with 5 agar discs (8mm) of *Streptomyces flavotricini* culture taken from 5-7 day old culture on starch nitrate agar and incubated at 28^{o}C for 9 days on rotary shaker (150 rpm). The medium was centrifuged at 13000 *g* for 15 min then filtered using Whatman paper No.1. Agar well diffusion method used for test the filtrate of fermented broth for antagonistic activity. The organic solvent (ethyl acetate) was used for extraction of antifungal compounds from culture filtrate (Three times).The upper layer was separated. Agar well diffusion method was used for testing the antifungal activity of the crude antifungal substance.

Purification of the antifungal compounds were carried out by silica gel column chromatography (60-120 mesh). Column (50 × 2.5 cm) using chloroform: methanol (95:5) as solvent system. The crude extract was loaded on the top of the column and eluted using chloroform: methanol gradient (5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%, vol/vol) as solvent system. Fractions were collected then detection by TLC of each fraction was performed using precoated TLC plates (Sigma 20×20 cm) to detect the different fractions. Similar fractions having same R_f value were combined together and the solvent was evaporated at 40^{o}C on rotary evaporator. These fractions were tested for it is antifungal activity using the agar well diffusion method. The fractions that showed antifungal activity were repurified by silica gel column chromatography. Preparative Thin Layer Chromatography was used to visualize different compounds under UV light at 365 nm. The activity of the purified compounds was tried against tested fungus by agar well diffusion method.

The IR, NMR spectrum and Mass spectrum were used to reach the final chemical formula of the antifungal compound.

RESULTS AND DISCUSSION

Thirty six percent of tested actinomycetes isolates did not reveal any antifungal activity against *Pyricularia grisea*, 34% of tested organisms gave less than 25 mm inhibition zone while 8% gave 25-34 mm inhibition zone and 22% of tested actinomycetes isolates gave more than 35 mm inhibition zone. The highest antifungal activities were obtained from the culture of Isolate No. 16 that gave the highest inhibition zone (40 mm), therefore this isolate was chosen for further investigations.

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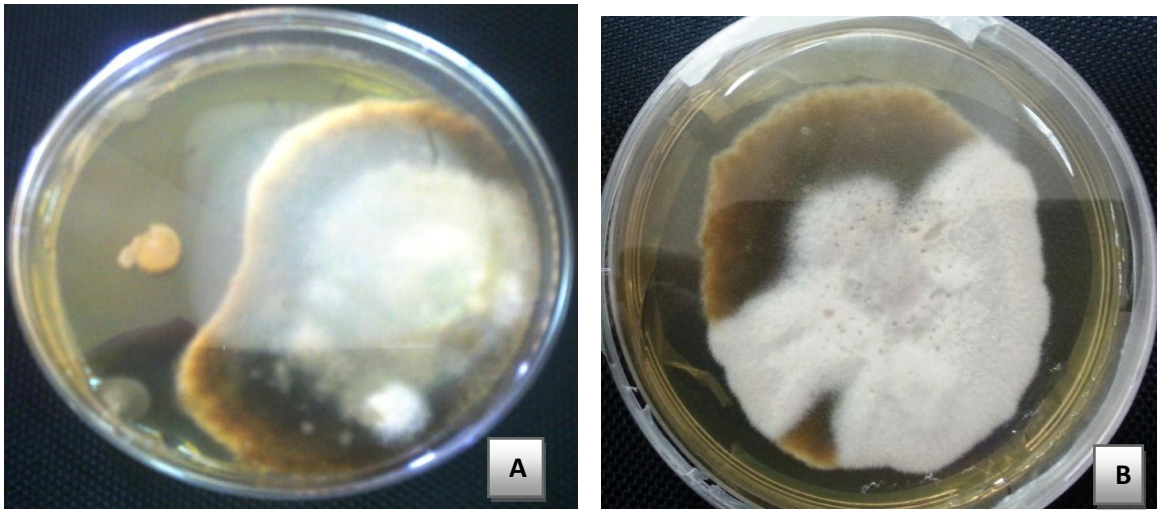

Fig. 1. A- Antagonism test No. 16 isolate against *Pyricularia grisea* (Dual culture) 7 days old culture on PDA medium, B- *Pyricularia grisea*.

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* but they did not suppress the development of *Penicillium* sp. [5]. *Streptomyces* sp. isolated from the surface of a pear leaf was strongly antagonistic against several plant pathogenic fungi such as *Pyricularia oryzae* [21].

The isolate No. 16 to be *Streptomyces flavotricini* proved, aerobic, spore-forming, Gram positive. Table 1 shows morphological and physiological characterization and Table 2 show cultural properties of *Streptomyces flavotricini* on different medium that was done according to (ISP) [16, 17] and was confirmed using molecular identification.

Table 1: **Morphological and physiological characterization of *Streptomyces flavotricini*.**

Parameters	**Results**
Soluble pigments	ND*[1]
Spore chains	Straight
Spore surface	Smooth
Gelatin liquefaction	+ve
Carbon source utilization	
D-glucose	+4*[2]
D-fructose	+4
Sucrose	+3
D-mannitol	+3
Raffinose	+3
Inositol	+3
Rhamnose	+3
Arabinose	-ve
Xylose	-ve
Coagulation and peptonization of milk	+ve
Decompose of cellulose	+ve
Starch-hydrolysis	+ve
Produce melanin pigments	Produced dark brown pigment

***[1]ND: No produced.; *[2] +4 very good and+3 good growth, -ve No growth.**

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Table 2: Cultural properties of *Streptomyces flavotricini* on different medium.

Type of Medium	Type of growth	Color of colony			Growth intensity
		Aerial mycelium	Reverse side of colony	Diffusible pigments	
Starch-nitrate agar	elevated- good sporulation	pink/pink brownish	yellow	non pigmented	*+ 5
Glycerol-asparagin agar	elevated- good sporulation	yellow	yellow whitish	non pigmented	+4
Malt-yeast-extract agar	thin-good sporulation	yellow	off white yellowish	non pigmented	+3
Glucose-nitrate agar	thin-good spoulation	yellow whitish	yellowish	non pigmented	+3

*** (+5) very good growth, (+4) good growth, (+3) moderate growth.**

The result of PCR 16S rRNA of isolate No, 16 gave a single band at about 1500 bp. The partial sequences analyses for isolate No. 16 was confirmed with results of morphological and physiological identification that this isolate is *Streptomyces flavotricini* with 98%.

250 mg of crude compounds were produced from cultured *Streptomyces flavotricini* on liquid shaken medium (PDB). The bioassay of the filtrate was performed using agar well diffusion method gave inhibition zone of 33mm after 7 days old.

The environmental and nutritional conditions have profound influence on the level of secondary metabolites production [22]. The results showed that *Streptomyces flavotricini* produced high antifungal activity which is in an agreement with the results obtained [23] found that *Streptomyces sp.* DPTB16 produced detectable quantities of antifungal compounds on starch casein broth.

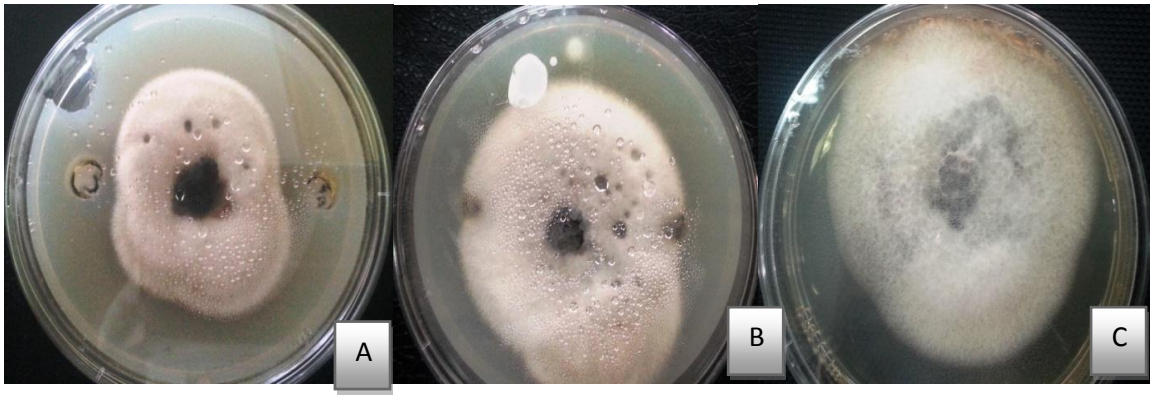

Fig. 2. A- Antifungal test for the purity compounds against *Pyricularia grisea* 7 day old culture on PDA medium, B- Control with DMF, C- Control.

The results of antifungal production that obtained with incubation temperature and agitation of the culture medium were in agreement with several workers [24] whom revealed that the best temperature and agitation for *Streptomyces flavotricini* growth for antimicrobial production at 28^{o}C at 180 rpm, while the optimum pH was in agreement [25] whom indicated that the best pH value for antibiotic production between 7.0- 7.5. While the results of incubation period on antifungal production were in agreement [26] whom showed that the highest antifungal compounds production by *Streptomyces* sp. were obtained after 8 days of incubation.

During our work the crude extraction was tested against rice blast disease fungus, the inhibition zones was measured after 7 days of incubation at 28^{o}C which gave inhibition zone 21 mm.

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The crude extract of antifungal compounds were purified using chromatographic techniques, the fraction 1 (95:5) and fraction 2 (90:10) (chloroform: methanol) which gave antifungal activity against *Pyricularia grisea* both fractions were repurified and confirmed using TLC plates. The purified compounds were tested for their antifungal activity against *Pyricularia grisea* again by agar well diffusion. The results show an inhibition zone of diameter (16 mm) after 7 days old culture (Plate 2) which showed on TLC yellow whitish compound under UV light and R_f values at 0.3. The antifungal compounds produced by *Streptomyces flavotricini* were oily material and tend to be soluble in ethyl acetate, chloroform, methanol, DMSO and DMF. Elucidation of the antifungal compound was carried out IR, NMR and mass spectrum (EI-MS) to reach the final chemical formula of the antifungal compound. A combination of several techniques is often for structure elucidation for unknown compound.

Infra-Red spectrum

The Infra Red (IR) spectrum of the pure antifungal compound of *Streptomyces flavotricini* (Fig. 3) showed absorption band at 3432 cm^{-1}, that band revealed the presence of hydroxyl group (NH and OH), absorption band at 2921 cm^{-1} corresponding to aliphatic group (-C-H, aliphatic), absorption band at 1599 cm^{-1} corresponding to (C=O of COOH) group, absorption band at 1563 cm^{-1} corresponding to (C=O) group of NH-C=O, absorption band at 1423 cm^{-1} corresponding to (C=C) group and absorption band at 1120 cm^{-1}, that band revealed presence of (C-O) group.

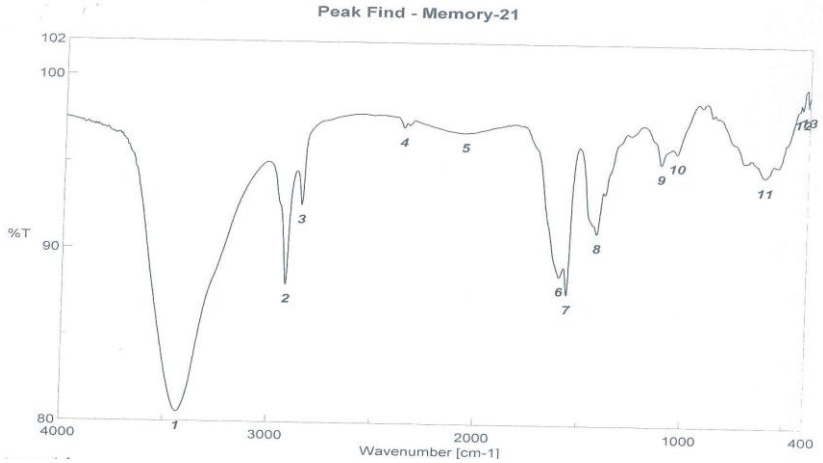

Fig. 3. Infra Red (IR) spectra of the antifungal compound produced by *Streptomyces flavotricini*.

^{1}H NMR spectrum for antifungal compound:

The antifungal pure compound produced by *Streptomyces flavotricini* was dissolved in $CDCl_3$, (Fig. 4) explain the ^{1}H NMR of this compound and showed tow Para aromatic peaks at value 7.20, 721 ppm (1H) and 7.33, 7.35 ppm (1H). Three olefinic double bond of side chain moiety appear as 7.27, 7.28, 7.29 ppm (m, 3H). The CH proton of furan ring appear at 2.33, 2.36, 2.38 ppm (m) and 4CH at 1.2 ppm, 19CH_2 at 1.6-1.7, 3 CH_3 at 1.3.

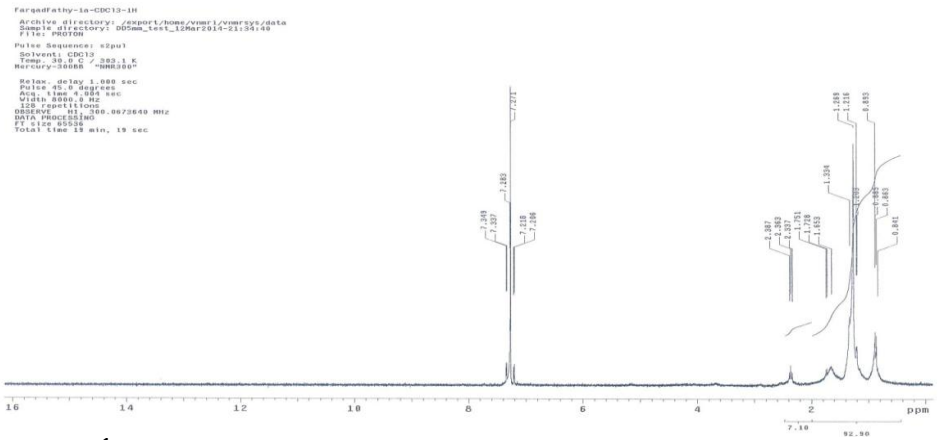

Fig. 4. ^{1}H NMR spectrum of antifungal compound produced by *Streptomyces flavotricini*.

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Mass spectra for antifungal compound

In mass spectrometry (MS) the investigated species is ionized and detected by its specific mass to charge ratio (*m*/*z*).

In electron impact (EI), major of the molecular ions are fragmented and ions of these fragment are presented in the mass spectrum, the high fragmentation pattern reproducibility in (EI-MS) makes it possible to use the compound mass spectrum as fingerprint. The mass spectrum of the pure antifungal compound of *Streptomyces flavotricini* (Figure 5) and the molecular weight is probably 692.

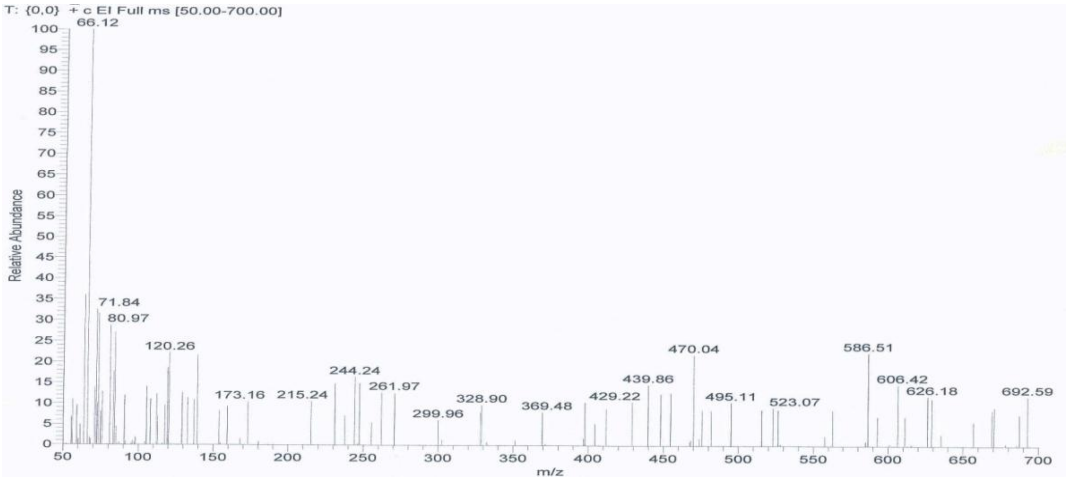

Fig. 5. Mass spectrum of the antifungal compound produce by *Streptomyces flavotricini*.

The results of analyses of IR, NMR spectra and mass spectra for the identification of antifungal compounds, suggested that the molecular formula of the antifungal compound produced by *Streptomyces flavotricini* $C_{37}H_{58}N_2O_{10}$,the suggested of being dihydroxy viridiofungin and the chemical structure was indicated in (Fig. 6). Viridiofungins members of a novel family of amino alkyl citrates [27]. They are broad spectrum antifungal agents that inhibit the squalene synthase *in vitro*, but do not specifically inhibit fungal ergosterol synthesis in whole cells [28].

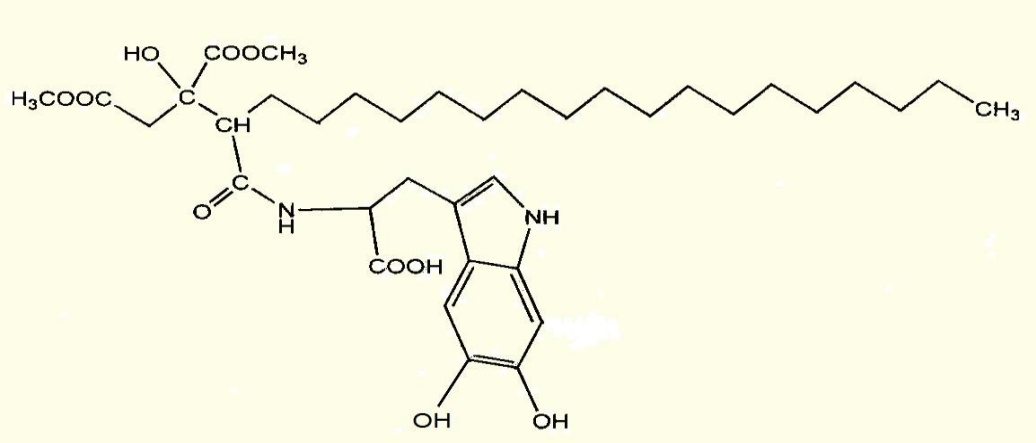

Fig. 6. Chemical structure of dihydroxy viridiofungin.

The minimum inhibitory concentration (MIC) was tested with different concentration of antifungal activity and showed the inhibition activity at 55µg/ml.

The MIC is not a constant for a given agent, because it is affected by the nature of the tested organism used, the inoculum size, and the composition of the culture medium, the incubation time, and aeration. The MIC values of the crude extract produced by *Streptomyces flavotricini* NBRC 12823^T (AB 184173) against *Bacillus subtilis, Klebsiella pneumonia,* methicillin-resistant *Staphylococcus aureus* and *Pseudomonas auroginosa* were found to be 15.6, 31.2, 62.5 and 125 µg/ml respectively [24].

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