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AN ANTIFUNGAL COMPOUND: 4' PHENYL -1-NAPTHYL -PHENYL ACETAMIDE FROM STREPTOMYCES SP. DPTB16

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Summary. *The identification of antifungal compound was made by determining the melting point, by means of elemental analysis and spectral analysis such as UV, IR, NMR and Mass spectra. Based on the spectral characteristics, the antifungal compound was identified as 4' phenyl-1-napthyl-phenyl acetamide from Streptomyces sp. DPTB16. It showed antifungal activity against Candida albicans followed by Aspergillus niger, A. fumigatus, A. flavus and minimum inhibitory activity was observed with Mucor sp. and Penicillium sp.*

Key words: *Marine Streptomyces, Sea shore soil, Antifungal assay*

Introduction

Among the different types of drugs prevailing in the market, antifungal antibiotics are a very small but significant group of drugs and have an important role in the control of mycotic diseases. The need for new, safe and more effective antifungal compounds are a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immunocompromised host.

The history of new drug discovery processes shows that novel skeletons have come, in the majority of cases, from natural sources (1). This involves the screening of microorganisms and plant extracts (2). The search for new, safer, broad-spectrum antifungal antibiotics with greater potency has been progressing. One reason for this is that when compared to antibacterials, fungi are like mammalian cells, eukaryotes and therefore agents that inhibit protein, RNA or DNA biosynthesis in fungi have greater potential for toxicity as well (3). The other reason is that, until recently, the incidence of life threatening fungal infections was perceived as being too low to warrant aggressive research by the pharmaceutical companies (4). In the course of our screening programme for new antifungal antibiotic, we found that *Streptomyces sp*. DPTB16 isolated from Cuddalore sea shore soil, Tamilnadu, India is capable of producing an antifungal antibiotic against *C. albicans*. In the present study, we report the isolation, purification and characterization of the antifungal antibiotic from *Streptomyces* sp DPTB16.

Materials and Methods

Streptomyces isolates. About 48 *Streptomyces* strains were isolated from cuddalore coastal soil, Tamil nadu, India. All these strains were screened for their antifungal activity against pathogenic fungi. Among the *48 Streptomyces,* the broad spectrum strain *Streptomyces sp*. DPTB16 was selected for compound characterization and purification.

4' phenyl-1-napthyl-phenyl acetamide Production. The antifungal compound production was evaluated by the method of Abou-Zeid *et al.* (5). The medium starch casein broth (starch 5g,casein 0.3g, Ferrous sulphate 0.01g, Calcium carbonate 0.02g, potassium nitrate 2g, magnesium sulphate 0.05g,sodium chloride 2g, agar 20g, distilled water 500ml, sea water 500ml) was prepared and sterilized in a 250 ml Erlenmeyer flask. After the sterilization the broth was inoculated with heavy spore suspension of *Streptomyces* sp and incubated in a rotary shaker (operated at 200 rpm) for seven days at 28±2°C.

Extraction of 4' phenyl-1-napthyl-phenyl acetamide. The culture broth of *Streptomyces* sp. DPTB16 (5 litres) was centrifuged at at 4°C, 10,000 rpm for 10 min. The mycelium was separated with 3 volumes of methanol and the filtrate with n-butanol (2:1). The active solvent extracts were combined and evaporated to dryness under reduced pressure. The crude extracts were dissolved in a small amount of methanol, then filtrated and precipitated with acetone-ether (10:1,v/v). The precipitate was left to stand for 24 hrs at 5-10°C, filtered and washed with acetone and ether. Five grams of crude power were obtained from 5 litres of culture broth. A methanolic solution of the powder (1.0g) was chromatographed on silicagel 60 (70-325 mesh) columns. The 4'

phenyl-1-napthyl-phenyl acetamide was eluted with the lower phase of a mixture of chloroform/methanol/water (175:100:50). The active eluates were combined and evaporated *in vacuo* to dryness. A methanolic solution was precipitated with acetone-ether (10:1,v/v) and filtered to give 200mg of the 4' phenyl-1-napthyl-phenyl acetamide.

Purification of 4' phenyl-1-napthyl-phenyl acetamide. The obtained 4' phenyl-1-napthyl-phenyl acetamide was purified by silica gel column chromatography. Two grams of crude powder were dissolved in 10 ml ethyl acetate. The solution was passed through a silica gel column in benzene. The active fractions were pooled and subsequently subjected to analytical thin layer chromatography. 200g of silica gel was stirred into 500ml of distilled water. The mixture was shaken mechanically for 0.5h and then left to stand. 50ml of mixed slurry was used to coat the five 20x 30 cm glass plates. The coated plates were left to stand until the slurry set. The coated plates were then oven dried. Using a capillary tube a row of spots of the 4' phenyl-1-napthyl-phenyl acetamide sample was applied along a line, 1.5 cm above from the bottom of TLC plate. The spots were left to dry. The TLC plate was placed vertically in a trough containing the solvents (n-butanol-ethylacetate-water (9:9:1). When the solvents moved up to 80% of TLC, the plate was taken out and dried, then sprayed with ninhydrin.

Characteristics of
4' phenyl-1-napthyl-phenyl acetamide

The antifungal compound from *Streptomyces* sp. DPTB16 was characterized by the Harindran *et al.,* method (6)

Quantitative analysis of the 4' phenyl-1-napthyl-phenyl acetamide.

Solubility test. The solubility of the substance was tested using various solvents like ethyl acetate, pyridine, aniline, chloroform, methanol, acetone, butanol, diethyl ether and petroleum ether

Melting point. The melting point was determined using an apparatus consisting of a round bottom flask filled with conc. H_2SO_4. It was fitted with a thermometer. One side opened capillary tube with powdered antifungal compound along the with 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.

Ultra Violet spectrum. The ultra violet spectral measurement of the pure 4' phenyl-1-napthyl-phenyl acetamide was made 200-400nm by using Shimadzu (UV1601) instrument, ethanol was used as a solvent.

FT-Infra Red spectrum. The FT-Infra Red spectrum of 4' phenyl-1-napthyl-phenyl acetamide was analysed by Fukuda *et al*. (1990) method (7). The pure compound of *Streptomyces* sp. DPTB16 was subjected to IR spectral analysis. IR spectrum was recorded on a Bruker FT-IR instrument equipped with AT-XT Golden gate accessories.

Mass spectrum. The mass spectrum was recorded using Finnigan MAT 8230 Mass spectrometer under the current (MA) 100 and the temperature at 90^oC.

Table 1. ^{1}H-NMR Chemical shift data for 4' phenyl-1-napthyl-phenyl acetamide (400 MHz)

Proton	1 H ppm
H-1	8.4809
H-2	8.0473
H-3	6.5597
H-4	5.4422
H-5	4.8339
H-6	4.4284
H-7	4.2012
H-8	4.1829
H-9	4.0144
H-10	3.8812
H-11	3.8263
H-12	3.7848
H-13	3.7554
H-14	3.7335
H-15	3.6748
H-16	3.6565
H-17	3.4855
H-18	3.4623
H-19	3.4403
H-20	3.3035
H-21	2.5365
H-22	2.4229
H-23	2.2397
H-24	2.1872
H-25	2.1701
H-26	2.0516
H-27	2.0113
H-28	1.8489
H-29	1.4935
H-30	1.3860
H-31	1.3689
H-32	1.1820
H-32	1.1674
H-34	0.9671

NMR.^{1}H NMR spectra were analysed by Ivanova and Schlegel method (8) and measured in $CDCI_3$ on a JEOL GSX-400 NMR spectrophotometer at 400 MHZ for ^{1}H.

Antifungal assay of 4' phenyl-1-napthyl-phenyl acetamide. The assay of *Streptomyces* antifungal compound was evaluated by Lyons and Pridham, method (9).

Antifungal activity. Antifungal property of the 4' phenyl-1-napthyl-phenyl acetamide was determined by agar diffusion assay method using the following pathogens such as *Aspergillus flavus, A. niger, A. fumigatus, Mucor* sp. *Penicillium* sp and *Candida albicans*. The compounds were dissolved in solvents namely aniline, pyridine and ethyl acetate and petroleum ether. The potato dextrose agar plates were prepared and lawn cultures of fungi were made. The 5mm diameter wells were made using a cork borer. The mixtures (solvents + antifungal compounds) were poured into the well and incubated at 28±2^oC for 72 hours. The solvents alone were used as control.

Thermo stability of the 4' phenyl-1-napthyl-phenyl acetamide

The antifungal compound solutions were taken in test tubes and heated by keeping them in a thermostat at various temperatures for 10 min. The tubes were subjected to a temperature below that of the room temperature by placing them in a refrigerator. After the treatment, the solutions were cooled or warmed to the room temperature (28 to 30°C) and tested for their antimicrobial activity by an agar diffusion assay method.

pH stability of4' phenyl-1-napthyl-phenyl acetamide. The stability of the antifungal compounds was tested at different pH levels. The compound solutions were distributed into 100 ml Erlenmeyer flasks and their pH level was adjusted from 4.0 to 9.0 by using 0.1N HCl or 0.1N NaOH. The flasks were kept at the room temperature (28 to 30°C) for 3 hours and then the samples were tested for their antimicrobial activity by the agar diffusion assay method.

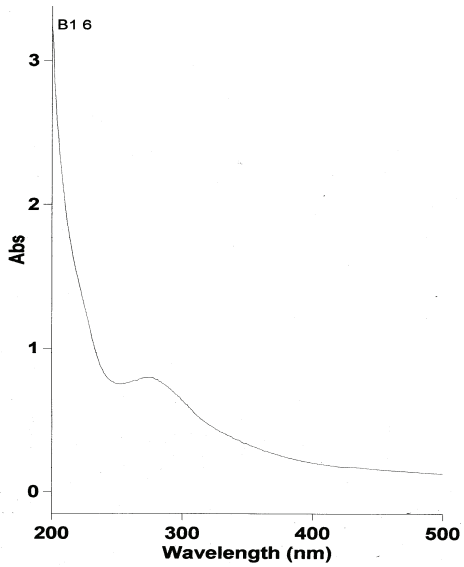

Fig. 1. UV-spectrum of 4' phenyl-1-napthyl-phenyl acetamide

Results

The fermented broth of *Streptomyces* sp. DPTB16 was extracted with nine different solvents. The 4' phenyl-1-napthyl-phenyl acetamide from *Streptomyces sp.* DPTB16 extract was dissolved in ethyl acetate, pyridine, methanol, n-butanol and aniline, whereas it could not be dissolved in chloroform, acetone, diethyl ether and petroleum ether.

The extracted compound was purified and separated by column and thin layer chromatography. Single separated band was observed in both samples in thin layer chromatography. The Rf value of 4' phenyl-1-napthyl-phenyl acetamide was 0.40cm in a thin layer chromatographic separation.

The compound obtained from *Streptomyces* sp. DPTB16 was brownish in colour and the melting point was 240°C. The UV spectrum of the compound of *Streptomyces* DPTB16 showed that the absorption maximum was at 230nm in ethyl acetate (Fig. 1). IR spectrum of 4' phenyl-1-napthyl-phenyl acetamide showed two absorption peaks in the region of 3399 and 2927 cm^{-1}. The spectrum indicates that the compound had

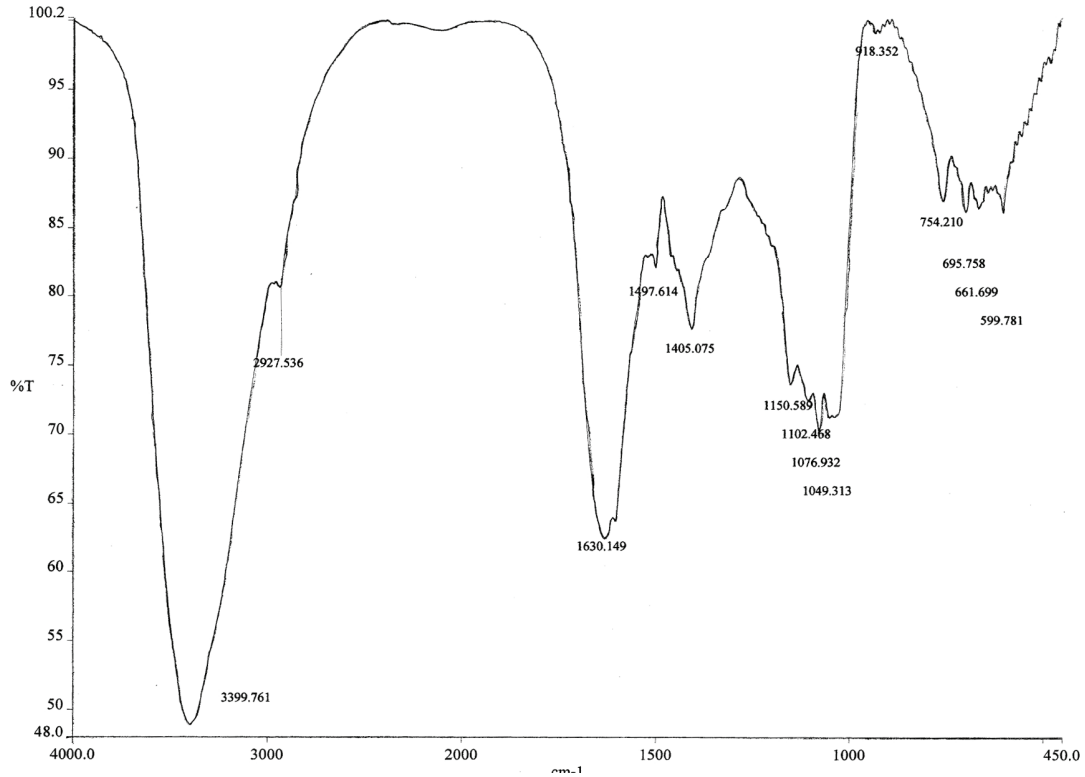

Fig. 2. FT- IR-spectrum of 4' phenyl-1-napthyl-phenyl acetamide

NH_2 and O-H group. The absence of carboxylic acid (COOH), ester (COOR), and alkyne (C=C-), was confirmed by the lack of a band in the region of 1670-1740, 1700-1750 and 2100-2260cm^{-1} respectively (Fig. 2).

The large number of peaks through out the δ value of 0-10 were observed in the ^{1}H NMR spectrum of purified 4' phenyl-1-napthyl-phenyl acetamide. The peaks of δ value at 7.08 to 8.5s, 1H of C=C-H (m,16H) due to aromatic protons including periprotons of napthalein ring 1.9δ due to NH_2 group, it was D_2O exchangeable, because of usage of D_2O during the time of sample preparation (Fig. 3, Table 1). The mass spectrum showed that the molecular ion peak recorded was 323m/z and the molecular weight of the compound was determined as 337 (Fig. 4). The molecular formula of the compound was established from the elemental analysis as $C_{24}H_{19}NO$. The quantitative chemical analysis of the antifungal compound showed the presence of the methyl group in phenyl acetamide compound. Thus, the chemical structure of the compound obtained from *Streptomyces* sp. DPTB16 was derived as $C_{24}H_{19}NO$ and the estimated molecular weight was 337. This compound was named as 4' phenyl-1-napthyl-phenyl acetamide (Fig. 5).

The *in vitro* antifungal activity of 4' phenyl-1-napthyl-phenyl acetamide showed maximum inhibitory activity against *Candida albicans* (25.05±0.81) followed by *Aspergillus niger , A. flavus* (13.6±1.23), *Mucor* (13.27±0.44), *A. fumigatus* (10.8±0.49), and minimum inhibitory activity was observed with and *Penicillium* sp. (Table 2). On testing the thermostability and pH stability of 4' phenyl-1-napthyl-phenyl acetamide, we found that compound was stable at pH from 4.5 to 7.5 and the temperature ranging from 25 to 35°C.

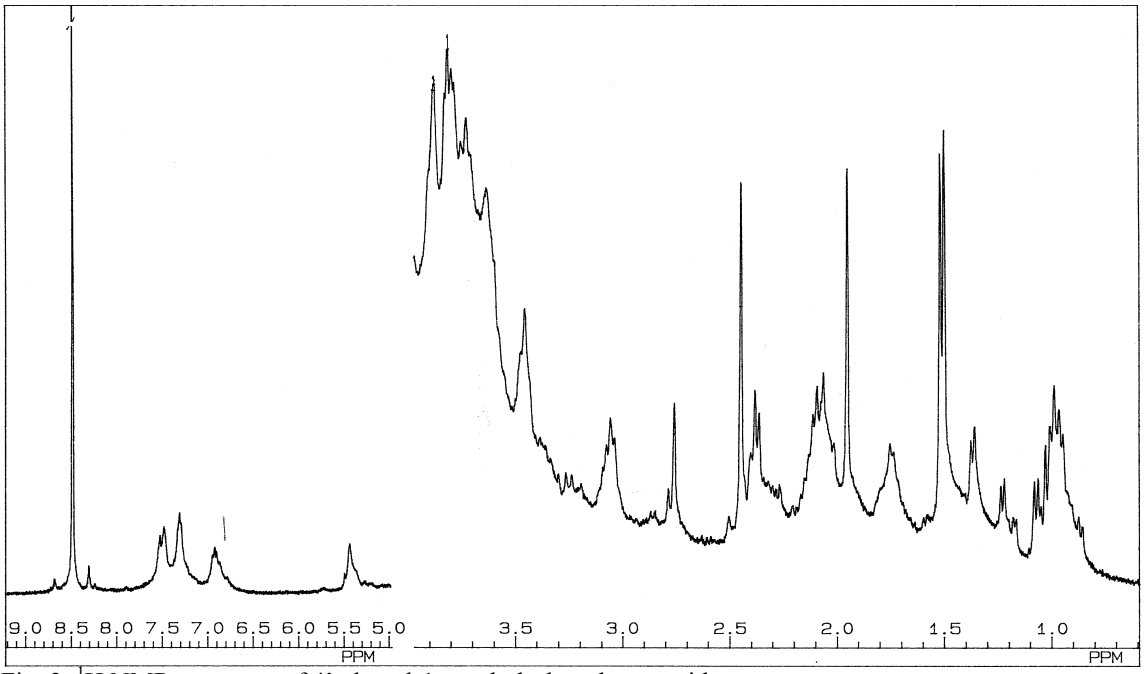

Fig. 3. ^{1}H NMR spectrum of 4' phenyl-1-napthyl-phenyl acetamide

Table 2. Mean deviation of antifungal efficacy of 4' phenyl-1-napthyl-phenyl acetamide

S. No.	Name of the fungi	Zone of inhibition (mm)		
		Aniline	Pyridine	Ethyl acetate
1.	*Aspergillus flavus*	5.13±0.24	5.37±0.45	13.66±0.54
		(4.9±0.10)	(3.70±0.17)	(4.73±0.24)
2.	*A. niger*	10.57±0.2	8.3±0.46	13.6±1.23
		(4.33±0.68)	(6.67±0.01)	(6.4±0.73)
3.	*A. fumigatus*	3.63±0.35	5.17±0.42	10.8±0.49
		(1.73±0.26)	(3.4±0.36)	(6.8±0.37)
4.	*Mucor* sp.	7.40±0.56	4.9±0.1	13.27±0.44
		(5.03±1.40)	(2.7±0.36)	(5.03±0.74)
5.	*Penicillium* sp.	10.76±0.42	6.17±0.17	7.8±0.22
		(4.7±0.28)	(4.1±0.22)	(5.5±0.41)
6.	*Candida albicans*	10.77±0.17	14.83±0.7	25.0±0.81
		(8.3±0.8)	(7.8±0.17)	(6.43±0.4)

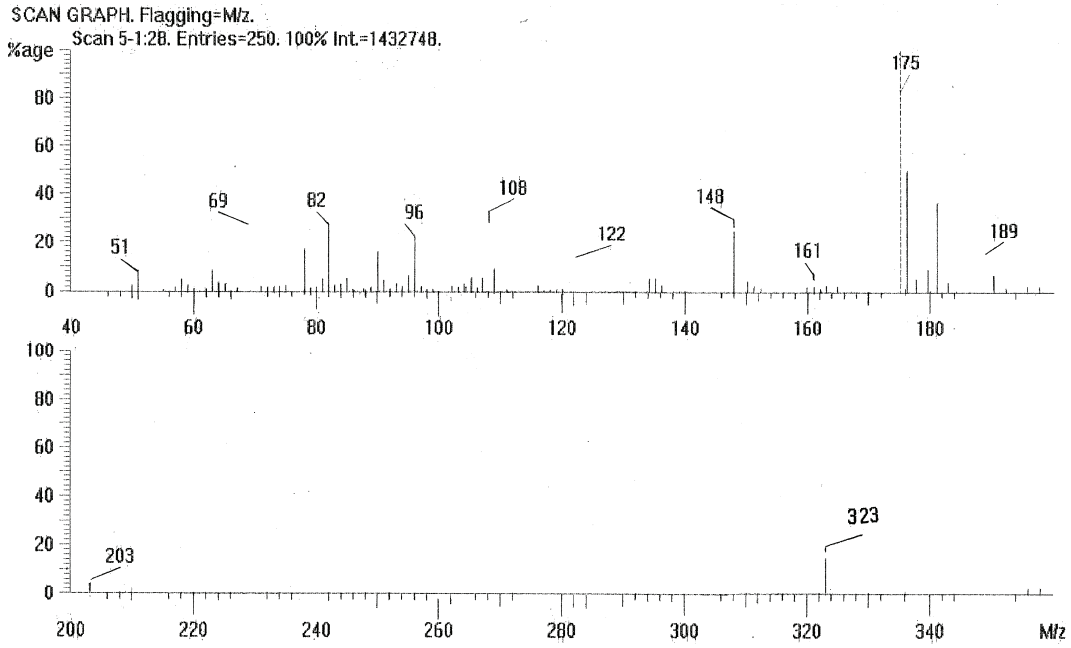

Fig. 4. Mass spectrum of 4' phenyl-1-napthyl-phenyl acetamide

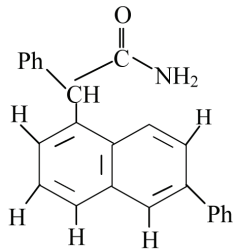

Fig. 5. Structure of 4' phenyl-1-napthyl-phenyl acetamide

Discussion

A search for new antibiotics from *Streptomyces sp.* DPTB16 resulted in the discovery of new antifungal 4' phenyl-1-napthyl-phenyl acetamide antibiotics. The number of antibiotics reported so far has already exceeded 50,000 and it is increasing year by year. However, the probability of discovering new antibiotics by conventional methods has been rapidly declining. In order to find out the new antifungal antibiotic, our approach was focused on marine *Streptomyces* in coastal soil. Earlier study results indicate that marine environment provides a good source of new antibiotics producing strains (10,11,12,13).

In the present research, *Streptomyces sp.* DPTB16 could produce detectable quantities of antifungal compounds in starch casein broth. 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 ^{1}H NMR elemental analysis were carried out for the isolate of *Streptomyces* sp. DPTB16. Based on the spectral characteristics, the compound was identified as 4' phenyl 1-napthyl phenyl acetamide from *Streptomyces* sp. DPTB16.

Otani *et. al* (14) and Sakai *et al* (15) isolated and characterized the antifungal compound from *Streptomyces* sp. and showed the narrow spectrum activity, while our results showed the broad spectrum activity of 4' phenyl 1-napthyl phenyl acetamide against *Candida albicans*, *Aspergillus niger, A. fumigatus A. flavus*, *Mucor sp.* (14,15). In this study it was reported that it is not closely related to the antifungal pyrolnitrin compound [3-chloro-4-(2'-nitro – 3' chloro – phenyl) pyrole] from *Pseudomonas cepacia* and phenyl acetic acid and sodium phenyl acetate from *Streptomyces humidus* (16)

Further investigation should address the relationship between the structure of 4' phenyl-1-napthyl-phenyl acetamide and the broad spectrum activity, as well as a rapid method for large scale production and purification and whether this group of antibiotics has any application in managing human infectious fungal disease.

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ANTIGLJIVIČNO JEDINJENJE: 4' FENIL – 1 – NAFTALIN – FENIL ACETAMID IZ VRSTE STREPTOMYCES DPTB16

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Kratak sadržaj: *Identifikacija antigljivičnih jedinjenja izvršena je odredjivanjem tačke topljenja, elementalnom analizom i spektralnom analizom kao sto su UV, IR, NMR i spektri mase. Na osnovu spektralnih karakteristika, antigljivično jedinjenje je identifikovano kao 4' fenil – 1 – naftalin – fenil acetamid iz vrste Streptomyces DPTB16. Pokazalo je antigljivičnu aktivnost na Candidu albicans koju su pratile Aspergillus niger, A. fumigatus, A. flavus a minimalna inhibitorna aktivnost je primećena sa Mucor sp. i Penicillium sp.*

Ključne reči: *morske Streptomyces, obalna zemlja, antigljivični test*