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CHARACTERIZATION OF BIOACTIVE COMPOUND OBTAINED FROM *STREPTOMYCES COELICOFLAVUS* NBRC (15399^T) AND ITS ANTICANCER ACTIVITY

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ABSTRACT

To characterize the anticancer activity of bioactive compound obtained from Streptomyces coelicoflavus NBRC (15399^T). It was isolated from oil contaminated naval dockyard soil near Visakhapatnam. It was identified by 16S rRNA sequencing and was used for screening their biosurfactant production. Characters of the biosurfactant were analyzed and their anticancer activity was performed in human breast carcinoma cell line (MCF-7) at different concentrations. The biosurfactant were characterized by TLC, FTIR, ^{1}H and ^{13}C NMR and Mass spectroscopic methods, identified as mono-rhamnolipid. Anticancer activity performed in the human breast carcinoma cell line at different concentrations of biosurfactants. Among the various concentrations of biosurfactants 80.54% cell inhibition was observed and the IC_{50} value was 88.602 µg/ml. Based on the findings, the present study concluded that, there is a possibility to develop eco-friendly anticancer drugs from oil contaminated soils.

Keywords *– Biosurfactant; Streptomyces; Anticancer activity; Spectroscopic methods; Bioactive compound..*

1. INTRODUCTION

Biosurfactants produced by microorganisms[1], are amphipathic surface active molecules containing hydrophilic and hydrophobic moieties that act by emulsifying hydrocarbons, increasing their solubilisation and subsequently rendering them available for microbial degradation[2]. They can be glycolipids, lipopeptides, lipopolysaccharides, polysaccharide protein complexes, fatty acids and lipids[3].

Bioemulsifiers generally include low-molecular-weight compounds, such as lipopeptides and glycolipids and bioemulsans include high-molecular weight polymers of polysaccharides, lipopolysaccharides, proteins or lipoproteins[4]. Biosurfactants are the most important and valuable products of biotechnology for industrial and medical applications. In recent years, these biomolecules were also found to possess several interesting properties of therapeutic and biomedical importance[5]. They have several applications including agriculture, bioprocessing, pharmaceuticals, food industry, dermatology and cosmetics industry. In pharmacological field, the biosurfactants act as antibacterial, antifungal, antiviral, anticancer, immunomodulator, anti-adhesive, antioxidants, stimulate dermal fibroblasts, vaccines and gene therapy[6]. Microbes from extreme environments have attracted considerable attention in recent years. This is primarily due to the secret that they hold about the molecular evolution of life and stability of the macromolecules[7]. They are often under extreme conditions of pressure, temperature, salinity, and depletion of micronutrients, with survival and proliferation often depending on the ability to produce biologically active compounds.

Biosurfactants are classified according to their molecular structure into mainly glycolipids (e.g., rhamnolipids (RLs) and sophorolipids), lipopeptides (e.g., surfactin), polymeric biosurfactants (e.g., emulsan and alasan), fatty acids ((e.g., 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs)), and phospholipids (e.g., phosphatidylethanolamine)[8,9].

Rhamnolipids are surface-active glycolipids. They have been intensively investigated and extensively reviewed[10-14]. However, two questions about this class of biosurfactants are emerging as important topics that have yet been poorly reviewed. First, what is the intrinsic, natural role of RLs for the producing organisms? Second, what actually are these producers? Indeed, while the opportunistic pathogen *Pseudomonas aeruginosa* has traditionally been considered the primary RL-producing microorganism, many other bacterial species, especially in recent years, have been reported to produce RLs as well.

The search for novel biosurfactants in oil contaminated soils seems to be particularly promising since they have particular adaptations to increase stability in adverse environments and the microbial products are highly stable and important in medical biotechnology. The present study point out the screening, characterization and its pharmaceutical importance of biosurfactants extracted from the actinomycetes *Streptomyces coelicoflavus* which was isolated from oil contaminated soils.

2. MATERIALS AND METHODS

2.1 Selection of soil samples

The soil sample was collected in sterile plastic bags from oil contaminated soil near naval dockyard in Visakhapatnam, India. This soil sample was found to be rich in fats and oils, and hence was used for the screening of biosurfactant producing microorganisms.

2.2 Isolation of actinomycetes

The collected soil sample was air dried at room temperature for one week, then preheated at 55ºC in a hot air oven for three hours, and was stored at room temperature in sterile bags labelled 'NDYS'. One gram of soil sample was serially diluted and 100 µl aliquot was applied to Humic-acid-Salts-Vitamin-agar plates[15] with pH adjusted to 7.0. Humic acid used here was synthesised in the laboratory using modified Essington method[16]. These plates were then supplemented with 50 µg/ml of rifampicin and cycloheximide, and were incubated at 28ºC for seven days for the growth of actinomycetes colonies. On eighth day of incubation, actinomycetes colonies were preliminarily selected based on colony morphology, and a small portion of them was streaked on the Bennets agar medium.

2.3 Identification of actinomycetes

The molecular identification and characterization of the actinomycetes NDYS-4[17] was carried out by 16S rRNA gene sequencing performed at Institute of Microbial Technology (IMTECH), Chandigarh (India). The similarity search was conducted insilico using the Basic Local Alignment Search Tool (BLAST) database of National Centre for Biotechnology Information (NCBI) of United States of America. The scanning electron microscope of the actinomycetes was done by Ruska Laboratory at the College of Veterinary Sciences, Sri Venkateswara Veterinary University, Rajendra nagar, Hyderabad (India).

2.4 Production and extraction of biosurfactants

Streptomyces coelicoflavus (NBRC 15399^T) was cultivated in 500 ml Erlenmeyer flasks containing 160 ml of medium. Cultures were incubated at 30°C on a rotary shaker at 220 rpm for 7days. The culture broth was centrifuged at 10000 rpm for 20 min at 4°C. The supernatant was adjusted to pH 2.0 with 6M HCl and then incubated at 4°C overnight. The total broth was extracted twice with chloroform–methanol (2:1, v/v). The mixture was stirred with magnetic stirrer for 20 min. The organic phase was concentrated by the vacuum distillation at 40°C. After evaporation, the resulting yellowish, oily crude product containing biosurfactants was obtained.

2.5 Purification of biosurfactants

The crude extract was purified by silica gel column chromatography which was eluted with chloroform and then with a sequence of chloroform–methanol 10:1 (v/v) 100 mL, 4:1 (v/v) 100 ml, 2:1 (v/v) 100 ml, 1:1 (v/v) 100 ml. The different glycolipidic types were separated by analytical thin-layer chromatography (TLC), carried out on silica gel plates GF-254 using chloroform/methanol/acetic acid (65:15:2, v/v/v) as developing solvent. Then the plates were visualized with sprayed phenol–H_2SO_4 and developed at 100°C for 5 minutes.

2.6 Physico-chemical characterization

2.6.1 Thin layer chromatography detection for purified biosurfactants

TLC has been used for detection and composition of rhamnolipids in culture broth extracts[18]. Under normal phase TLC conditions monorhamnolipids and dirhamnolipids are separated into two bands. Dissolve a small quantity of crude extract in chloroform and apply 10 µl onto a TLC plate and apply at point of origin near the bottom of the plate. Previously purified rhamnolipids should be applied as standards for comparison. Once dried, develop plate in solvent system of chloroform: methanol: acetic acid (6.5:1.5:0.2, v/v/v)[19]. When developed remove plate and allow air-drying in a fume cupboard. Anthrone reagent was sprayed evenly and place in an oven at 110ºC for 20 min. On visualisation the spot (green colour) nearer the point of origin corresponds to the dirhamnolipids, while the spot further from the point of origin represents the monorhamnolipid.

2.6.2 Fourier transform infra-red spectroscopy (FTIR) spectrum

The biosurfactant extract was investigated to FTIR spectral analysis in the mid IR region of 400-4000/cm. FTIR spectrum was recorded on a Thermo Scientific (USA), Nicolet 6700. The FTIR spectrum in 80% methanol confirmed the presence of functional groups in the absorption peaks.

2.6.3 Electrospray ionisation–mass spectrometry (ESI–MS)

ESI tandem mass spectra were acquired by mass-selecting the target ion using a quadrupole mass analyzer. The conditions of the analyses were: TSQ Quantum ultra (AM) quadruple instrument (Finnigan Ltd., Foster City, CA, USA). Negative ion mass spectra were used. The scanning mass range was 30~800 Da.

2.6.4 ^{1}H and ^{13}C-Nuclear Magnetic Resonance Spectrum

^{1}H and ^{13}C NMR spectrum were measured in DMSO-*d*6 on a Bruker (Germany), advance 600 and probe head: 5mm PABBO BB, 500 NMR Spectrophotometer at 500 MHz for 1hr.

2.7 Antitumor activities

The MTT assay developed by Mosmann[20] was modified and used to determine the inhibitory effects of test compounds on cell growth *in vitro*. In brief, the trypsinized cells from T-25 flask were seeded in each well of 96-well flat-bottomed tissue culture plate at a density of $5x10^3$ cells/well in growth medium and cultured at 37°C in 5% CO_2 to adhere. After 48hr incubation, the supernatant was discarded and the cells were pretreated with growth medium and were subsequently mixed with different concentrations of test compounds (12.5,25,50,100,200 µg/ml) in triplicates to achieve a final volume of 100 µl and then cultured for 48 hr. The compound was prepared as 1.0 mg/ml concentration stock solutions in DMSO. Culture medium and solvent are used as controls. Each well then received 5 µl of fresh MTT (5mg/ml in PBS) followed by incubation for 2 hr at 37°C. The supernatant growth medium was removed from the wells and replaced with 100 µl of DMSO to solubilize the colored formazan product. After 30 min incubation, the absorbance (OD) of the culture plate was read at a wavelength of 570 nm on an ELISA reader, Anthos 2020 spectrophotometer.

3. RESULTS AND DISCUSSION

A total 10 actinomycetes colony was isolated from the naval dockyard soil which was contaminated with petroleum oil near Visakhapatnam. Out of these 10 NDYS-4 showed promising biosurfactant[17].

3.1 Identification and characterization of the isolate NDYS-4

Outer surface of colonies were perfectly round initially, but later were developed into thin wavy mycelium. The colour of the aerial mycelium observed was white or grey and colour of the substrate mycelium was yellowish pink by studying the morphology and SEM (Figure 1), 16s rRNA gene sequencing, homology and Phylogenetic tree (Figure 2); the isolated strain was found to be *Streptomyces coelicoflavus* (NBRC 15399^{T}).

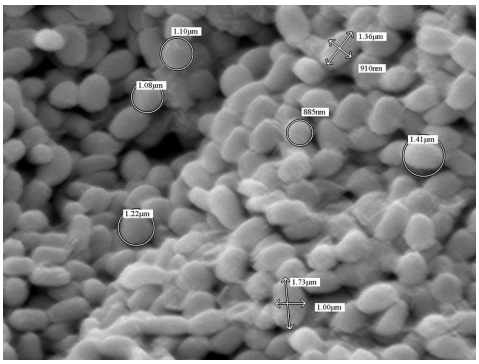

Figure 1 : Scanning electron microscopic image of NDYS-4 isolate

3.2 Solvent extraction

The solvent extraction using the mixture chloroform-methanol (2:1 v/v) gave the highest recovery percent, approximately 90% of recovery. Similar results were seen from rhamnolipid produced by *Pseudomonas aeruginosa* USMAR-2[21].

3.3 Purification of biosurfactants

Nearly 60 mg of the pure compound was obtained. This was further used for spectral analysis and identification of the compound. The pure compound was tentatively named as NDYS-4 E.

3.4 Characterization of bioactive compound NDYS-4

The compound NDYS-4 E was isolated as white waxy solid. IR spectrum of compound NDYS-4 E (Figure 3) showed signal at 173 δ (-C-O), 2853 (H-C=C), 3004 (H-C-), 3406 (br, -OH).

The NMR spectrum of compound NDYS-4 E and its chemical shifts was shown in Table 1. The chemical shifts of 1H protons (Figure 4) in the mono Rhamnolipid was exhibited 2.34(t,2H) ,1.65(t,2H), 1.33(d,2H), 1.28 (d,2H), 0.89(t,2H), 2.01(d,2H), 5.36(t,2H) ,5.36(t,2H) 4.19(h,2H), 3.91(d,2H), 3.60(dd,2H), 3.82(dd,2H), 3.60(dd,2H), 0.89(t,2H), 1.25(dd,2H) in the aliphatic region, no signals were observed in the aromatic region. Analysis of 13C (Figure 5) give the number of carbon atoms and 2 methyl groups, 6 methylene groups, 4 methane groups which were compared by heteronuclear multiple quantum coherence. 2D NMR spectra (1H-1H), of compound (NDYS4 E) has rhamnose sugar ether linked with monosubstuited lipid.

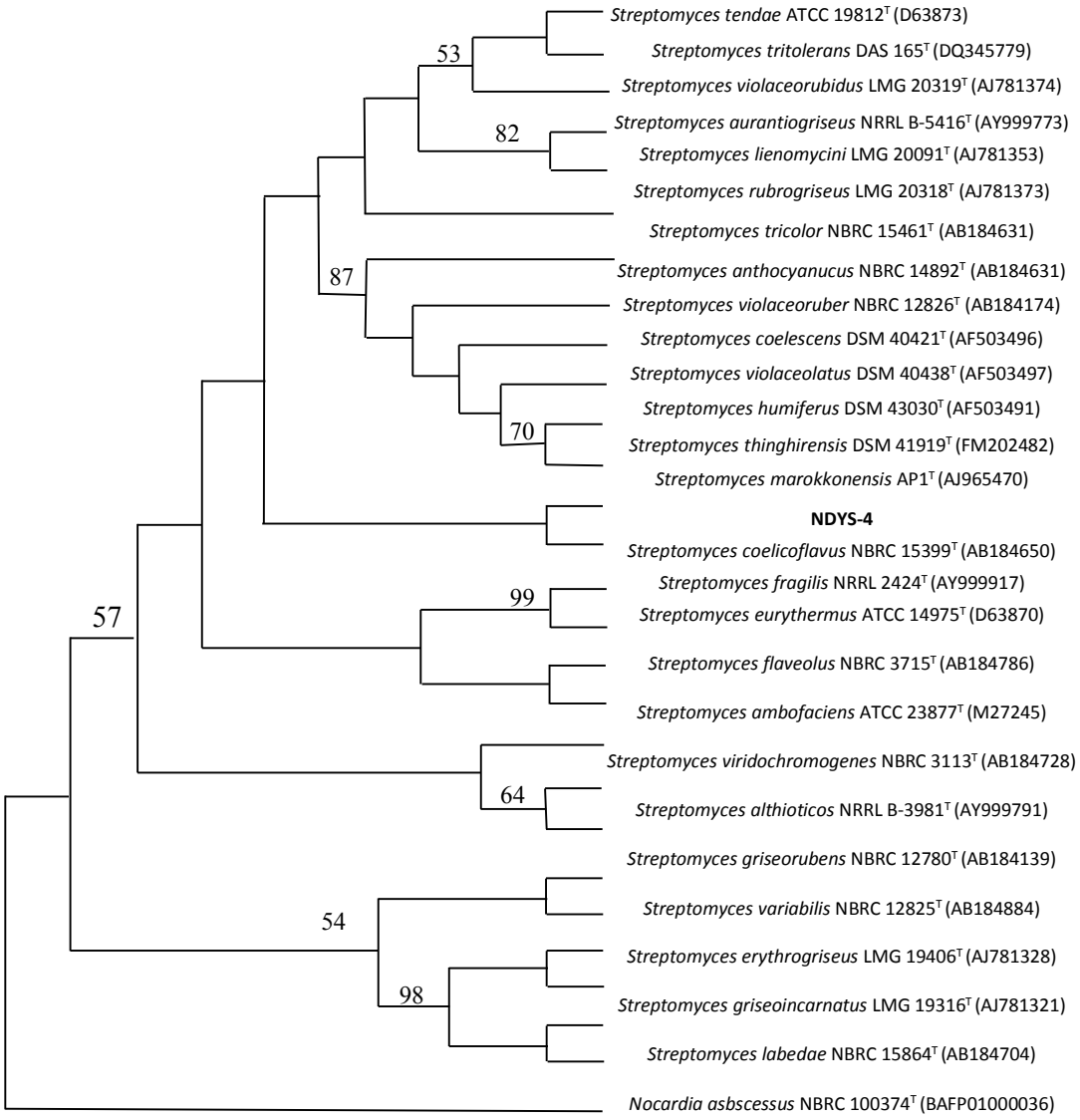

Figure 2 : Neighbour-joining Phylogenetic Tree of the isolate NDYS-4 made by IMTECH.

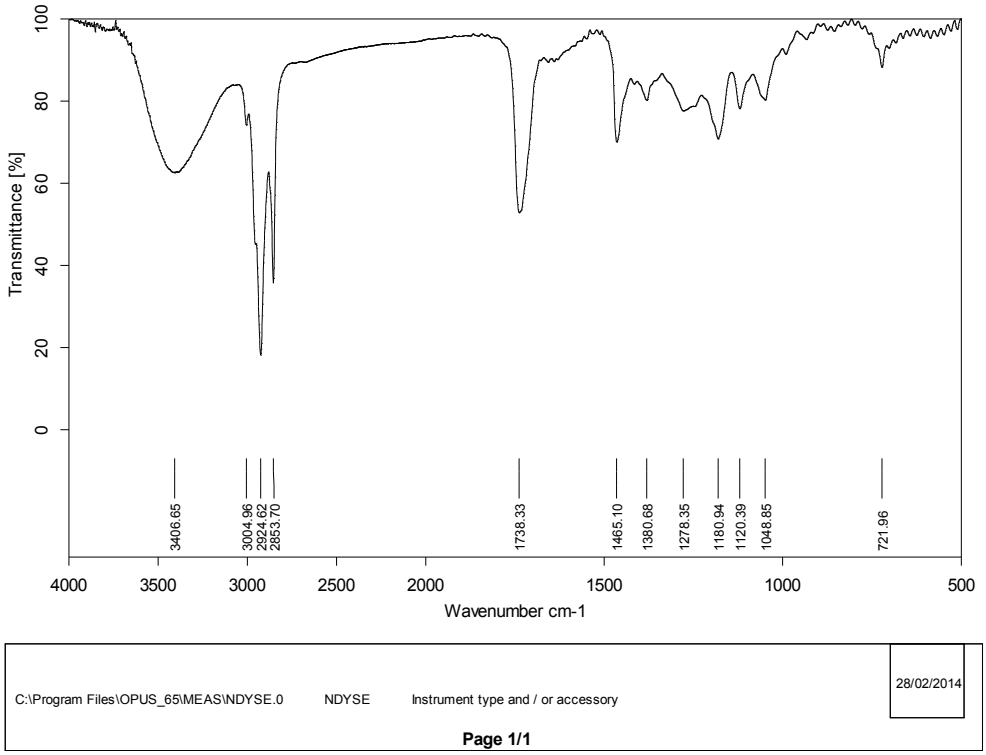

Figure 3: IR Spectrum of the compound NDYS-4 E

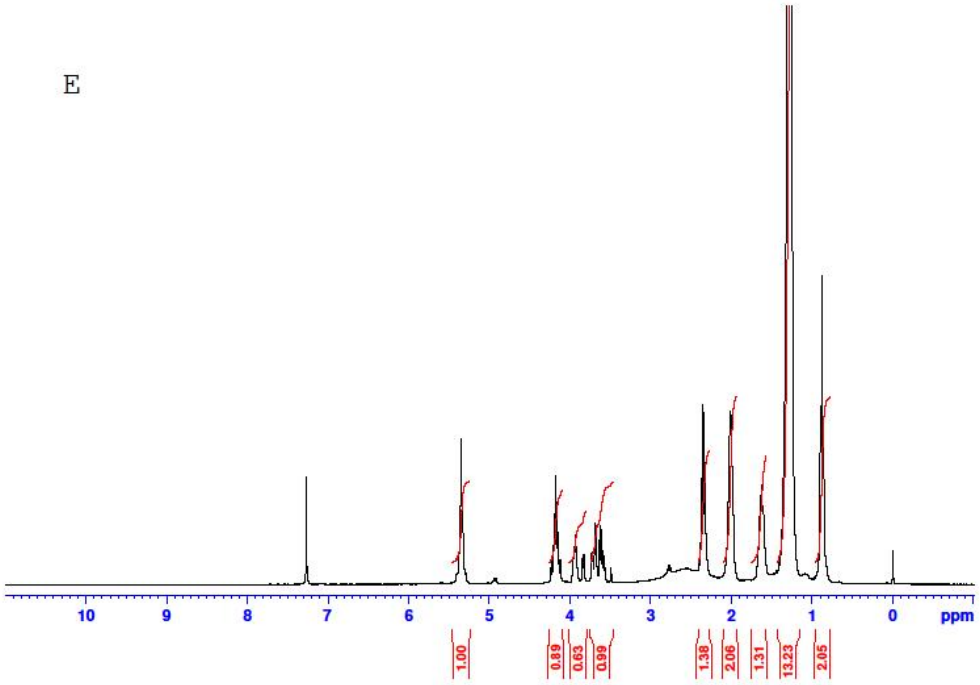

Figure 4 A : ^{1}H Proton NMR Spectrum of the compound NDYS-4 E

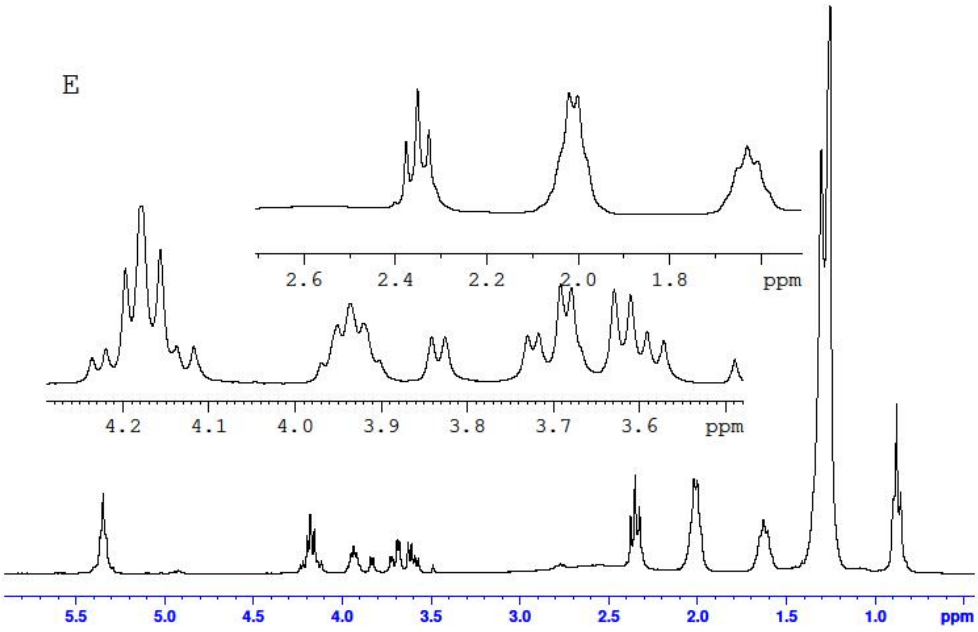

Figure 4 B : (Extension) ^{1}H Proton NMR Spectrum of the compound NDYS-4 E

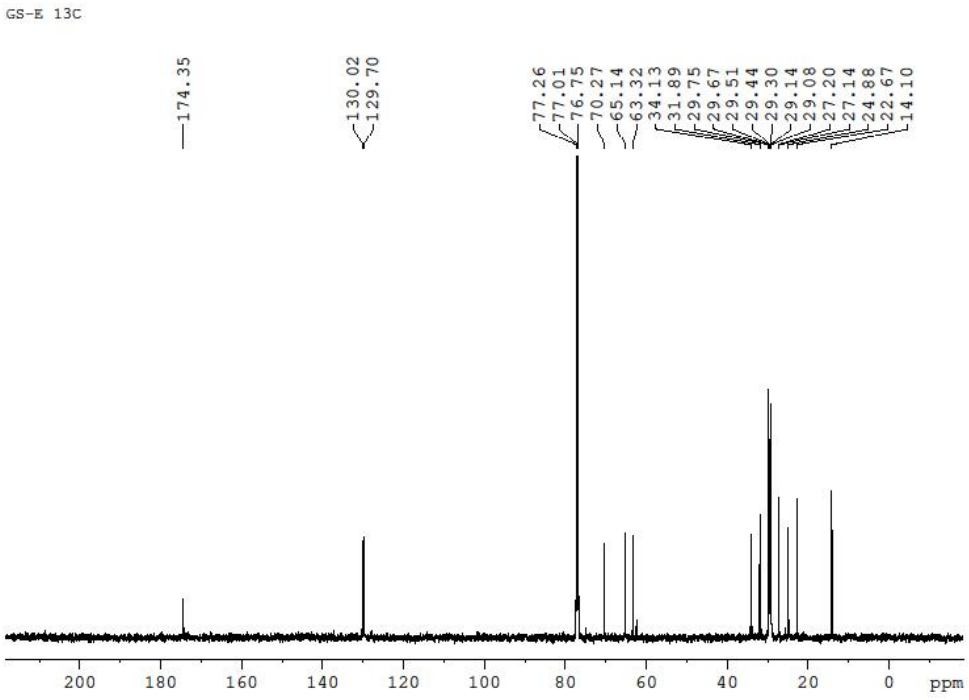

Figure 5A : ^{13}C NMR Spectrum of the compound NDYS-4 E

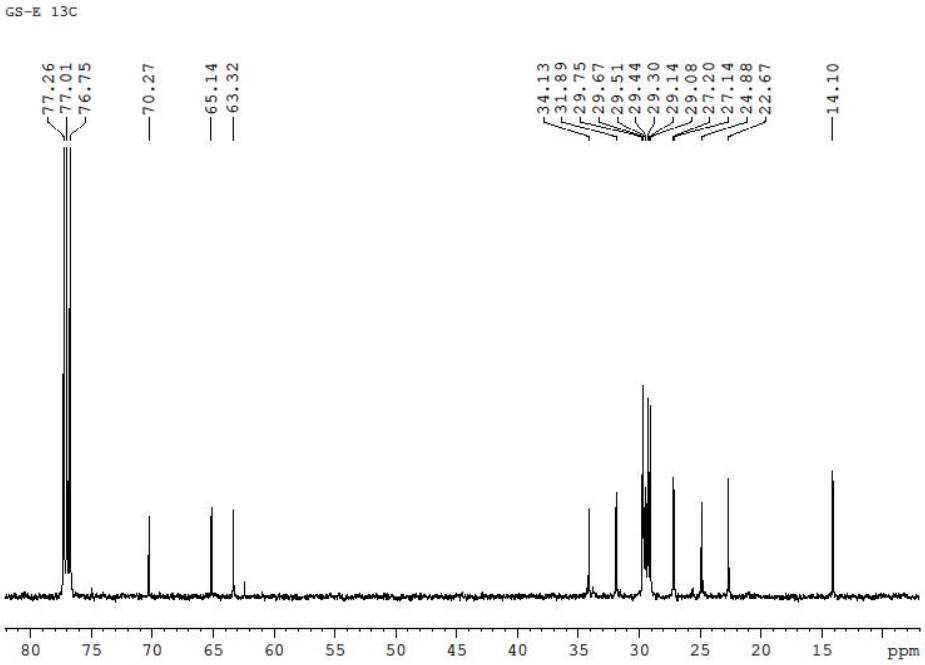

Figure 5B : (Extension) ^{13}C NMR Spectrum of the compound NDYS-4 E

Table 1 : The NMR spectrum of compound NDYS-4 E and chemical shifts

Position		*δ*H (*J* in Hz)	*δ*C	COSY	HMBC
1.		COOH	174.1	H6-H5-H3-H2,	--
2.	CH2	2.34(t,2H)	34.27	H4-H7-H8,	C3, C4, C1.
3.	CH2	1.65(t,2H)	24.98	H12-H11.	C4, C2, C1.
4.	CH2	1.33(d,2H)	29.62		C6, C3, C7, C2, C8.
5.	CH2	1.28 (d,2H)	32.01		C6, C3, C7, C2, C8.
6.	CH3	0.89(t,2H)	14.01		C5, C4.
7.	CH2	2.01(d,2H)	27.18		C4,C8
8.	CH	5.36(t,2H)	129.1		--
9.	CH	5.36(t,2H)	129.1		--
10.	CH2	4.19(h,2H)	65.23		--
11.	CH	3.91(d,2H)	70.33		--
12.	CHOH	3.60(dd,2H)	63.25		C10,C11
13.	CHOH	3.82(dd,2H)	62.5		C10,C11
14.	CHOH	3.60(dd,2H)	63.25		--
15.	CH3	0.89(t,2H)	22.09		--
16.	CH	1.25(dd,2H)	22.7		--

The ^{1}H-^{1}H COSY NMR correlations between H-6-H-5-H-3-H2 and H-4-H-7-H-8 supported that this chain was lipid moiety and rhamnose proton were confirmed by H-11-H-12 and H-13-H-14. By the ^{13}C NMR spectrum of (NDYS-4 E) aided by heteronuclear multiple quantum coherence (HMQC), showed sp carbon ten methine CH signals at aliphatic region.

In HMBC correlations of H-6 (δH 0.89) with C_5,C_4, H-5/4 (δH 1.28/1.33) with C_6,C_3,C_7,C_2,C_8; H-3 (δH 1.65) with C_4,C_2,C_1; H-2 (δH 2.34) with C_3,C_4,C_1; H-7 (δH 2.01) with C_4, C_8; H-12/13 (δH 3.6/3.8) with C_{10}, C_{11}. Based on the biochemical characteristics of compound NDYS-4 E, 1D and 2D NMR data and mass spectrum (Figure 6) the isolated compound NDYS-4 E (Figure 7) molecular formula is established as $C_{16}H_{28}O_7$, Molecular weight is 332.1D.

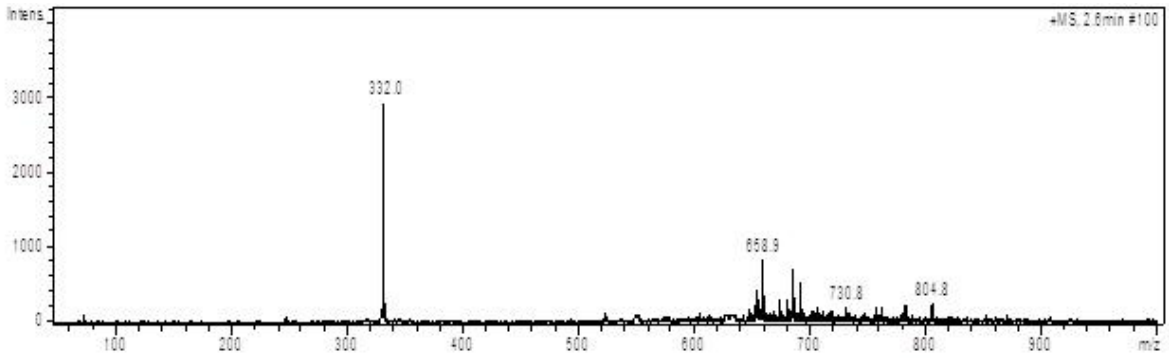

Figure 6 : Mass Spectrum of the compound NDYS-4 E

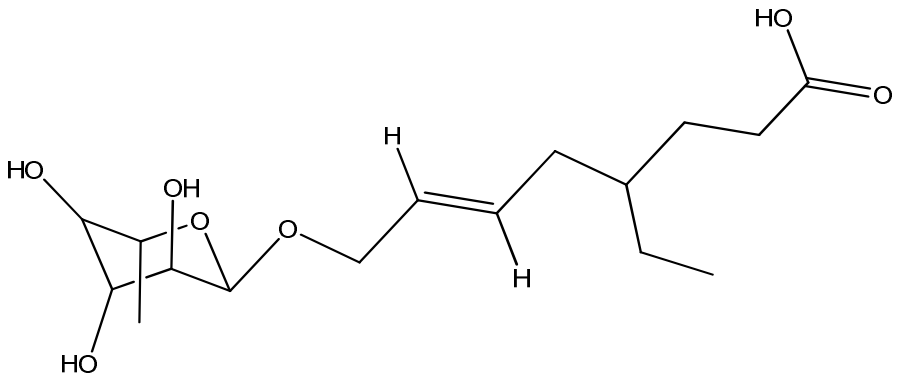

Figure 7 : Mono Rhamnolipid

3.5 TLC Analysis

By TLC analysis we can estimate the type of glycolipid primarily. If the spot appears near the origin it indicates dirhamnolipids type where as far away from origin, indicates monorhamnolipid type of biosurfactant after spraying anthrone reagent. Out of 2 isolates PLS-1 and NDYS-4, only NDYS-4 showed clear differentiation in TLC analysis as shown in Figure 8. Similar studies were reported on the purification and characterization of rhamnolipid biosurfactants produced through submerged fermentation using orange fruit peelings as sole carbon source and Kim *et al.*, 2000 purified and characterized biosurfactants from *Nocardia* sp. L-417[22,23].

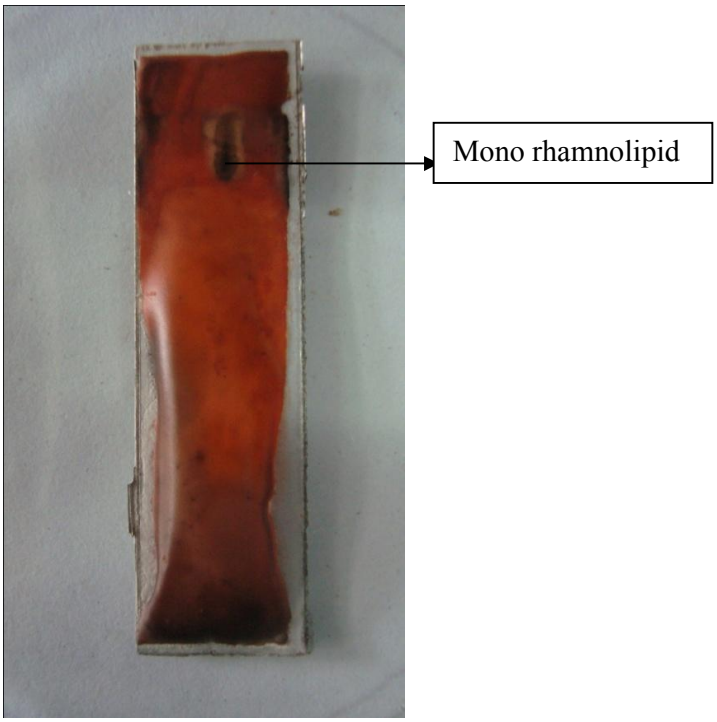

Figure 8: TLC of purified fraction of the isolate NDYS-4

3.6 Antitumor activity analysis

Using MTT assay, the *in vitro* cytotoxicity effect of the purified biosurfactant NDYS-4 (E) mono rhamnolipid on the growth of tumor cell line was studied. The LC_{50} values were calculated against Human Breast cancer MCF7 cells as shown in Table 2 and Figure 9 The incubation of MCF7 cells with gradual doses of purified biosurfactant (NDYS-4 (E)) mono rhamnolipid leads to a gradual inhibition in the cell growth as concluded from its low LC_{50} values 88.602 µg/ml respectively.

The MTT assay showed that purified biosurfactant (NDYS-4 (E)) possessed high anti tumor activity with low LC_{50} values of 88.602 µg/ml compared to the anti tumor activity of Tamoxifen (Sigma) having LC_{50} values 17.68 µg/ml of against MCF7 cells respectively.

Table 2 Dose Response of Sample on MCF-7 (Breast Cancer) Cell line

Conc. (ug/ml)	OD of Tamoxifen at 570 nm	% Cell Survival	% Cell Inhibition	OD of extract at 570 nm	% Cell Survival	% Cell Inhibition
6.25	0.323	79.18	20.82	0.378	94.06	5.94
12.5	0.263	62.97	37.03	0.332	81.62	18.38
25	0.182	41.08	58.92	0.291	70.54	29.46
50	0.138	29.19	70.81	0.253	60.27	39.73
100	0.086	15.14	84.86	0.189	42.98	57.02
200	0.051	5.67	94.33	0.164	36.22	63.78
400	0.042	3.24	96.76	0.102	19.46	80.54

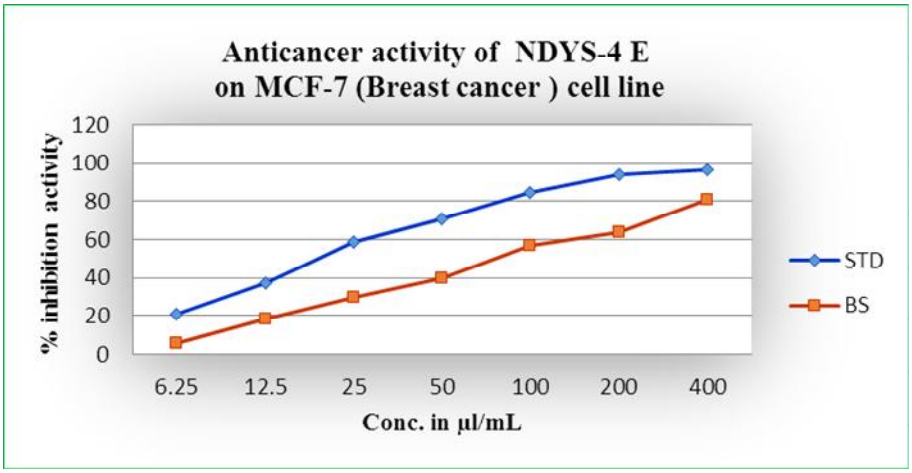

Figure 9 : Anti cancer activity of NDYS-4 E on MCF-7 cell line

4. CONCLUSION

After the characterization of the purified metabolins of *Streptomyces coelicoflavus* NBRC (15399^T) by TLC, FTIR, ESI-MS and ^{1}H and ^{13}C-NMR spectra's it was found that there were two major products, mono- rhamnolipids, di-rhamnolipids. Rha-C10-C10 and Rha2-C10-C10 were respectively verified as the major components of the mono- and di-rhamnolipids. Mono- rhamnolipids showed significantly anti-proliferative activity while the other two fractions and the crude extract didn't have this potential. The IC_{50} value of mono-rhamnolipid to MCF-7 was 88.602 µg/ml compared to the standard. Thus, mono-rhamnolipids produced by *Streptomyces coelicoflavus* NBRC (15399^T) could have potential applications as anticancer drug.

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