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Act-2 A, B and C New Antibiotics from Soil Screened *Streptomyces* species

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The primary objective is to isolate and characterize soil *Streptomyces* having the potential to synthesis new antibiotics. In this study, initially eighty nine isolates, then only seven were selected based on its antibacterial activities, among seven isolates, the *Act-2* was selected as the best organism based on their prominent antibacterial and antifungal activity. The selected *Act-2* isolate was undergone taxonomical and microscopical studies which were confirmed to the typical biochemical behavior resembling of *Streptomyces* species. These studies helped to design the bioprocess media for antibiotic production. The products such as *Act-2* A, B and C were recovered by using the selected *Act-2* strain for production of antibiotic from the designed production medium in bioprocess. The recovered antibiotics were purified by column chromatographic technique. The purified antibiotics were characterized by UV, FTIR, ^{1}H NMR and mass spectroscopic techniques. The purified compounds of Act-2A, B and C were found to be better antibacterial (25-200μg ml^{-1}), antifungal (25-200μg ml^{-1}) and cytocidal effects (1-40μg ml^{-1}). Since, the fractions of *Act-2* A, B and C were found to be better antimicrobial activities, even though it has significant cytocidal activity. Therefore it is worthwhile to study this compound for further work will be required to understand the antitumor activity.

Key words: *Streptomyces*, Act-2 A, B and C, bioprocess, antibacterial, antifungal and cytocidal activities.

Programmes aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have found applications in human medicine and agriculture. Among antibiotic compounds from microorganism has to combat opportunistic pathogens such as bacteria, fungi, protozoa and viruses. Development of resistance in pathogenic microorganisms to most, if not all, antibiotics, together with increasing emphasize research has resulted in an intensified search for alternative new antibiotic compounds. So the emergence of multiply antibiotic resistant human pathogens has resulted in an urgent need for new antibiotics[1].

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Streptomyces species is well known history for production of antibiotics. *Streptomyces* produces approximately two-third of all known antibiotics of microbial origin, including over 6,000 different chemical structures, and they continue to be an excellent source of novel compounds[2]. Many of these natural products are commercially important medicinal compounds with a variety of therapeutic uses. Frequently, one of the hurdles in the development of a newly discovered natural product of antibiotic or in the development of a novel antibiotic is the ability to generate sufficient quantity of the compound for further study[3]. The A*ctinomycetes*, which includes the genus *Streptomyces*, are soil bacteria that are well known for their exceptional ability to produce biologically active compounds[4]. The pharmaceutical industry, over several decades, has probably isolated and *screened* millions of *Streptomyces* strains. Consequently, the chances of isolating a novel *Streptomyces* strain have substantially diminished, and so has the probability of discovering a novel compound. Therefore, it has been estimated that only a fraction of the antibiotics produced by *Streptomyces* strains have been discovered[5], identifying the undiscovered portion will require a substantial effort. Hence, based on the above said objective, an attempt was made to select *Streptomyces* species *Act-2* strain as a suitable organism from soil for production of antibiotics *Act-2* A, B and C to conform their efficacy on antibacterial, antifungal and cytocidal activities.

MATERIAL AND METHODS

Screening of soil isolate

All the soil samples were collected from in and around Ooty (Tamil nadu, India). The screening methods were described by following the standard procedure as follows[6,7] Waksman (1945) and Emerson et *al.,* (1946).One gram of each sample was added to 1gm of calcium carbonate ($CaCO_3$) in an air-dried flask which was dispersed in 100ml of sterile water with 0.1ml of Tween 80. The flasks were kept on a shaker (Remi Vortex Shaker, India) for half an hour. These flasks were considered as stock cultures. From the stock cultures, 1ml was taken and diluted with 9ml of sterile distilled water in 6 culture tubes to get 10^{-1} to 10^{-5} concentrations of original sample. Then the last three dilutions were plated on sterile petridishes for crowded plating, the dilutions were mixed with antibacterial (Ampicillin 25µg ml^{-1}) and antifungal (Clotrimazole 50µg ml^{-1}) agents in starch casein medium. All plates were kept at 37°C in the incubator and observed for growth every day. After five days, each plate was observed, few *Actinomycetes* colonies which were isolated, based on pin point colonies behavior were preserved[8] in *Actinomycetes* agar slants for further studies.

Screening *Actinomycetes* for antibiotic activity

To obtain pure cultures, the isolates were streaked into Kenknight's agar plates by multiple streaking methods[9,10]. Based on the preliminary morphology observation (zone of inhibition), seven isolates were selected and tested for microbial sensitivity using different stains of bacteria and fungi by cross-streak method[11]. Each isolate was grown on nutrient agar medium while testing against bacteria at 37°C and in YEME agar (yeast extract-malt extract) medium in case of yeast and fungi for seven days at 28°C. Using the following test organisms such as: Gram positive bacteria: *Bacillus subtilis, Staphylococcus aureus,* Gram negative bacteria: *Escherichia coil, Pseudomonas aeruginosa,* Fungi*; Aspergillus niger, Aspergillus fumigates,* Yeasts: *Saccharomyces cerevisiae, Candida albicans* (Ca_5). The selected isolates of the *Actinomycetes* were streaked on their respective media in Petridishes. After 7 d, the test organisms were streaked at right angle to the original streak of *Actinomycetes*. For 24 h culture of bacteria and 48 h culture of fungi and yeast were used as inocula. These petridishes were kept for 24 h incubation at 37°C after inoculation for bacteria while for fungi and yeast 48 h incubation at 28°C. Based on their antibacterial and antifungal properties, *Act-2* isolate was chosen for taxonomical characterization.

Taxonomical studies

Taxonomical studies were performed by following methods such as melanin formation, gelatin liquification, acid production and hydrogen sulfide production tests, along with nitrate broth technique[12]. The cultural, physco-chemical and taxonomical characteristics[9] were observed on melanin formation, gelatin liquification, hydrogen

sulfide production and acid production tests, along with nitrate broth technique. Microscopical studies of isolated strain *Act-2* were carried out by (i) agar block method and (ii) inclined cover slip method. In the agar method, the isolated strain was prepared in thin agar block and was examined under high magnification (phase contrast microscopy, 100X) and in the inclined coverslip method the mycelia adhering to cover slips placed at an angle in growing culture was examined at high magnification. Twenty seven isolates which were taken for primary screening[13,11] to identify antibiotic productivity, only *Act-2* isolate having prominent antibiotics producing capacity was selected for the morphological and cultural characteristics studies, which were performed the method described by (ISP) International *Streptomyces* Project[14,12] . The selected *Act-2* strain was inoculated and incubated at 30°C for 21 d in different media described as follows: ISP cultural media: ISP medium-1 Tyrosine-yeast extract broth, ISP medium-2 Yeast extract- malt extract agar, ISP medium-3 Oat meal agar, ISP medium-4 Inorganic salts starch agar, ISP medium-5 Glycerol-asparagines agar, ISP medium-6 Peptone-yeast extract iron agar, ISP medium-7 Tyrosine agar and Bennett agar. Assimilation is the utilization of carbon source[15] (Dextrose, sucrose, lactose, maltose, D-mannitol, fructose and sorbitol) by microorganisms in the presence of oxygen. Positive assimilation of growth was identified by the change in pH of the medium.

Bioprocess for antibiotic production of *Streptomyces Act-2* strain

Bioprocess for *Streptomyces* strain *Act-2* was carried out by using cultivation media with different ratios of carbon and nitrogen source in the cultivation broth in seed medium[16] and production medium. A seed medium (200 ml) consisted of glucose -1%w/v, soluble starch-1% w/v, yeast extract powder-0.5w/v, beef extract-0.3w/v, calcium carbonate ($CaCO_3$)-0.2%w/v, which was adjusted to pH 7.2 prior to sterilization, was dispersed into each of two 500 ml Erlenmeyer flasks and sterilized. A loopful of *Streptomyces Act-2* (ISP medium-4 slant) was inoculated to each of the medium and cultured under shaking condition at 30° C for 3 d. The production medium three liter (3 l) consisted of soluble starch-20g, sucrose-15g, soya bean meal-20g, yeast extract powder-5g, calcium carbonate ($CaCO_3$)-3.2g, magnesium sulfate ($MgSO_4.7H_2O$)-2.5g, potassium mono hydrogen phosphate (K_2HPO_4)-5g, manganese sulfate ($MnSO_4.H_2O$)-0.2g, sodium chloride (NaCl)-0.01g, ferrous sulfate ($FeSO_4.4H_2O$)-0.002g, Silicone oil-0.3ml, which was adjusted pH 7.0 prior to sterilization. The resultant seed broth (20ml) was inoculated to the production medium and cultured at 30°C for 3 d, the second stage seed culture 180ml was used as an inoculum to initiate the cultivation in five liter (5 l) bioreactor containing three liter (3 l) of a cultivation medium. The cultivation was carried out at 28°C with 1200ml (1.2 l) of air per min and agitation at 200rpm.Culture growth was evaluated, using the reported method[17] by centrifuging untreated and treated bioprocess broth at 5000rpm for 10 min.

Product recovery for *Streptomyces Act-2* strain bioprocess

The recovery of *Act-2* A, B, and C is explained as a schematic diagram in fig 1. Since the antibiotic activity was observed in broth filtrate and mycelia, the active compounds were extracted from broth filtrate (2.6 l) and mycelia after separation by centrifugation from the cultured broth (3 l).One part of the filtrate (1.3 l) was extracted three times with (1.3 l) of n-butanol. The extract was concentrated in vacuum at 40° to obtain a crude oily product 3.11g (crude extract I). Another part of the filtrate (1.3 l) was extracted three times with 1.3 l of ethyl acetate. The extract was concentrated in vacuum at 40°C to obtain crude oily product giving 2.45g (Crude extract II). Both the crude extracts II and I were subjected to silica gel chromatography separation technique using[18, 19] chromatography column (length 15 cm × 3 cm diameter, silica gel 60-120 mesh column). The chromatography column was cleaned using water and rinsed with acetone, after drying, a small piece of cotton was plugged at the bottom of the column. Silica gel (60-120 mesh) was then packed in the column by using methanol: chloroform (1: 9) as solvent system. The crude antibiotic was loaded at the top of the column and eluted using methanol: chloroform (1:9) as solvent system giving A and B fractions at 20 min interval. Thin layer chromatography (TLC) of each fraction was performed using precoated TLC plates and simple glass plates to detect the antibiotic[16]. The TLC plates were exposed to iodine vapors to develop

the antibiotic, if any. The fractions having same Rf value were mixed together and the solvent evaporated at 40°C in a vacuum oven. These fractions were tested for their antibacterial activity by using the agar well diffusion method[19, 16]. The fractions showing antibacterial activity were again purified by using the same above mentioned column chromatography system and purity was

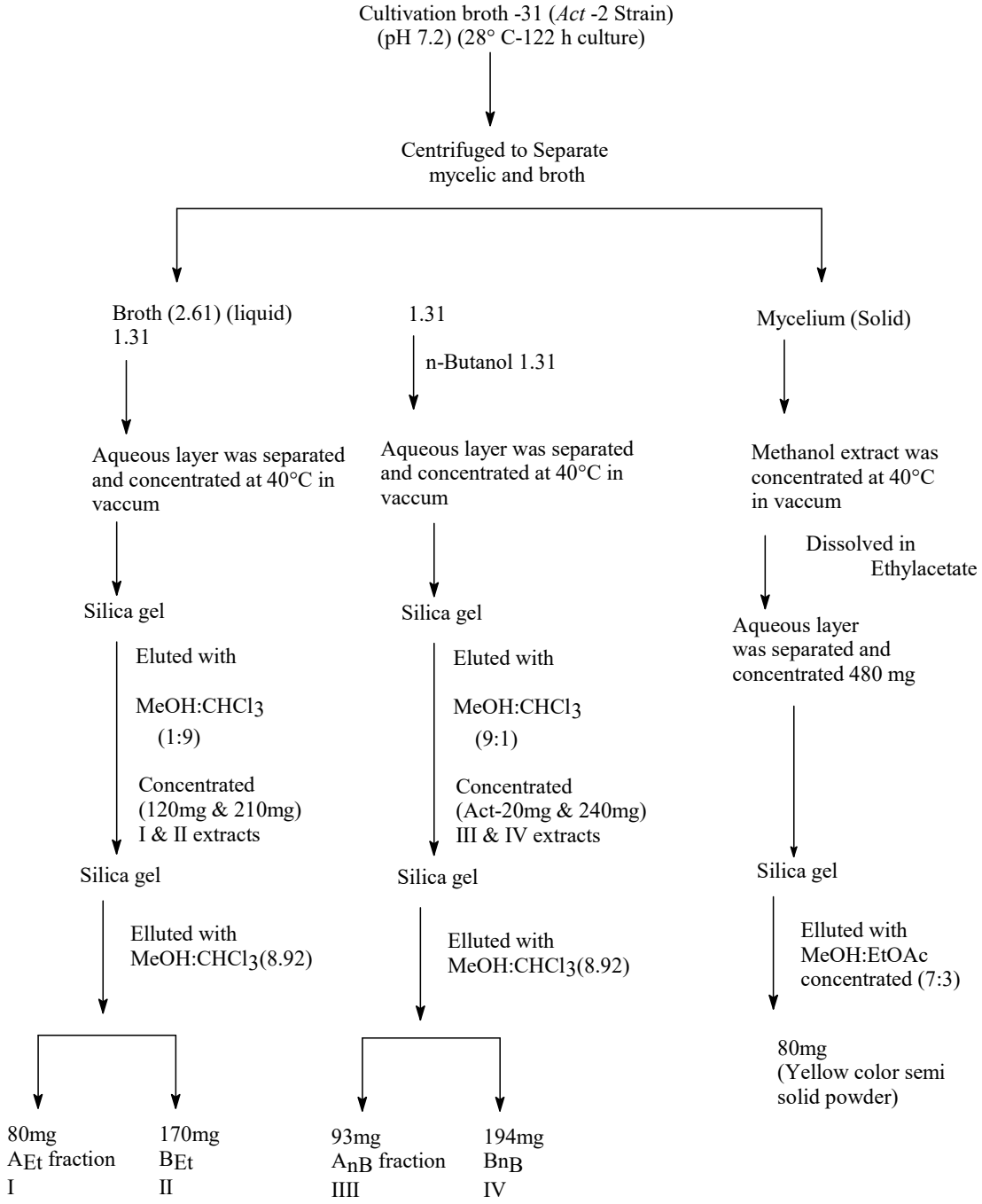

Fig. 1. Schematic diagram for product recovery of *Act-2* A, B &C

was stored on *Actinomycetes* agar slants for preservation for further studies. The 27 isolates of *Actinomycetes* were then observed microscopically in the presence of filamentous, spore and fragmentation characters of *Actinomycetes* as described by Bergey's Manual of Determinative Bacteriology[23]. These isolated strains were tested for microbial sensitivity using different strains of bacteria and fungi which were described in materials and methods by cross streak method. The results showed that only 7 isolates were found to possess better antimicrobial activity. Among 7 isolates, strain *Act-2* was selected, based on prominent antibacterial activity and antifungal activity. The strain *Act-2* showed significant activities against *Pseudomonas aeruginnosa* (32mm), *E.coli* (30mm), *Bacillus subtilis* (31mm) and *Staphylococcus aurues* (29mm). All other isolates exhibited low to moderate activities as shown in Table 1. The *Act-2* strain was very effective towards *Aspergillus niger* (20mm) and *Candida albicans* (31mm) while others exhibited moderate to low activity. The antifungal activity and antiyeast activity are shown in Table 2. Based on the prominent antibacterial and antifungal activities, the selected *Act-2* strain was taken for the taxonomical studies, showed positive results in nitrate reduction, H_2S production, melanin formation, gelatin liquification, starch hydrolysis, and peptonisation tests which were carried out by procedure discussed in materials and methods, the results are shown in Table 3. It showed the positive reaction in gelatin liquification, melanin formation test, nitrate broth technique, acid

Table 1. Microbial sensitivity using different strains of bacteria (Cross streak method) (Zone of inhibition in mm)

Isolate name & Collected place in Ooty	*E. coli*	*P. aeruginosa*	*B. subtilis*	*S. aureus*
105-SM Sixth mile	25	30	24	27
JSSCP-G Garden, JSS College of Pharmacy campus	15	20	25	10
RGICRose garden	27	32	26	24
Act-2Rockland forest	30	32	31	29
Act-1Rockland forest	27	30	21	22
GBG Govt botanical garden	10	7	21	22
104-SMSixth mile	25	26	Nil	Nil

Table 2. Antifungal activity using different strains of fungus (Cross streak method) (Zone of inhibition in mm)

Isolate name and Collected place in Ooty	*A. niger*	*A. flavus*	*S. cerevisiae*	*Candida albicans*		
				Ca_5	Ca_6	Ca_{27}
105-SMSixth mile	20	19	20	Nil	Nil	Nil
Act-2 Rockland forest	10	5	32	30	31	30
Act-1 Rockland forest	Nil	Nil	10	Nil	Nil	Nil
RGICRose garden	Nil	Nil	Nil	Nil	Nil	Nil
JSSCP-G Garden, JSS College of Pharmacy campus	Nil	Nil	Nil	Nil	Nil	Nil
104-SM Sixth mile	10	20	10	Nil	Nil	Nil
GBGGovt botanical garden	5	7	12	Nil	Nil	Nil

production, and negative reaction in hydrogen sulfide production. Formation of melanoid pigment was observed on peptone-yeast extract agar. The selected *Act-2* strain was subjected to morphological, cultural and physiological characteristics (Growth characteristics, reverse color, presence of aerial mycelia with spores, soluble pigment and the microorganisms showed growth, which having a spreading nature and having a matte surface) were observed in ISP media (ISP-2,3,4,5,7 and Bennett's agar), are summarized in Table 4.The aerial mycelium was pale white color on yeast malt extract agar, grayish white on Oct meal agar, greenish gray color on inorganic salts agar, pink white color on tyrosine agar and reddish white on Bennett's agar. Reverse side of the growth culture was pale white on yeast extract-malt extract agar, pale white on oct meal

Table 3. Results of taxonomical studies of *Act-2* strain

Organism Strain no: Name	Melanin formation test	Nitrate reduction	Hydrogen suphide production	Starch hydrolysis test	Gelatin liquification test	Acid production
Act-2	+ +	-	-	+ + +	+	+
	- Negative indicates no melanin formation + Positive melanin pigment formed	Negative No pink color+Positve pink color formed	+Positive yellow color - Negative No yellow color	± clear zone between range -Negative 1-5mm + clear zone between 5-10 mm + + Clear zone between 10-15 mm + + + Clear zone more than 15 mm	+ Positive Gelatin liquefied - No gelatin liquification	+ Positive acid production – Negative No acid production

Table 4. Cultural characteristics of *Act-2* strain

Medium	Cultural characteristics	
ISP-2(YEME)	G: Good slight pink color	R : Pale white-collar
Yeast extract malt extract agar	AM: pale white color	SP :Pale yellow brown color
ISP-3	G: Good	R: Pale white
Oct meal agar	AM: Grayish white	SP: Brown (Mustard)
ISP-4	G: Good	R: Pale white
Inorganic salts agar	AM: Greenish gray	SP: Slightly pink pigmentation
ISP-5	G: Good (Pinkish and white)	R: Rose color with pale
Glycerol asperagine agar	yellow	
	AM: White with pinkish rose	SP: Yellow
ISP-6	G: Good Pale yellow color	R: Pale blackish yellow
Peptone –yeast extract iron agar	AM: Whitish gray	SP: Slightly blackish yellow
ISP-7	G: Good dark pink	R: Light rose color with white
Tyrosine agar	AM: Pink with slightly white	SP: Brick red
Bennett agar	G: Good	R: Brick red
	AM: Reddish white	SP: Brick red

G- Growth, AM- Aerial mycelium, R-Reverse color , SP: Soluble pigments

agar, brick red color on Bennett's agar, rose color on glycerol asperagine agar, pale blackish yellow on peptone yeast extract iron agar .Rose color to brick reddish soluble color pigments was several ISP media. Positive assimilation found to be on various carbon sources with *Act-2* strain in carbon utilization tests. These physico-chemical properties of *Act-2* strain helped to differentiate the species and subspecies in S*treptomyces*. Based on the utilization of carbohydrate sources, protein hydrolysis and peptonization properties and also the antimicrobial activity, *Streptomyces Act-2* strain was selected, for parameters also include the cheaper utilization of the cultivation media. The seed medium and cultivation medium were designed based on taxonomical studies, which gave information about the nutritional requirements of the S*treptomyces* strain *Act-2*. The bioprocess was monitored at every 12 h intervals for the packed cell volume and change in pH. The increasing acidity was neutralized by addition of 1N sodium hydroxide. The pH of broth becomes acidic (pH 6) up to 120 h after which it remained neutral. At 122 h of the bioprocess, the product yield showed a large zone of inhibition against the test organisms. The mycelia were

Table 5. Spectral data showing prominent of the compounds *Act-2* A, B and C

Compounds	UV spectra (solvent DMSO)		FTIR (KBr)	^{1}H NMR (δ) (ppm) (Deteuriated DMSO)	Mass spectra (M^+)	
	λ	Absorbance			Parent	Base
Act-2 A	267.6	1.860	3423.6,2927.9,1719.5, 1654.2,263.9,1379.2, 1350.4,1249.7,1108.5	1-2,2-3,3.5-4, 4-4.5,5-5.5,7-7.5	410.5	62
Act-2 B	273.4	2.137	3435.0,2925.11734.9, 1654.2,1758.4,1350.3, 1249.1,1106.6,951.5	1-2,2-2.5,35-4,4-4.5,5-5.5	305.0	54
Act-2 C	267.0	1.324	3356.9,2360.9,1638.1, Act-212.3,1015.9	1-2,2-2.5,3-4,4-4.5,5-6,7.5-8	209.0	40

Table 6. Antibacterial activities of antibiotic compounds isolated from *Streptomyces* strain *Act-2*

S. No.	Name of the antibiotic compounds of *Act-2*	Minimum Inhibitory Concentration (μg ml^{-1})			
		E. coli	*P. aeruginosa*	*B. subtilis*	*S. aureus*
1.	*Act-2* A	200	100	100	50
2.	*Act-2* B	200	100	100	50
3.	*Act-2* C	200	100	50	25

Table 7. Antifungal activity of antibiotic compounds isolated from *Streptomyces* strain *Act-2*

S. No.	Name of the antibiotic compounds of *Streptomyces* strain *Act-2*	Minimum inhibitory concentration (μg ml^{-1})					
		A. niger	*A. flavas*	*Candida albicans*			
				Ca_5	Ca_6	Ca_{25}	Ca_{27}
1.	*Act-2*A	200	200	50	50	50	50
2.	*Act-2*B	200	200	50	25	25	25
3.	*Act-2* C	100	200	50	25	25	25

separated from the bioprocess broth by centrifugation. The filtrate was extracted with n-butanol and ethyl acetate crude extract I (3.11gm) and crude extract II (2.73gm) respectively. On subjecting these crude extracts to column chromatography (length 15cm × 3cm diameter silica gel (60-120 mesh) column) by eluting with solvents chloroform: methanol (9:1 to 92:8) stepwise, fractions containing active material were obtained. The yields of active fraction were (n-butanol extract)A_{nB} = 93mg and B_{nB}(n-butanol extract) = 198mg and A_{Et} (acetate crude extract) = 80mg and B_{Et} (acetate crude extract)= 170mg from n-butanol crude extract I and ethyl acetate crude extract II respectively obtained by evaporation in vacuum. All these fractions were yellow in color and oily in nature and the Rf values were found be different. The mycelia were also extract first with methanol and then with ethyl acetate. A yellow color fraction was obtained by evaporation in vacuum. Products *Act-2* A, B and C were isolated from extra cellular and intracellular materials by the procedure shown in schematic diagram 1. In an attempt to establish the chemical structure of the antibiotic produced by strains *Act-2*; spectral studies such as UV, FTIR, FAB MS and ^{1}H NMR were performed and the characteristic peaks and their absorbance are shown in Table 5. The λ_{max} of *Act-2* A, B and C was found to be 267.6; 273.4 and 272.4nm respectively. The peaks in each case had a broad shoulder which indicates π- π* transition, n-π* transition which shows the presence of aromatic and ketonic transitory electron characters. The IR studies supported the UV, analysis where the λ_{max} of the first two compounds was almost the same. To confirm the results of the above studies, ^{1}H NMR and Mass spectroscopy were performed. However, it was seen that *Act-2*A differed from *Act-2*B and C. It showed a single peak at 78 indicating the presence of an aromatic group. A corresponding peak was not found in the spectra of *Act-2*C. However, complete structural elucidation to identify the compounds produced by *Act-2* is in progress. The antimicrobial activity of the isolated compounds *Act-2* A, B, and C is shown in Table 6. The antibiotics showed broad spectrum of activity A and B on gram positive and gram negative organisms. *Staphylococcas aureus* was found to be susceptible to the isolated antibiotic. The Antifungal activity of these compounds was also performed. The minimum inhibition concentration (MIC) values are tabulated in table 7. The fractions of *Act-2*A, B and C showed potent antiyeast activity but less antifungal activity in the filamentous fungi. The antibiotic fractions of *Act-2*, A, B and C showed potent cytotoxic activity invitro against *Hep-2 vero* cells event at a very low concentration of 1µg ml^{-1}. The results are shown in Table 8. Since, the fractions of *Act-2*A, B and C were found to be better antimicrobial activities but it has significant cytocidal activity, especially the fraction *Act-2* B which found to possess cytotoxic activity at a very low concentration of 1µg ml^{-1}. Even though it is worthwhile to study this compound, µr further work will be required to understand the antitumor activity.

Table 8. Cytotoxic activity of *Act-2* compounds using *HEp-2 vero* cells

Drug No.	Concentration (µg ml^{-1})	%Viability of cells
Act-2 B	1 µg ml^{-1}	0
	10 µg ml^{-1}	0
	20 µg ml^{-1}	0
	30 µg ml^{-1}	0
	40 µg ml^{-1}	0
Act-2 C	1 µg ml^{-1}	17.30
	10 µg ml^{-1}	4.116
	20 µg ml^{-1}	2.94
	30 µg ml^{-1}	0
	40 µg ml^{-1}	0
Control	-	97.43
Act-2 A	1 µg ml^{-1}	20.00
	10 µg ml^{-1}	17.08
	20 µg ml^{-1}	7.40
	30 µg ml^{-1}	5.88
	40 µg ml^{-1}	3.12

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