ANTIMICROBIAL AND ANTIFUNGAL ACTIVITY OF ENDOPHYTIC ACTINOMYCETES AND ENDOPHYTIC FUNGUS ISOLATED FROM THE ROOTS OF CATHARANTHUS ROSEUS

[1]Jacintha Jasmine.D and [2]Paul Agastian

[1]Research Scholar and [2]Associate Professor

[1] PG & Research Department of Biotechnology, Women's Christian College, Chennai- 600006, India

[2] Department of Plant Biology and Biotechnology, Loyola College, Chennai - 600 034, India

Abstract

Antibiotic resistant pathogens are increasing at an alarming rate globally due to their sustainability to most commonly available antibiotics. Mining for novel antibiotics from unexplored habitats could pave way to combat the problem. Endophytic organisms are found to be beneficial sources of secondary metabolites which can be used against antibiotic resistant pathogens. The increasing demand for novel antibiotics led to the isolation of endophytic actinomycetes and fungi from the roots of *Catharanthus roseus.* The present study was carried out to screen the antimicrobial and antifungal potentials of the ethyl acetate extracts of endophytic actinomycetes and fungi. Surface sterilization technique was performed and plating on starch casein agar for endophytic actinomycetes and water agar plates for endophytic fungi. Morphological, cultural and taxonomic properties for endophytic actinomycetes and fungi were studied. Antimicrobial studies showed that crude extracts of endophytic fungi showed high activity when compared to the ethyl acetate extracts of endophytic actinomycetes. In the case of antifungal activity, using broth micro dilution method, actinomycetes extracts showed a minimum inhibitory activity.

Keywords Endophytic actinomycetes, endophytic fungi, antimicrobial activity, antifungal activity.

1. Introduction:

Pathogenic microorganisms are turning resistant to antibiotics discovered from bacteria and fungi, or developed by chemical synthesis and have become effective chemotherapeutic options. However, the misuse of antibiotics has lessened the efficacy of many commonly used antibiotics. The emergence of resistant strains of bacteria has seriously limited our ability to treat bacterial illness, and new antibiotics are desperately needed (Mathew et al., 2017). This emerging trend is concerning and is considered by the World Health Organization (WHO) to be perhaps the most urgent issue facing medical science (WHO 2016).

Catharanthus roseus commonly called Madagascar periwinkle is an herbaceous sub-shrub of latex producing plants belonging to the family *Apocynaceae* (Gajalakshmi et al., 2013). This plant is a native to Madagascar but also found in Malaysia, where it is called Kemunting Cina and is popularly employed in landscaping or gardening due to its colorful flowers. This ornamental plant is also reported to be used as anticancer where it produced the alkaloids called vincristine and vinblastine (Balaabirami and Patharajan, 2012). The report on the medicinal efficacy of this plant incurs the current surge in its global market, and thus the flower of this plant was chosen as a logo for the National Cancer Council Malaysia (MAKNA).

The plant associated microorganisms are believed to produce similar metabolites as their host plant. Endophytes, microbes that colonize healthy tissues of the plant for at least part of their life cycle without causing any apparent disease symptoms in their host (Petrini, 1991). These endophytes are also recognized as rich sources of secondary metabolites of multifold importance (Tan and Zou, 2001) including enzymes and plant growth

hormones (Carol, 1988). Some of these metabolites are bioactive compounds that demonstrated potent anticancer, antibacterial and antiarthritis activities.

Endophytic fungi spend the whole part of their life cycle living symbiotically within the healthy tissues of the host plant (Tan & Zou, 2001; Ravindra et al., 2014). Some chemical defenses once thought to be produced by the plant have since been shown to be synthesized by endophytic fungi. The chemical basis of insect resistance in endophyte-plant defence mutualisms has been most extensively studied in the perennial ryegrass and three major classes of secondary metabolites are found: indole diterpenes, ergot alkaloids and peramine (Betina, 1984, Rutschmann et al., 1978 and Zhang et al., 2009). Related compounds are found across the range of endophytic fungal associations with plants. The terpenes and alkaloids are inducible defences which act similarly to defensive compounds produced by plants and are highly toxic to a wide variety of phytophagous insects as well as mammalian herbivores (Zhang et al, 2009).

A wide spectrum of actinomycetes have been found to produce key drugs that have been used as biomedical agents but it is difficult to find novel compounds that are of in great demand. It is well known that many kinds of bacteria live in and around plant roots, including the phyla Proteobacteria, Actinobacteria (Gram-positive high G+C bacteria), Bacteroides and Verrucomicrobia, which have been detected by pyrosequencing (Romero et al, 2014). Over 40 genera of filamentous Actinobacteria (actinomycetes) have been detected from Triticum aestivum roots alone by terminal restriction fragment length polymorphism (Conn et al, 2004). Notably, the genus Streptomyces in the family Streptomycetaceae accounted for only 26%, meaning that rare actinomycetes (non- Streptomyces) make up 74% of the total and other reports also indicated that rare actinomycetes are majority in plant, (Li, 2012) although Kaewkla and Franco reported that Streptomyces strains accounted for 72% of endophytic actinomycetes isolated from Australian native trees (Kaewkla et al, 2013).

Breast cancer is a first killer for women in the world followed by cervical and ovarian cancer. World Health Organization (W.H.O) has estimated that by the year 2030, 12 million people will be diagnosed with the breast cancer. Unfortunately, some people refused to get a cancer treatment since they were afraid that they could not afford to pay for the high cost. Due to very expensive vinblastine and vincristine used in chemotherapy process for certain types of cancer including breast cancer. Naturally, these alkaloids were produced by the Catharanthus roseus. However, this plant takes about one year before it is ready to be harvested and lead the high cost of production. It is reported that it needs 500 kg of Catharanthus roseus's leaves to produce 1 g of purified vinblastine and vincristine that cost ranging from 1 mil USD to 3.5 mil USD/kg (Chandra, 2012).

Secondary metabolite production from endophytic fungi and actinomycetes which dwell in *Catharanthus roseus* provides various insights in the design of novel drug which can be used in the treatment of breast cancer patients.

2. Materials and Methods:

2.1 Isolation and Identification of Organisms:

The roots of healthy *Catharanthus roseus* plants were collected from Loyola College campus located in the Garden, were taken to the laboratory and processed immediately by surface sterilization technique. Each root was split into pieces of 1.0 cm to exposing the cortex and vascular bundles. They were then aseptically plated on starch casein agar medium for actinomycetes and 2.5% water agar medium for fungal isolation. The organisms were identified based on morphological, cultural and biochemical characterization.

2.2 Production of secondary metabolites from endophytic actinomycetes and endophytic fungi:

The isolated actinomycetes and fungi were mass produced by inoculating them in Modified Nutrient Glucose broth (MNGB) and Potato dextrose broth (Himedia, Mumbai) respectively. The inoculated actinomycetes flasks were kept in rotatory shaker at 120 rpm about 10 days for its growth and as still culture for inoculated fungus. Full growth occurred after 10 days and then the broth was centrifuged at 8000 rpm for 10 minutes at 4ºC (Fig 4 & 5). The supernatant was collected and dissolved in equal volume of ethyl acetate and the organic layer was separated using the separating funnel. The solvent was subjected to Rota vacuum evaporator for getting concentrated crude extracts and stored at 4ºC until further use.

2.3 Antibacterial activity:

2.3.1 Test organisms:

The following test organisms commonly used to test antimicrobial activity using disc diffusion method and minimum inhibitory concentration of the extracts were collected from Department of Microbiology, Vellore Institute of Technology, Vellore : *Staphylococcus aureus* ATCC 25923, *Yersinia enterocolitica* MTCC 840, *Pseudomonas aeruginosa* ATCC 27853, *Vibrio parahaemolyticus* MTCC 451, *V. fischeri* MTCC 1738, *Enterobacter aerogens* MTCC 111, *Bacillus subtilis* MTCC 441, *Escherichia coli* ATCC 25922, *Proteus vulgaris* MTCC 1771 and *Candida albicans* MTCC 227.

Apart from this, the extracts were also tested against clinical isolates (Table 3) which were obtained from the Department of Microbiology, Christian Medical College, Vellore and Tamilnadu India. The cultures were maintained by inoculating in 3ml of Mueller Hinton Broth (Himedia) and incubated at 37ºC for 24 h.

Antibacterial activity was carried out using disc-diffusion method (Murray *et al*. 1995). Petri plates were prepared with 20 ml of sterile Mueller Hinton Agar (MHA) (Hi-media, Mumbai). The test cultures (100µl of suspension containing 10^8 CFU/ml bacteria) were swabbed on top of the solidified media and allowed to dry for 10 min. The tests were conducted at three different concentrations of the crude extract (5mg, 2.5mg and 1.25mg per disc). The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Negative control was prepared using respective solvent. Streptomycin (10µg /disc) was used as positive control. The plates were incubated for 24 h at 37ºC. Zone of inhibition was recorded in millimeters and the experiment was repeated twice.

2.4 Antifungal Activity:

2.4.1 Fungal Strains

The following fungi were used for experiments: *Aspergillus flavus, Botyritis cinerea, Curvularia lunata* 46/01, *Aspergillus niger* MTCC 1344, *Trichophyton rubrum* 57/01 and *T. mentagrophytes* 66/01 and *Candida albicans MTCC227.* These strains were maintained in the Department of Biotechnology, Loyola College, Chennai.

The filamentous fungi were grown on Sabouraud Dextrose Agar (SDA) slants at 28ºC for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on Sabouraud Dextrose Broth (SDB) at 28ºC for 48 h.

The antifungal activity was performed according to the standard reference method (NCCLS, 2002). The extracts were dissolved in water with 2% dimethyl sulfoxide (DMSO). The initial concentration of the extract was 1mg/ml. The initial test concentration was serially diluted two-fold. Each well was inoculated with 5 µl of suspension containing 104 spore/ml of fungi. The antifungal agents Fluconazole and Ketoconazole were included in the assays as positive controls; MIC was defined as the lowest extract concentration, showing no visible fungal growth after incubation time.

3. Results and Discussion:

The endophytic actinomycetes and fungi isolated from the roots of *Catharanthus roseus* (Fig 1) were subjected to identification morphologically, culturally and biochemically. Gram staining test performed indicated that it was a Gram positive organism. Lacto phenol cotton blue staining was performed on to the colonies on Potato Dextrose agar (PDA) and *Fusarium oxysporum* was observed based on colony morphology microscopic examination showed colourless or with a tinge of pink, purple or yellow and become dark coloured at maturity (Fig 3). The dark mycelium produces thick bands which plug the vascular tissues and produce serious toxic secretions. Based on microscopic examination and the growth on Starch casein agar (SCA), Actinomycetes was identified based on colony morphology showing vegetative hyphae with extensive branching, aerial mycelium with round shaped sporangia is borne terminally and pinkish red pigment diffused in the surrounding media (Fig2) and biochemical tests like methyl red, indole, citrate, starch hydrolysis, catalase were performed etc.

The present study was to evaluate antimicrobial activity of two crude ethyl acetate extracts of endophytic fungi and actinomycetes from the roots of C*atharanthus roseus* tested against selected reference cultures (Table 1). Ethyl acetate extracts of endophytic fungus(EAF) was found to show high activity (23 mm) against *E. aerogens* when compared to positive control, followed higher activity (22mm) was shown against *B. subtilis*. In the case of the ethyl acetate extracts of endophytic actinomycetes (EAA), it showed lesser activity (10mm) against *Y. enterocolitica*, and showed no activity against the other reference cultures (Fig 6). Extracts from LGMB491 (closely related to *A. ponti*) showed great activity against MRSA, with inhibition zones higher than caused by vancomycin, the clinical antibiotic used for the treatment of this resistant bacterium (Francielly et al 2017). Based on the results obtained from the zone of inhibition, minimum inhibitory concentration for crude extract of endophytic fungus was found to be 0.312 mg/ml for both *B. subtilis* and *S. aureus* mentioned (Table 2, Fig 6&7).

The activity against clinical isolates as well as drug resistance microbes showed that EAF showed higher activity (27nm) against MRSA (clinical pathogen), followed by 25nm was against *Enterococcus durans* (P502), 22nm against MRSA [Methicillin Resistant S.*aureus*] ICMR-5, and tracer activity of 10nm against ICMR-19 *Acetobacter baumanii* (Carbapenem R). EAA showed activity (17nm) against *Proteus vulgaris* and tracer activity against S.*aureus*, ICMR-24 [*E.coli*] Cipro R, ICMR-19 *Acetobacter baumanii* whereas EAF showed no activity against some of the clinincal isolates (Table 3, Fig 8, 9 & 10). In another study, ‘‘differences in the relative abundance of endophytic fungi colonizing the roots’’ between transgenic plants and the control plants were observed (Gotz et al. 2006). From the total number of four isolates most were actinomycetes and fungi isolated from roots. Sardi et al (1992) found that from 499 endophytic actinomycetes isolated from 28 plant species about 98% *Streptomyces*, 0.2% *Streptosporangium* and no *Microbispora* were reported.

4. Conclusion:

Antibiotic resistant pathogens are increasing at an alarming rate. Emergence of novel antibiotics may be a striking solution to different diseases. Identification and characterization of isolated endophytic fungus and actinomycetes from the root of *Catharanthus roseus* were studied. Fungus was identified as *Fusarium oxysporum* and *Streptomyces* was identified belonging to the of Actinomycetes group. Ethyl acetate extract of fungus (EEF) and actinomycetes (EEA) were screened for its antimicrobial activity against reference cultures, clinical isolates, drug resistant microbes and pathogenic fungi. Based on the results obtained in the zone of inhibition, minimum inhibitory concentration for the extracts of endophytic fungus using broth micro dilution method, EEF showed a wider range of inhibition against organisms when compared to EEA. The approach is further being critically investigated for maximum yield of the bioactive compound to formulate a novel drug against antibiotic resistant pathogens.

Tables

Table 1: Antimicrobial activity of ethyl acetate extracts of endophytic fungi (EPF) and actinomycete (EPA) from *Catharanthus roseus.* (Zone of inhibition in mm)

Name of the microbe	Ethyl acetate extract						S (µg/disc)
	EPA (mg/disc)			EPF (mg/disc)			
	1.25	2.5	5.0	1.25	2.5	5.0	10
S. aureus (ATCC 25923)	-	-	-	15	16	17	13
Y. enterocolitica (MTCC 840)	10	10	10	10	10	10	24
P. aerugenosa (ATCC 15380)	-	-	-	-	-	-	
V. parahaemolyticus (MTCC 451)	-	-	-	-	-	-	24
V. fischeri (ATCC 1738)	-	-	-	-	-	-	13
E. aerogens (MTCC 111)	-	-	-	19	22	23	19
B. subtilis (MTCC 441)	-	-	-	15	18	22	24
E. coli (ATCC 25922)	-	-	-	-	-	-	10
P. vulgaris (ATCC 1771)	-	-	-	10	13	16	10
C. albicans (MTCC 227)	-	-	-	10	10	11	-

Table 2: Minimum inhibitory concentration of ethyl acetate extract of EPF from *C. roseus.*

Name of the Microbe	MIC (mg/ml of broth)
S. aureus (ATCC 25923)	> 0.312
Y. enterocolitica (MTCC 840)	0.625
P. aerugenosa (ATCC 15380)	ND
V. parahaemolyticus (MTCC 451)	ND
V. fischeri (ATCC 1738)	ND
E. aerogens (MTCC 111)	> 0.156
B. subtilis (MTCC 441)	> 0.312
E. coli (ATCC 25922)	ND
P. vulgaris (ATCC 1771)	0.625
C. albicans (MTCC 227)	> 0.625

MIC : Minimum inhibitory concentration

ND : Not done

Table 3: Antimicrobial activity of ethyl acetate extracts of EPF and EPA from *C. roseus* against clinical isolates. (Zone of inhibition in mm)

Code No	Name of Microbe	Ethyl acetate extract					
		EPA (mg/disc)			EPF (mg/disc)		
	Clinical isolates	**1.25**	**2.5**	**5.0**	**1.25**	**2.5**	**5.0**
17	*ESBL Klebsiella (ICMR-6)*	10	10	10	15	17	17
28	*ESBL, E.coli (clinical pathogens)*	-	-	-	-	-	-
41	*Proteus*	13	15	17	-	-	-
45	*Salmonella paratyphi*						
21	*Staphylococus aureus [Methiciline Sensitive S. aureus]*	10	10	10	11	13	14
32	*Eschericia coli (clinical pathogens)*	-	-	-	-	-	-
10	*Enterococcus durans (P 502)*	-	-	-	24	25	25
15	*MRSA [Methiciline Resistant S. aureus] ICMR - 5*	-	-	-	21	22	22
24	*MRSA(clinical pathogens)*	-	-	-	20	24	27
19	*ICMR-24 [E.coli] Cipro R*	10	10	10	-	-	-
7	*ATCC – 29213 [MSSA]*	-	-	-	14	17	18
20	*ICMR-19 Acetobacter baumanii (Carbapenem R)*	10	10	10	10	10	10
37	*ESBL [Klebsiella pneumonia]*	-	-	-	-	-	-

Table 4: Antifungal activity of EPF and EPA of *C. roseus* using broth micro dilution method MIC (µg/ml)

Tested fungi	EPA (mg/ml)	EPF (mg/ml)	Fluconazole (mg/ml)	Ketoconazole (mg/ml)
Aspergillus flavus	2000	1000	50	<12.5
Botyritis cinerea	1000	125	100	<12.5
Curvularia lunata 46/01	1000	125	<12.5	<12.5
Aspergillus niger MTCC 1344	125	500	100	<12.5
T. rubrum 57/01	2000	62.5	25	<12.5
T. mentagrophytes 66/01	2000	62.5	25	<12.5

Figures

Fig 1a : Isolation of Endophytic actinomycetes from the root of *Catharanthus roseus*

Fig 1b: Isolation of Endophytic fungus from the root of *Catharanthus roseus*

Fig 1: Isolation of endophytes from the root of *Catharanthus roseus*

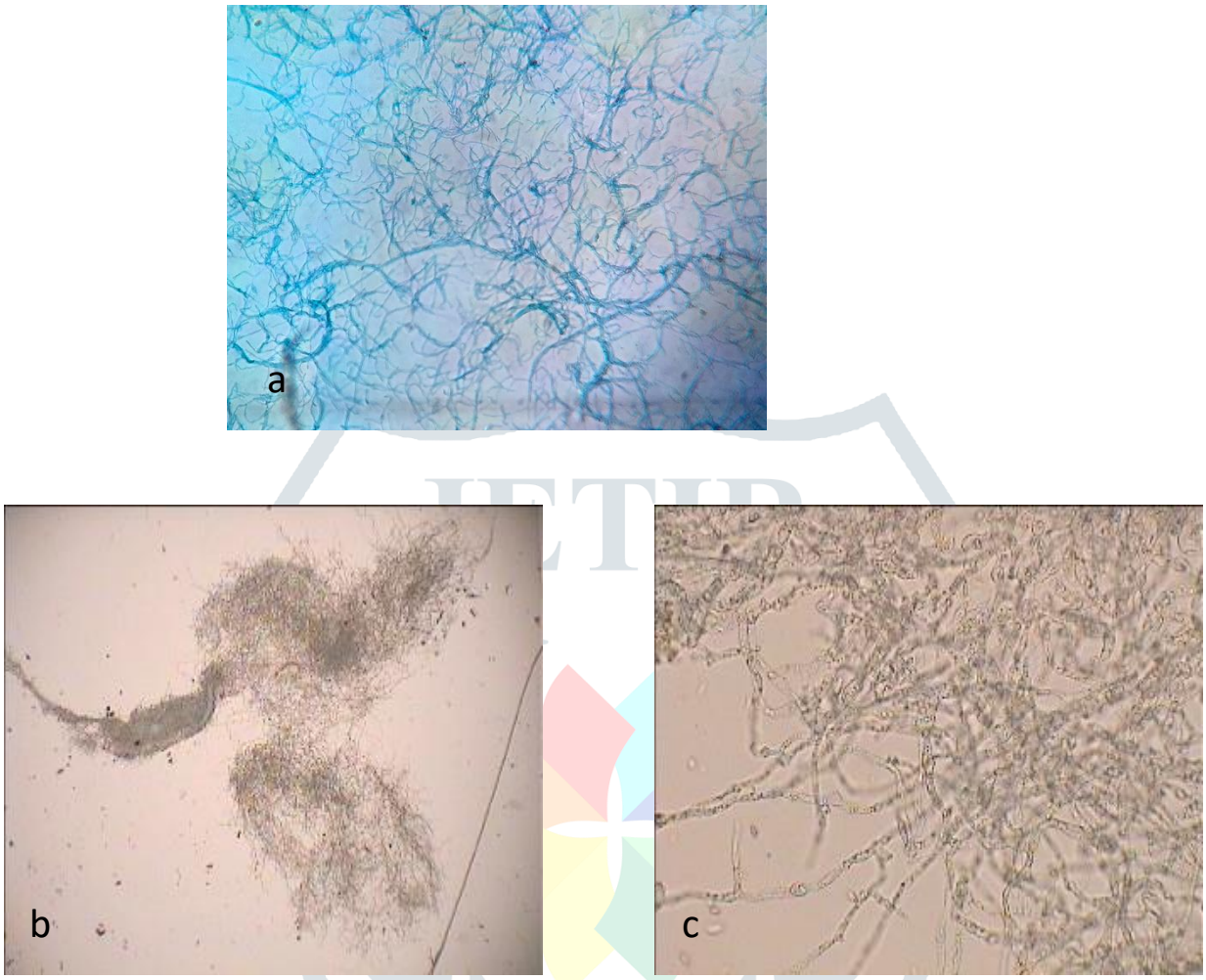

Fig2: Identification of EPA isolated from the root of C.roseus

(a) Gram Staining of endophytic actinomycetes
(b) Photomicrograph of active actinomycete culture at 40x and 400x magnification

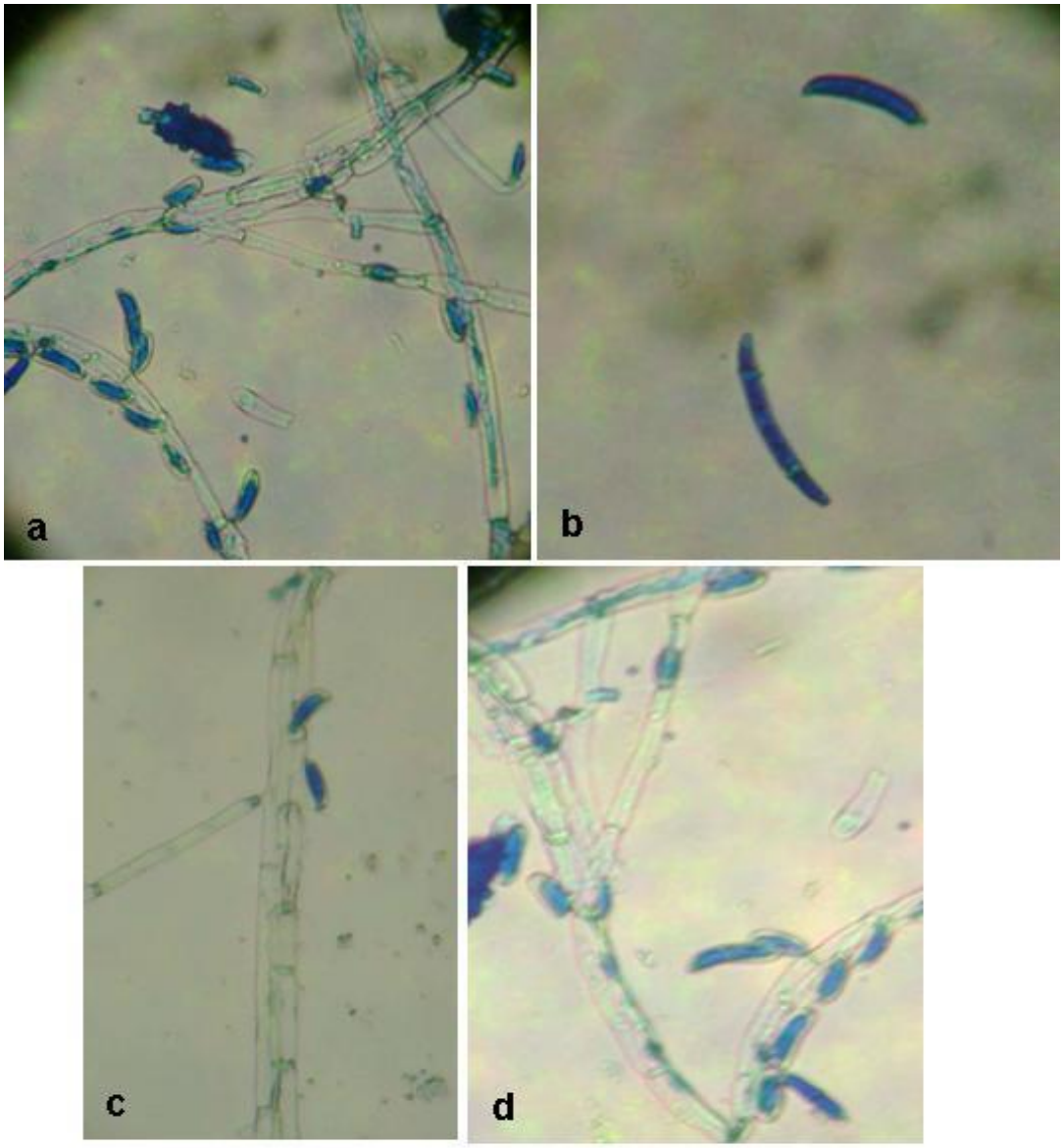

Fig 3: Identification of EPF from the root of *C. roseus*. Lactophenol cotton blue staining EPF filament slide culture with different magnification

a) Slide showing Chlamydospores on hyphae

b) Slide showing Macroconidia

c&d) Slide showing septate and branched hyphae

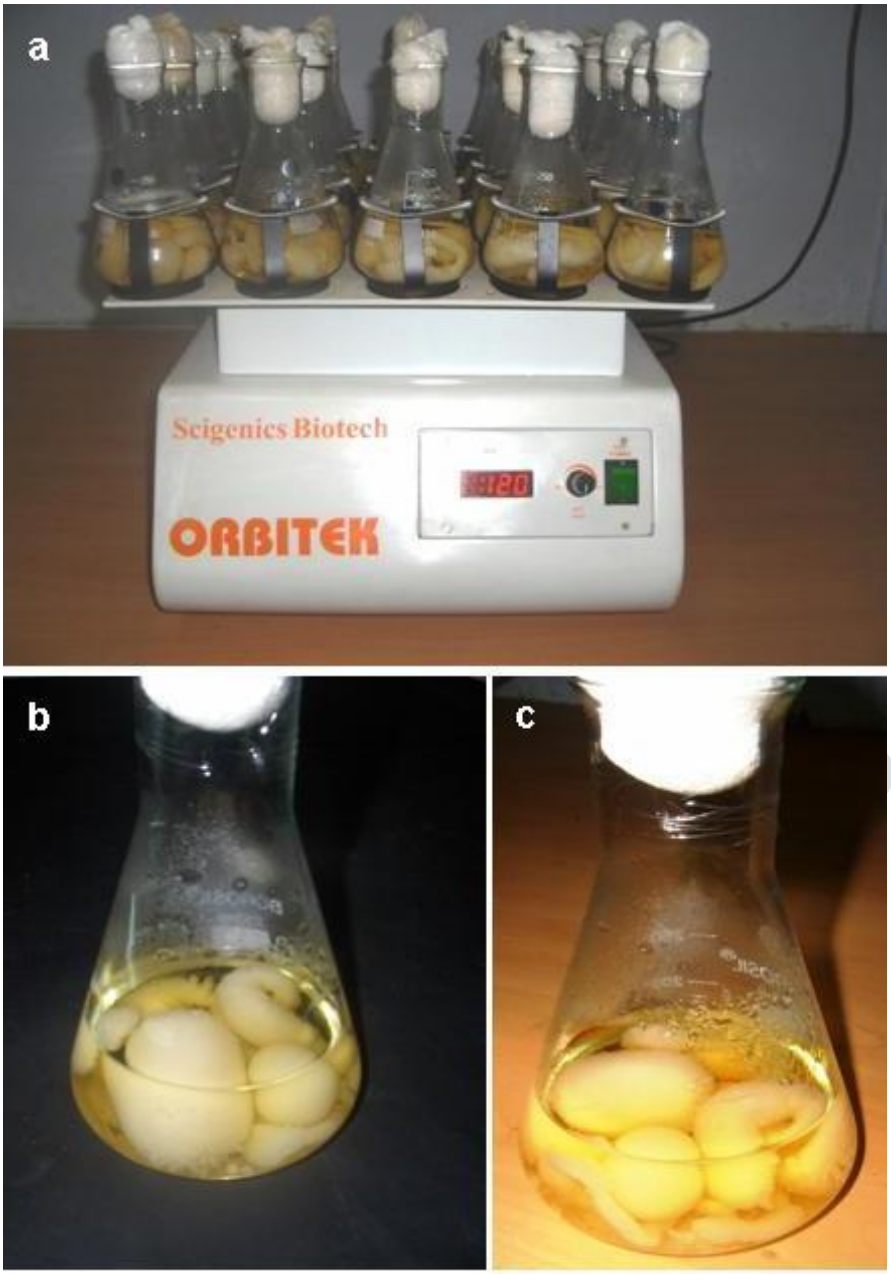

Fig 4: Mass production of EPA of *Catharanthus roseus*

A. Mass produced endophytic actinomycetes in rotatory shaker at 120rpm maintained at 37°C.

B. Clear view of the mass produced endophytic actinomycetes.

C. Well grown spore formed cells of endophytic actinomycetes.

Fig 5: Mass production of EPF using potato dextrose broth

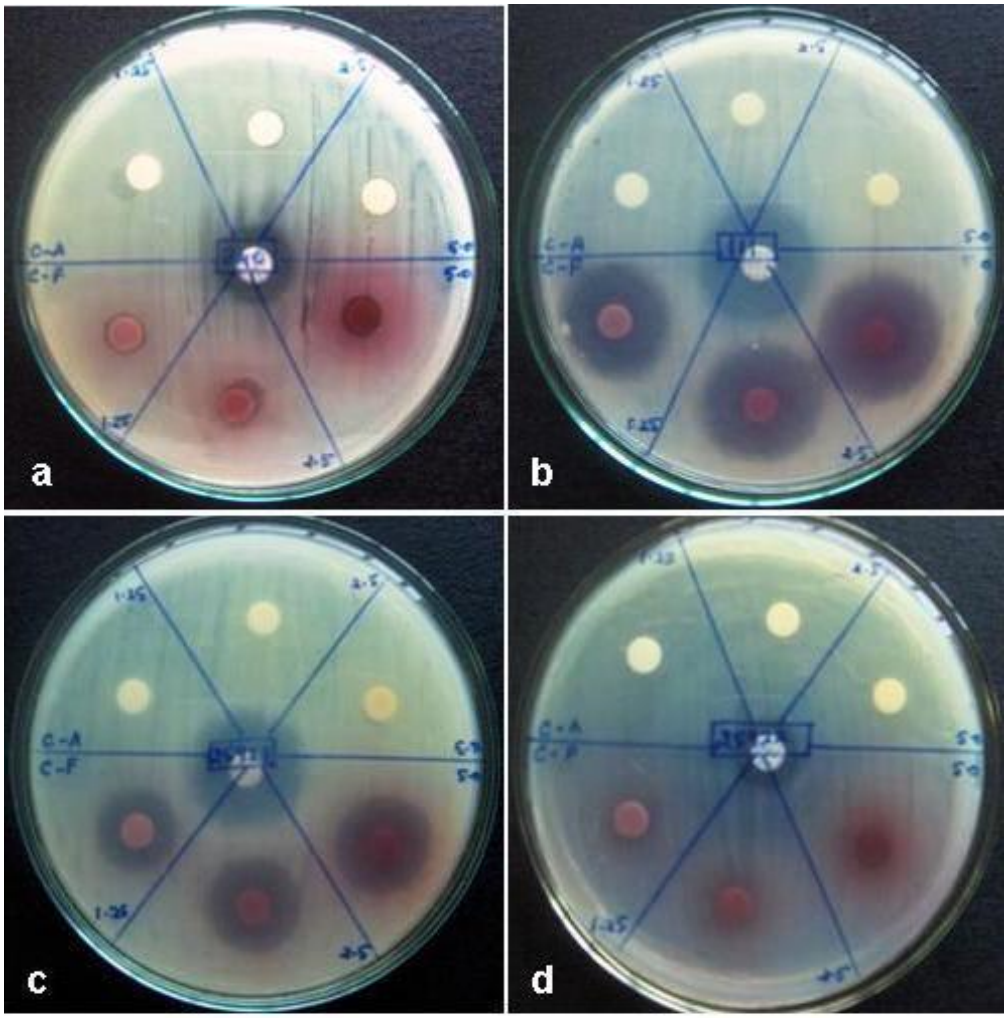

Fig 6: Antibacterial activity of ethyl acetate extract of EPA and EPF from the root of *C. roseus*

A. Crude ethyl acetate extract against *Y. enterocolitica*

B. Crude ethyl acetate extract against *E. aerogens*

C. Crude ethyl acetate extract against *S. aureus*

D. Crude ethyl acetate extract against *E. coli*

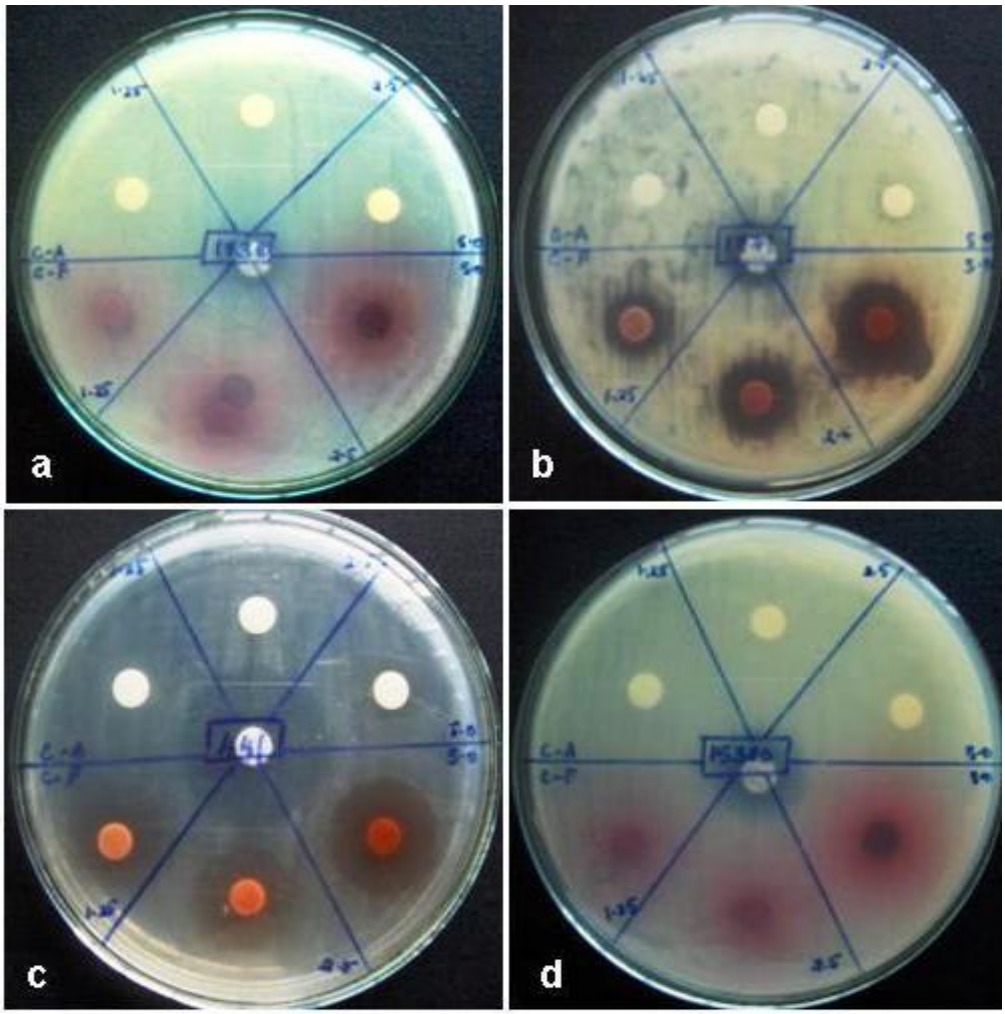

Fig 7: Antibacterial activity of ethyl acetate extract of EPA and EPF from the root of *C. roseus*

A. Crude ethyl acetate extract against *V.fischeri*

B. Crude ethyl acetate extract against *P.vulgaris*

C. Crude ethyl acetate extract against *B.subtilis*

D. Crude ethyl acetate extract against *P.aerogenosa*

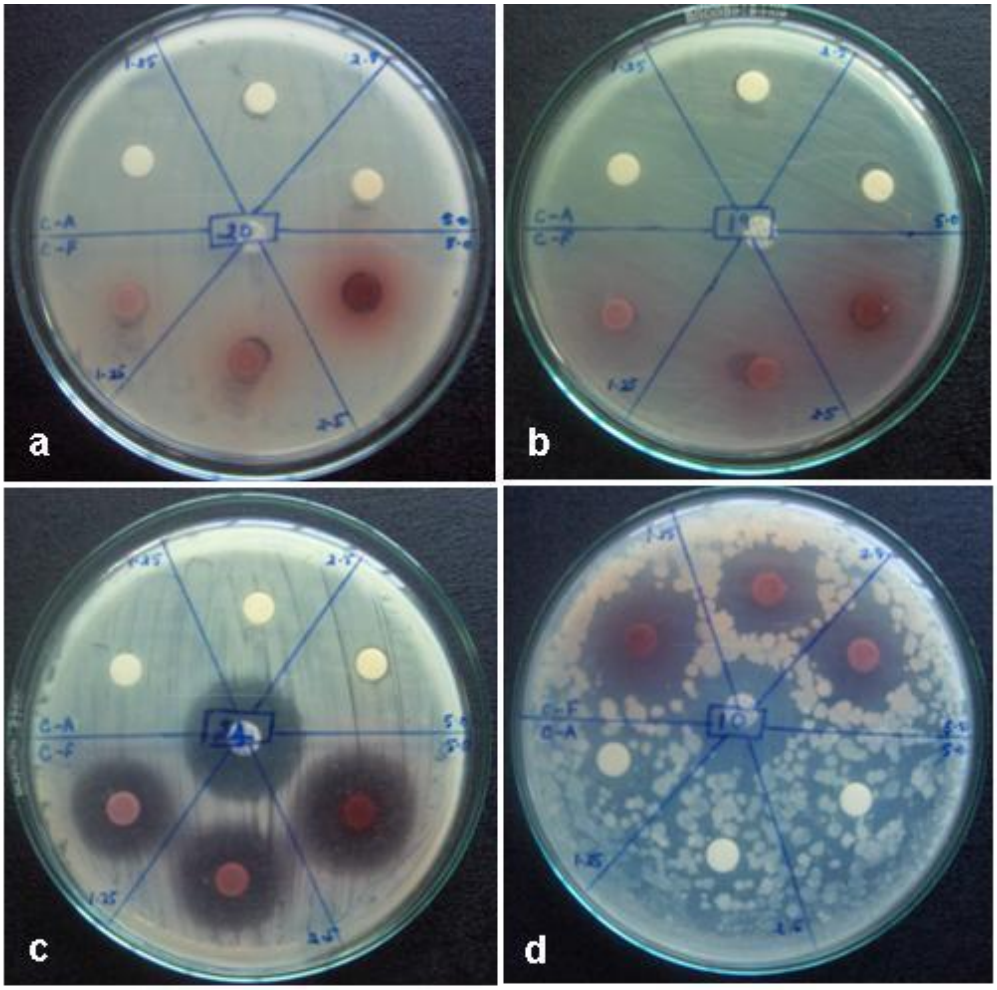

Fig 8: Antibacterial activity of ethyl acetate extract of EPA and EPF from the root of *C. roseus*

A. Crude ethyl acetate extract against *Acetobacter baumanii*

B. Crude ethyl acetate extract against [*E. coli*] Cipro *R*

C. Crude ethyl acetate extract against *MRSA (clinical pathogens)*

D. Crude ethyl acetate extract against *Enterococcus durans*

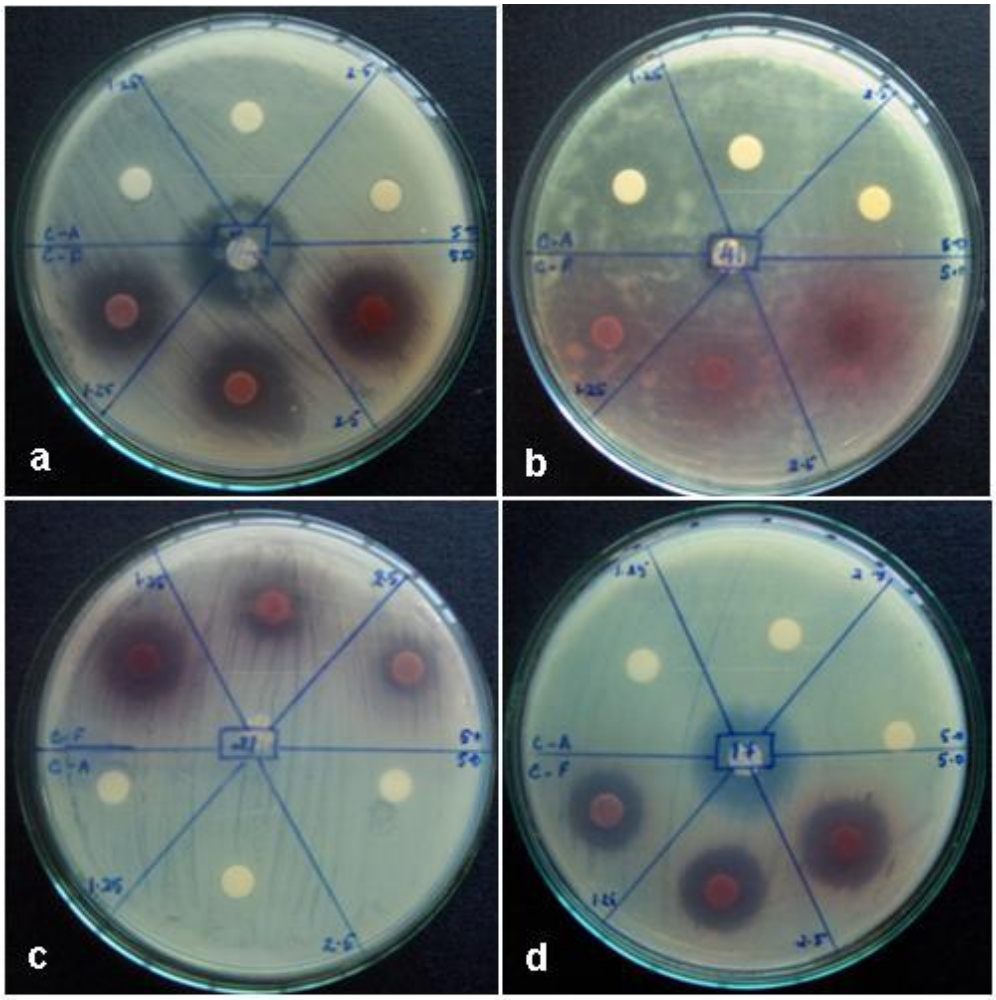

Fig 9: Antibacterial activity of ethyl acetate extract of EPA and EPF from the root of *C. roseus*

A. Crude ethyl acetate extract against [*MSSA*]

B. Crude ethyl acetate extract against *Proteus*

C. Crude ethyl acetate extract against *Staphylococcus aureus*

D. Crude ethyl acetate extract against *Klebsiella*

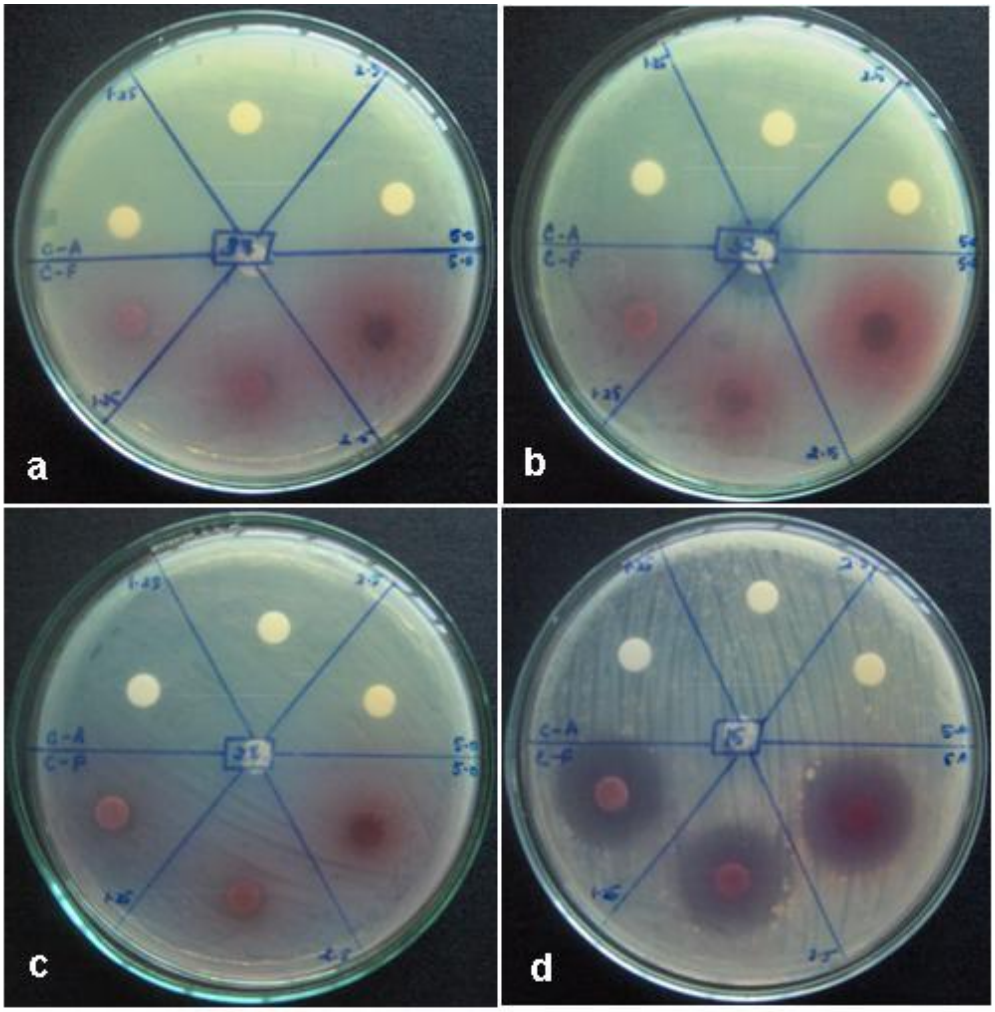

Fig 10: Antibacterial activity of ethyl acetate extract of EPA and EPF from the root of *C. roseus*

A. Crude ethyl acetate extract against *ESBL [Klebsiella pneumonia]*

B. Crude ethyl acetate extract against *Eschericia coli (clinical pathogens)*

C. Crude ethyl acetate extract against *ESBL, E.coli (clinical pathogens)*

D. Crude ethyl acetate extract against *MRSA* [Methiciline Resistant *S. aureus*]

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