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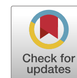
Original article

Screening and identification of novel isolate *Streptomyces* sp., NLKPB45 from Nellore costal region for its biomedical applications



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ABSTRACT

Actinobacteria, which are the prolific producers of antibiotics and significant suppliers to the pharmaceutical industry, can produce a wide variety of bioactive metabolites. An actinomycete strain designated NLKPB45 was isolated from mangrove soils samples of Nellore coastal regions Andhra Pradesh and assessed for antibiotic production and activity against pathogenic bacteria. From a total of 9 mangrove soil samples, 143 acinomycetes were isolated. Among the isolated them 6 actinomycetes strains showed potential antibacterial activity against at two tested pathogens gram positive and gram negative bacteria *E. coli and S. aureus*. The potent strain NLKPB45 was identified by 16S gene isolation and sequencing to the Streptomyces genus. The ethyl acetate extracts also as shown excellent antimicrobial activity against *Salmonella* sp., *staphylococcus aureus, E. coli, and B. subtilus* were detected in both the supernatant extract samples from fermentations of culture NLKPB45. The anticancer activity of extracts in the HeLa with IC_{50} value of 37.1924 µg/ml, MCF-7 IC_{50} value of 40.9177 µg/ml and HT 29 IC_{50} value of 43.3758 µg/ml.

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1. Introduction

It is certain that new antibiotics are immediately needed to arrest and reverse the persistent spreading of antibiotic resistant pathogens which cause life-threatening infections and risk undermining the viability of health care systems (Talbot et al., 2006). Marine microbes represent a promising source for commercially important bio-active compounds. Among marine microorganisms, bacteria have gained a special importance as the most potent source of anti-biotics and other bio-active secondary metabolites. While most of the studies on bacteria have focussed on antibiotic production (Basha et al., 2009). Around 23,000 bioactive secondary metabolites from microorganisms have been reported, and over 10,000 of these are from actinomycetes, constitute 45% of all proactive microbial metabolites. So, the searching for novel actinomycete constitutes an essential component of natural product-based drug discovery is appreciably in recent years. Actinobacter from marine source provide many important bioactive compounds that have high profitable value. Actinobacteria are a productive source of structurally diverse secondary metabolites; many of these possess pharmaceutically important biological activities. Such bacteria which are ability to produce a variety of bioactive compounds has been utilized in a complete series of researches in numerous institutional and industrial laboratories. This has resulted in the isolation of certain agents, which have found application in combating a variety of human infections (Kamble et al., 2012).

That is the reason over 70% of in nature occurring anti-infection agents have been isolated from various genus of actinomycetes (Vining, 1992). Out of these diverse variety, Streptomyces is the biggest genus known for the generation of numerous bioactive metabolities (Edwards, 1992), which have biological activities, for example antifungal, antibacterial, antiparasitic, anticancer, and immunosuppressive activities (Demain, 1995; Xu et al., 2005; Watve et al., 2001). The screening of microbial compounds continues to signify an significant route to the innovation of valuable chemicals, for the improvement of new remedial agents and for assesses of the potential of lesser known and new bacterial taxa are of growing interest (Kurtboke 1998). In spite of above studies on bioactive compounds production by actinobacteria from marine

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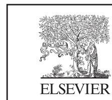

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areas, Hence the present study aimed to isolate actinobacteria from marine water for and screening of antagonistic activity.

2. Materials and methods

2.1. Isolation of marine actinomycetes

Mangrove soil tests were gathered Krishna patnam port territory at Nellore region, Andhra Pradesh, India. One gram of soil test were blended with 10 mL sterile refined water in a test tube and fomented for 10 min. The suspension was serially diluted by exchanging 1 mL aliquots to a progression of test tubes; each containing 9 mL sterile refined water and from separate respective 1 mL test was in to the laminar wind current (Vimal et al., 2009; Krishna et al., 2019). The samples were spread over the entire surface of plates consisting of Casein powder 1.000, Starch 10.000, Sea water 37.000, Agar 15.000, Final pH (at 25 °C) 7.2 ± 0.2 After incubation at 25 °C for 48 h, all colonies were screened and those with various morphology.

2.2. Screening of potential Actinomycetes

The isolated marine actinomycetes were screened for their antogonestic activity by Cross streak technique against the bacterial pathogen. The lawn culture was prepared by using sterile cotton swab and allowed to remain for 1 min. The actinomycetes were streaked on middle of the Starch Casein agar and incubated at room temperature for four days and observed the zone of inhibition.

2.3. Secondary screening

About 6 mm diameter wells were made on plates using sterile steel cork borer. Further, each well were loaded with 60 μl of crude extracts and incubated at 37 °C for 24 h. The antibacterial activities of *Actinomycetes* culture filtrate were compared with known antibiotics streptomycin as positive control. After incubation, the zone of inhibition was evaluated and expressed as mm in diameter. Based on the results of potential actinobacterial strain was selected for molecular identification.

2.4. Molecular-based characterization

The actinomycete strain which shown the better inhibition against the selected pathogens, was subjected for molecular identification by using 16 s rRNA sequencing. The DNA sample of the actinomycete culture, which had been amplified by using primary forward (5′-TCACGGAGAGTTT-GATCCTG-3) and the reverse (5′-GCGGCTGCTGGCACGTA GTT-3′) primers of 16S rRNA was then sequenced to produce gene sequence of 16S rRNA. The resulting sequence was then analyzed using similarity search program called BLAST (Basic local alignment search tool) and compared with nucleotide on the database "GenBank" (NCBI) (Krishna et al., 2019). A phylogenetic tree showing the evolutionary relationships between the selected sequences was obtained with the maximum likelihood.

2.5. Fermentation process

The fermentation carried out for the selected active actinomycete strains using 250 mL capacity Erlenmeyer flasks, containing 100 mL of Starch casein broth medium. For the sterilized fermented broth the pure actinomycete strain was inoculated with 1 mL culture suspension. Inoculated flasks were incubated at 28 °C for five days on a rotary shaking incubator at 250 rpm. After incubation the fermented media was centrifuged at 10,000 rpm or 20 min for crude extract preparation.

2.6. Metabolites extraction

After fermentation, the culture was harvested and centrifuged to remove cells and debris. Then filtrate was used for extraction by adding equal volume of ethyl acetate for three successive times with vigorous shaking for 15–30 min. The ethyl acetate layer containing organic layer was separated and concentrated under reduced pressure. The fractions were then transferred into pre-weighed vials, evaporated under vacuum and then dissolved in Dimethyl sulfoxide (DMSO) to get a final concentration of 1 mg/ml, for in vitro antimicrobial and anticancer activity.

2.7. Antimicrobial activity

2.7.1. Antibacterial activity

Anti bacterial activity of crude extracts studied by the Agar well-diffusion method with four concentrations (10, 25, 50 and 100 μl) were tested against different bacterial pathogens such as *Salmonella* sp., *staphylococcus aureus, E. coli, B. subtilus*. The plates were incubated at 37 °C for 18–24 h and end of the experiment the diameter of the inhibition zone (mm) was measured and the activity index was also calculated. The readings were taken in three different fixed directions and the average values were recorded (Alzoreky, 2009).

2.7.2. Antifungal activity

Anti fingal activity of crude extracts were tested by well diffusion method with four concentrations of test compound (10, 25, 50 and 100 μl). The PDA media plates were spreaded with different fungal strains such as *Aspergillus niger* and *Candida albicans* (Alzoreky, 2009). The petri plates were incubated at 37 + 2 °C for 48 h for fungal activity. After 48 h, the plates were observed for zone formation around the wells and the zone of inhibition (mm) was measured and the activity index was also calculated.

2.8. Anticancer activity

2.8.1. Cytotoxicity assay

After 48 h of incubation, to each well added 15 μl of MTT (5 mg/ml) in phosphate buffered saline (PBS) and incubated at 37 °C for 4 h. The medium with MTT was removed and the formed formazan crystals were solubilized in 100 μl of DMSO solution. Using a micro plate reader the absorbance was measured at 570 nm (Mosmann, 1983). The percentage cell inhibition was determined using the formula. Percentage Cell Inhibition = $[100 - \text{Abs (sample)}/\text{Abs (control)}] \times 100$.

3. Results and discussion

Marine actinomycetes have been more deeply studied in several unexplored environments and extreme habitats of seashore in different parts of the world over the most recent couple of years There are few reports shown actinomycetes isolation in Nellore district located at Andhra Pradesh state, so there was an endeavor has been made to separate the actinomycetes from this few locations of Nellore district region in order to find novel species for antibiotic production. From a total of 9 mangrove soil samples, 143 actinomycete strains were found at different places and locations of Nellore district (Table 1) of these few of the samples were found to be active after sub culturing. Similarly Mantada et al. (2013) isolated 97 actinomycete strains from 24 marine samples such as sea water, sea sediments, sponges, and corals obtained from

Table 1
Actinomycetes in different areas of marine water sample in Nellore district.

S. No	Colletion areas	Colonies found
1	Kodur beach	18
2	Katepelly beach	20
3	Krishna patnam beach	6
4	Krishna patnam beach site 1	17
5	Krishna patnam beach site 2	19
6	Krishna patnam port	12
7	Site 1	6
8	Mypadu beach	25
9	Thupilipalyam beach	20
Total strains found		143

Tiruchendur and Kulasekarapattinam. The Bioactive screening has likewise centered around microorganisms related with such host surfaces and the different natural products isolated from marine invertebrates often show structural similarities to known metabolites of microbial starting point (Arpigny and Jaeger, 1999; Haygood et al., 1999). Organic compounds from aquatic to terrestrial microorganisms have wide-ranging of use in the cure of many diseases and serve as compounds of interest in commercial synthetics. These compounds provided important contributions to the discovery of antibacterial agents (Angel et al., 2009).

Among the samples 143 actinomycetes isolated from different mangrove areas of Nellore district the 6 strains showed potential antibacterial activities against at two tested pathogens gram positive and gram negative bacteria *E. coli, S. aureus*. Out of six actinomycetes strains two were selected for secoundary screening. The results of those isolates, NLKB27 and NLKPB45 exhibited highest activities against tested bacteria. Furthermore in the secondary screening the selected NLKB27 and NLKPB45 actinomycete culture filtrate were subjected to antibacterial activity in well diffusion assay with different volumes shown in the Fig. 1 and Table 2.

The selected strain subjected to Hexane, Acetone, methanol and ethyl acetate extraction which were tested for antibacterial activity against some test bacteria, when the EA-ethyl acetate and methanol of each isolate was browbeaten for antibacterial activity assay using well diffusion method. It was noted that a momentous result was obtained only from ethyl acetate extract of NLKPB45 against *Salmonella* sp., *staphylococcus aureus, E. coli, B. subtilus* shown in the Fig. 2.

3.1. Antifungal activity

The antifungl activity of crude EA extract (10, 25, 50, and 100 μg/mL) of isolated actinomycetes NLKPB45 was evaluated against *Aspergillus niger* and *Candida albicans* using well diffusion method. The results shown that crude (100 μg/ml) extract exhibited 16 mm zone of inhibition against *Aspergillus niger* and 09 mm zone of inhibition against *Candida albicans* as shown in Fig. 3.

3.2. Molecular identification

The strain were identified by Performing PCR reactions using universal primers were used for amplification and subjected to sequencing the16S rDNA of NLKPB45 which displayed high antimicrobial activity evaluation. The isolate NLKPB45 was closely related to *Streptomyces* sp. strain of Accession no (MG241290.1) The biochemical investigations were given in Fig 4 and Table 3.

3.3. Anticancer activity

In order to study the growth inhibitory activity of the different cancer cell lines like HeLa, MCF-7 and HT 29 with crude extract of

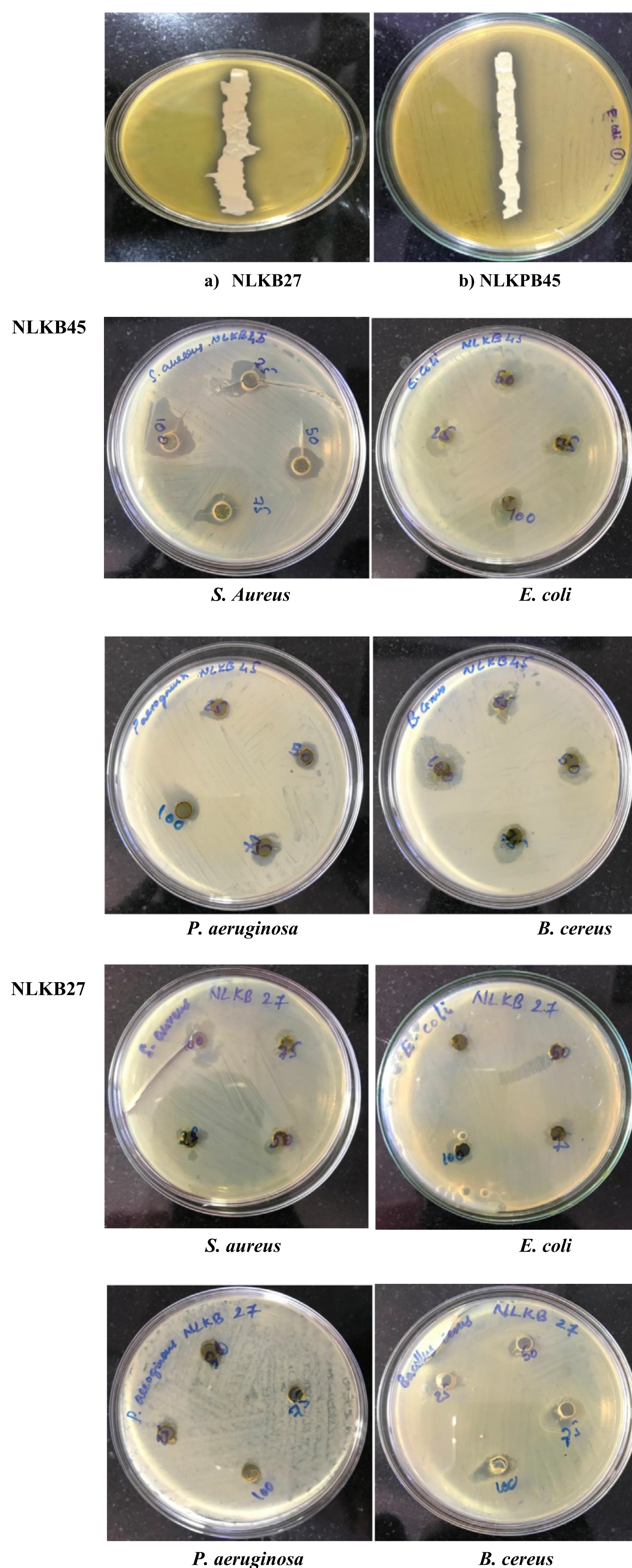
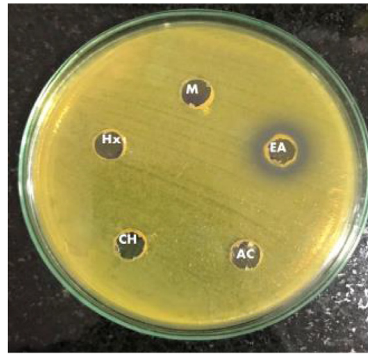


Fig. 1. Secondary screening by culture supernatant of NLKB45 and NLKB27.

NLKPB45 the anticancer activity was evaluated by MTT assay. The investigate for the marine microorganisms that they afford a potent source of biomedically valuable compounds obtained from studies showing that marine bacteria generate antimicrobial agents (Rosenfeld and Zobell, 1947). The result of MTT assays in

Table 2
Zone of inhibition for secondary screening by culture supernatant of NLKB45 and NLKB27.

TEST ORGANISMS	Zone Of Inhibition (mm)							
	NLKB45				NLKB27			
	10	25	50	100	10	25	50	100
E. Coli	03	04	06	09	01	02	03	04
P. aeruginosa	01	03	05	06	–	–	02	03
Bacillus cereus	01	02	03	08	–	–	–	–
S. aureus	03	04	06	09	00	00	02	01



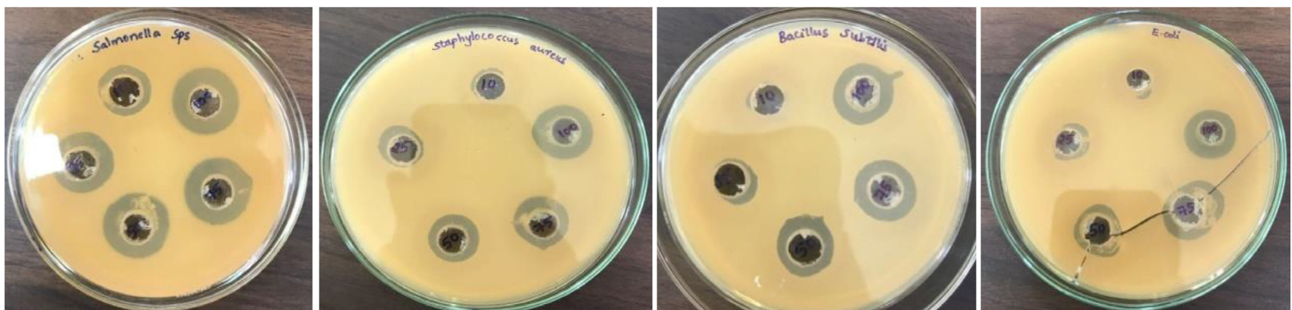


Fig. 2. Antimicrobial activity (in ZOI) against pathogenic bacterial strains (*Salmonella* sp., *Staphylococcus aureus, Bacillus subtilis, E. coli*) shown by NLKPB45.

Antifungal activity:

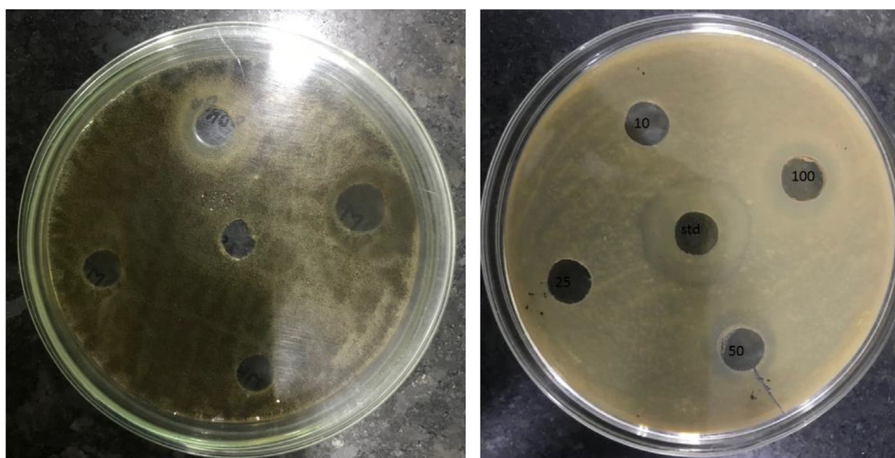


Fig. 3. (A) *Aspergillus niger* and (B) *Candida albicans.*

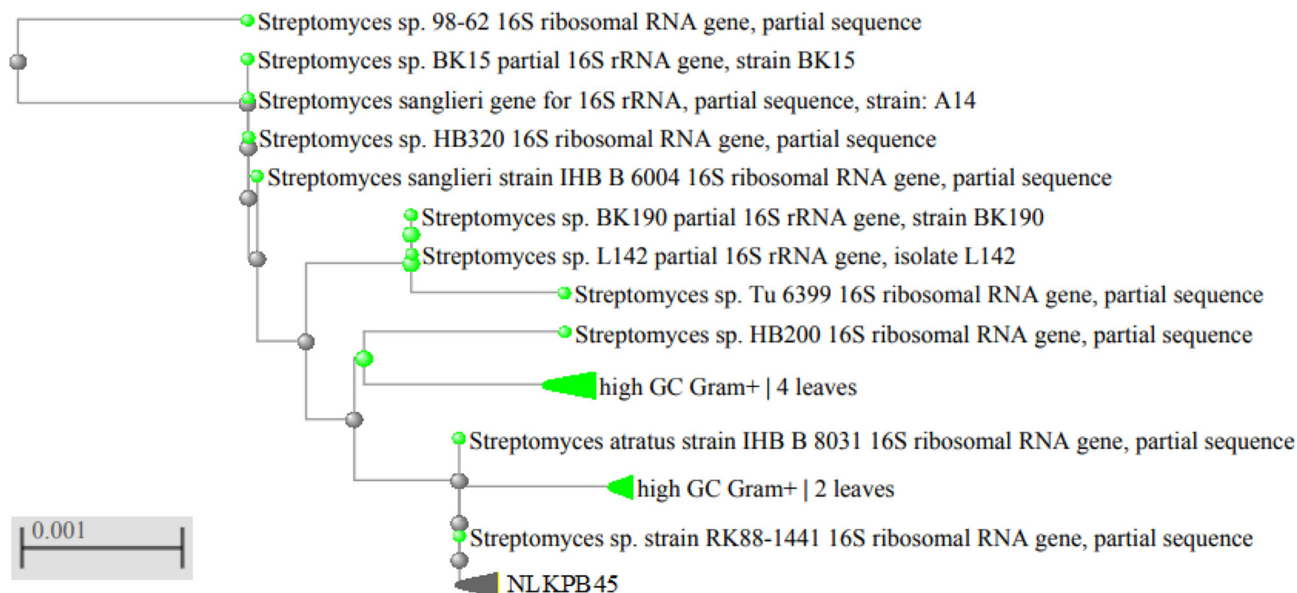


Fig. 4. The biochemical analysis of isolate NLKPB45.

Table 3
Biochemical Characterisation.

Test	+ve/−ve
Indole	−
Voges-Proskauer test	−
Citrate test	−
Lysine test	−
Ornithine test	−
Arginine test	−
Nitrate test	+
Malonate test	−
Urease test	−
Phenylalanine-deamination test	−
H_2S production test	−
ONPG test	−
Glucose	+
Mannitol	+
Xylose	+
Inositol	+
Sorbitol	−
Rhamnose	−
Sucrose	−
Lactose	−
Arabinose	−
Adonitol	−
Raffinose	−
Salicin	−

+: Positive; −: negative.

Table 4
Anticancer activity Ethyl acetate (NLKPB45) extract.

S. No	Cancer Cells	Ethyl acetate (NLKPB45) IC50
1	HeLa	37.1924 µg/ml
2	MCF-7	40.9177 µg/ml
3	HT 29 cells	43.3758 µg/ml
4	Standard drug Cisplatin	8.26 µg/ml

our study revealed that the ethyl acetate extract of *Streptomyces* sp.-NLKPB45 decreased the percent viability of all the cells but to different extent. Methanol and ethanol extract was found to induce more cytotoxicity towards cancer cell lines HeLa, MCF-7 and HT 29. These study results revealed morphological changes of cells leading to cell death induced by the *Streptomyces* sp.-NLKPB45 extracts in the HeLa with IC_{50} value of 37.1924 µg/ml, MCF-7 IC_{50} value of 40.9177 µg/ml and HT 29 IC_{50} value of 43.3758 µg/ml shown in the represented in (Table 4) The marine bacteria were enormously difficult to isolate and culture, however, that bacteria are now known to be proficient of producing unusual bioactive natural products that are not observed from terrestrial sources (Fenical, 1993; Fenica and Jensen, 1993).

even though the growing curiosity in chemical biosynthesis, biological products from marine source of Actinobacteria are coming up to be discovered and developed. Nonetheless, the advancement of new development systems is as yet an extraordinary test. Improved and particular separation techniques can likewise be utilized to segregate uncommon Actinobacterias from marine natural specialties having the capacity to biosynthesize novel bioactive compounds. These strains may greatly affect human wellbeing and are a wellspring of mixes with exceptionally encouraging antitumor activities.

4. Conclusion

Among 143 isolates in primary screening 6 isolates showed activity against two test organisms *E. coli and S. aureus* by cross strak technique. From these 6 isolates two (NLKPB27 and 45) were showed best inhibition zone in secondary screening against *E. coli* and *S. aureus* by agar well diffusion method. To select the best isolate of these two again different amount of cell extract was checked on four different pathogens and we concluded NLKPB45 was showing best inhibition zone (Table 2). NLKPB 45 strain subjected to different solvent extractions and observed ethylacetate extract showed phenonymous activity against *Salmonella* sp., *staphylococcus aureus, E. coli, B. subtilus* as mentioned in Fig. 2. In molecular identification the isolate NLKPB45 was closely related to *Streptomyces* sp. strain of Accession no (MG241290.1).

The *Streptomyces* sp.-NLKPB45 ethylacetate also showed anticancer activity. Based on the results obtained in the present research study we concluded that the potent strain identified as *Streptomyces* sp. The ethyl acetate crude extract inhibited the growth of gram positive pathogenic bacteria. This again proved Actinomycetes are good sources of unique natural bioactive metabolites. In future, this may lead the way towards large scale profitable production of antimicrobials and bioactive compounds.

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