Antibacterial, antitubercular and anticancer activity of gut-associated *Streptomyces enissoceasilis* SFA isolated from marine fish *Carcharhinus amblyrhynchos*

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Abstract

The present study aims to investigate anticancer, anti TB and antibacterial activity of fish gut associated actinobacteria isolated from marine fish Carcharhinus amblyrhynchos. Actinobacterial strains are isolated by standard spread plate method and screened for antibacterial activity by agar plug method against the carbapenem resistant Klebsiella pneumonia ATCC 13882. LRP assay used to determine the antitubercular activity and anticancer assay was evaluated by MTT assay against MCF 7 breast cancer cell line. TLC and bioautogram were used to separate the chemical compounds and identify the active fraction. A total of sixteen gut-associated actinobacterial strains were isolated from three marine fishes viz. Carcharhinus amblyrhynchos, Sphyraena barracuda and Scomberomorus guttatus. Strain SFA was isolated from Indian shark fish; Carcharhinus amblyrhynchos was showed maximum of 26 mm inhibition against the test pathogen.

The extract showed 70% inhibition against Mycobacterium tuberculosis H37Rv at 500µg/ml concentration. In MTT assay, the ethyl acetate extract showed 79% inhibition against breast cancer cell line MCF7 at 1000µg/ml. Based on their phenotypic and molecular characteristics, strain SFA was identified as Streptomyces enissoceasilis. The findings suggested that the fish associated actinobacteria is a promising source for multifunctional bioactive compounds for the development of novel therapeutics drugs.

Keywords: Antimicrobial, anti-TB, anticancer, *Streptomyces enissoceasilis*, fish gut, *Klebsiella pneumoniae*.

Introduction

Discovery of antibiotics to treat infectious diseases has revolutionized the field of medicine in the mid-twentieth century. However, due to overuse or misuse of antibiotics over a prolonged period, most of the pathogens have become resistant to multiple antibiotics. Thus, there is a dire need for the discovery and development of new antibiotics to effectively target the life threatening disease causing pathogens[1]. The emergence and spread of pathogens harboring extended spectrum β-lactamases like carbapenem-resistant *Klebsiella pneumoniae* (CR-KP), *Acinetobacter baumanii*, *Escherichia coli* and other gram negative bacteria causing major threat to public health[2,3].Carbapenem resistance is a major and an on-going public health problem globally which may be intrinsic or mediated by transferable carbapenemase-encoding genes.

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is among the oldest and most pervasive diseases. Treatment of TB has always been a challenge[4]**.** Tuberculosis (TB) also remains a leading cause of death in the world today as a result of the evolution of the multi-drug resistant TB (MDR-TB), extensively drug-resistant TB (XDR-TB) and TB/HIV co-infection. In order to combat MDR- and XDR-tuberculosis, new antibiotics especially those with new molecular scaffolds and unique mode of action are needed urgently.

Among the life style diseases, cancer still remains one of the most serious health problems in humans. Notably, breast cancer remains as the second most universal cause of cancer deaths in women[5]. Therapeutic options to treat cancer either fall into or the combination of approaches like surgery, radiotherapy, immunotherapy and chemotherapy[6].

Natural products are considered a valuable resource for drug discovery due to their diverse chemical scaffolds which cannot be matched by any synthetic libraries[7]. Currently, marine microorganisms comprise precious, vital source as new and promising bioactive metabolites with significant biological activities. Among the marine microbes, actinobacteria are the potential source of novel secondary metabolites notably antibiotics followed by anticancer agents[8]. Hundreds of compounds with different structure or remarkable bioactivity have been isolated from marine actinobacteria[9]. Historically, marine invertebrates have been a prolific source of unique natural products with a diverse array of biological activities.

Recent studies of invertebrate-associated microbial communities are revealing that the associated microorganisms are the true producers of many of these compounds. In general, marine sediment derived actinobacteria are reported as promising source for novel bioactive compounds[10] whereas reports on bioactive

compounds from fish gut associated actinobacteria are scanty. With this view, the present study explores the bioactive potential of fish gut associated actinobacteria with special reference to antimicrobial, antitubercular, anti-oxidant and anticancer properties.

Material and Methods

Collection of fish gut sample and processing: Marine fishes such as *Carcharhinus amblyrhynchos, Sphyraena barracuda* and *Scomberomorus guttatus* were collected from Kovalam (Lat: 12.7870^{0}N, Long: 80.2504^{0}E) costal area, Chennai, Tamil Nadu, India. The samples were transported to the laboratory under iced conditions. After transportation to the laboratory, fishes were anaesthetized with 0.01% benzocaine and surface sterilized by immersion in 70% ethanol for 30 seconds. The gut portion were dissected by according the method of Vijayabaskar and Somasundaram[11]. Gut was weighed and placed into a 10 ml sterile double strength phosphate-buffered saline (PBS) solution [disodium phosphate, 2-3% (w/v); sodium phosphate, 0-6% (w/v) and sodium chloride, 1-2% (w/v)]. Gut was homogenized in tissue homogenizer[12].

Isolation of actinobacteria: Actinobacteria were isolated from the processed fish gut samples by adopting standard spread plate method described by Vignesh et al.[13] Starch casein agar (g/lit: Starch 10.0, Casein 0.3, $MgSO_4.7H_2O$ 0.05, KNO_3 2.0, NaCl 2.0, $CaCO_3$ 0.02, $FeSO_4$ $7H_2O$ 0.01, Agar 25.0, pH 7.0 ± 0.2) and Kuster's agar (g/l: glycerol: 10 g, casein: 0.3 g, KNO_3: 2 g, K_2HPO_4: 2 g, soluble starch: 0.5 g, asparagine: 0.1 g , $FeSO_4.7H_2O$: 0.01 g, $CaCO_3$: 0.02 g, $MgSO_4$.7H2O: 0.05 g, Agar 25.0, pH 7.0 ± 0.1) were prepared with 50% sea water. Both the media were amended with nalidixic acid (20 µg.ml $^{-1}$) and nystatin (100 µg.ml $^{-1}$) to inhibit the growth of bacteria and fungi respectively. One ml of homogenate of pooled intestinal segments was mixed with 100 ml of 0.85% saline in 250ml conical flask.

The suspension was serially diluted up to 10^5 dilutions using sterile distilled water blank. Hundred microliter of aliquot from 10^3, 10^4, 10^5 dilutions was taken and spread over agar plates using sterile L-rod. Morphologically different actinobacterial colonies were selected, purified and sub cultured using yeast extract malt extract agar medium (ISP2) and 30% glycerol stored at -20°C.

Screening of actinobacteria for antibacterial activity: Antibacterial activity of actinobacterial cultures was tested against carbapenem resistant *Klebsiella pneumoniae* ATCC13882 by adopting agar plug method. Actinobacterial cultures were grown on ISP2 agar plates at 28ºC for 10 days. After incubation period, agar plug with 5 mm diameter was cut from the ISP2 agar by using well cutter and placed over nutrient agar plate swabbed with test pathogen. This experiment was performed in triplicate and mean value was taken. Plates were incubated at 37 ±2.0ºC for 24 hours. Antibacterial activity was evaluated in triplicate and recorded by measuring the diameter of inhibition zone (mm)[14].

Molecular identification of SFA by 16SrRNA sequencing: Microscopic, cultural and physiological characteristics of one potential strain SFA were studied by adopting the methods described by Shirling and Gottileb[15]. The potential actinobacterial strain SFA was processed for the genomic DNA extraction using solute ready genomic DNA kit for immediate use or for storage at -20 ºC. Concentration and purity of the extracted DNA were then evaluated by running on agarose gel and by Nano Drop (Thermo Scientific) readings.

The genomic DNA obtained from the actinobacterial strain SFA was further subjected to PCR amplification of 16S rRNA gene using the primers 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′)[16]. The reaction mixture (50µl) consisted of 100ng of 2µl genomic DNA, 5µl of 10x buffer, 4µl of BSA (1mg/ml), 1µl of dNTP mixture (2.5mM), 1µl of each primer (10µM) and 3.5U of Taq DNA polymerase.

PCR condition followed as 6 min at 94°C followed by 35 cycles of 45s at 94°C, 45s at 55°C and 1.5min at 72°C followed by an 8min extension at 72°C. PCR amplification was performed in an Eppendorf master cycler gradient. Purified PCR product was sequenced bidirectionally to obtain complete coverage at Eurofine genomics, Bangalore, India. Sequences were edited and contig was assembled in DNA baser v.3 and compared with Gen Bank sequences by BLAST analysis. The 16S rRNA gene sequence was multiply aligned with selected sequences obtained from the GenBank using CLC sequence viewer 6.0 program. Phylogeny prediction was done using MEGA 7 [17]. The confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses based on 1000 resampling of the neighbour joining data set.[18] The partial 16S rRNA nucleotide sequence of the potential actinobacteria strain SFA was deposited to GenBank database.

Production and extraction of bioactive metabolites: The selected potential actinobacterial strain SFA were inoculated into yeast extract-malt extract (ISP2) agar plates (25ml/plate) and incubated at 28±2.0ºC for 10 days. After incubation, mycelial growth was removed aseptically using sterile spatula. The agar medium was cut into small pieces and extracted using ethyl acetate at 1:2 ratio for overnight at room temperature[19].

Determination of antitubercular activity: The ethyl acetate extract of strain SFA was tested for antitubercular activity against standard laboratory strain *Mycobacterium tuberculosis* H37Rv by adopting luciferase reporter phage (LRP) assay[20]. About 350µl of G7H9 broth supplemented with 10% albumin dextrose complex and 0.5% glycerol was taken in cryo vials and added with 50 µL of crude extract in

order to get the final concentration of 250µg/ml and 500µg/ml. One hundred microliter of *M. tuberculosis* cell suspension was added to all the vials. DMSO (1%) was also included in the assay as solvent control. All the vials were incubated at 37°C for 72 hours.

After incubation, 50 µl of high titre phage phAE129 and 40 L of 0.1M $CaCl_2$ solutions were added to the test and control vials. All the vials were incubated at 37°C for 4 hours. After incubation, 100µl from each vial was transferred to luminometer cuvette. About 100 µl of D-luciferin was added and relative light unit (RLU) was measured in luminometer. RLU reduction by 50% or more when compared to control was considered as having antitubercular activity. The assay was performed by taking triplicate and the mean values of the results were calculated.

$$\% \text{ RLU reduction} = \frac{\text{Control RLU} - \text{Test RLU X100}}{\text{Control RLU}}$$

Anticancer activity of SFA: The ethyl acetate extract of strain SFA was tested for *in vitro* anticancer activity against MCF7 (breast cancer) cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay[21]. The MCF7 cell line was obtained from National Centre for Cell Science, Pune, India (NCSS) and maintained in Minimal Essential Media (MEM) supplemented with 10% FBS, penicillin (100µg/ml) and streptomycin (100µg/ml).MCF-7 were seeded at 5000 cells/ well in 96-well plates and both were incubated for 48hours. Twenty microliter of the ethyl acetate extract of strain SFA was added into each well with the final concentration ranging from 1 to 1000µg/ml.

Cells with the extract were further incubated for 72 hours before performing MTT assay and DMSO was used as negative control. Fifty microliter of MTT (5mg/ml) was then added to each well. The medium was then gently aspirated, and 100µl of DMSO was added. The absorbance was determined spectrophotometrically at 570 nm (with 650 nm as reference wavelength) using a microplate reader. The percentage of cell death was calculated as follows:

% inhibition= (C-T/C) x 100

TLC and bioautography: Chemical components from the crude ethyl acetate extract of strain SFA were separated by thin-layer chromatography (TLC) (pore size 60Å, mesh size: 230–400, particle size 40 63 µm, Merck) using organic solvents (chloroform: methanol) in ratio (9:1).The antibacterial active fractions were detected by bioautography[22]. For bioautography, the TLC plate was overlaid on nutrient agar medium supplemented with 0.1% (w/v) 2,3,5-triphenyltetrazolium chloride (tetrazodium red) and test pathogen *Klebsiella pneumoniae* ATCC 13882 at a final concentration of 10^{-7} CFU/ml.

The plates were incubated at 37°±2.0 C for 24 hrs. Clear zone of inhibition indicated the position of antimicrobial compounds on the TLC plates and the retention factor (Rf) value was calculated.

Statistical analysis: All the data were expressed as means ± SD (n=3). Differences were evaluated by Student's t-test and P values less than 0.05 were considered as significant.

Results and Discussion

Isolation and characterization of actinobacteria: Totally 16 morphologically different actinobacterial strains were isolated from three different marine fishes (*Carcharhinus amblyrhynchos*n=2, *Sphyraena barracuda* n=10 *scomberomorus guttatus* n=4) and preserved in ISP2 agar slants at 4°C. During recovery and preservation, all the actinobacterial cultures showed good growth with powdery and leathery consistency on ISP2 agar. All the actinobacterial cultures showed the presence of substrate mycelium and aerial substrate mycelium.

Based on this, most of the cultures were suspected as *Streptomyces* (Table 1). Several studies reported the distribution of actinobacteria from selected marine and fresh water fishes with reference to antimicrobial activity[23,24]. Previously, Chu et al[25] isolated 200 actinobacterial strains from the gut of *Carassius gibelio* for biomedical application.

Screening for antibacterial activity: In the preliminary screening, only one actinobacterial strain SFA isolated from *Carcharhinus amblyrhynchos* showed maximum of 26±0.4mm inhibition against carbaphenum resistant *Klebsiella pneumoniae* ATCC 13882 Table 2).Vignesh et al[13] have reported *Streptomyces sp* isolated from marine fish *Rastrelliger kanagurta* showing the antibacterial activity against *Staphylococcus aureus* MTCC96, *Escherichia coli* MTCC739, *Salmonella enterica* and *Candida albicans*.

Jami et al[26] isolated the *Streptomyces sp* and *Micromonospora sp* from gut of *Schizothorax zarudnyi* and *Schizocypris altidorsalis* showing antimicrobial activity against some fish pathogens.

Identification of potential actinobacterial strains SFA: Under microscopic observation, strain SFA showed the presence of both aerial and substrate mycelium with no fragmentation. Strain SFA showed good growth on ISP2 agar with powdery consistency (Fig. 1). Physiological characteristics of strain SFA was given in table 3. The obtained sequence of the 16S rRNA gene indicated that the promising actinobacterial strain SFA showed 98% similarity to *S.enissocaesilis*. The nucleotide sequence of strain SFA was deposited to GenBank sequence database with the accession number MH021966.

The neighbor-joining phylogenetic tree indicated the strain SFA to be a sister taxa to *S. diastaticus* within the genus *Streptomyces* (Fig. 2) previously reported by Chen et al.[27]

Table 1
Morphological characteristics of actinobacterial strains

S.N.	Strain. No	Cultural characteristics						
		Growth	Consistency	AMC	RSP	SP	AM	SM
1	SFA	Good	Rough	White	-	-	+	+
2	SFA1	Good	Rough	dirty white	-	-	+	+
3	SEFA1	Good	Rough	White	-	-	+	+
4	SEFA2	Good	Rough	Orange	-	-	+	+
5	SEFA3	Good	Powdery	dirty white	-	-	+	+
6	SEFA4	Good	Cottony	White	-	-	+	+
7	SEFA5	Good	Rough	Grey	-	-	+	+
8	SEFA6	Good	Rough	Pale pink	Light brown	-	+	+
9	SEFA7	Good	Powdery	Dirty white	-	-	+	+
10	SEFA8	Good	Leathery	Grey	-	-	+	+
11	SEFA9	Good	Rough	Pale yellow	-	-	+	+
12	SEFA10	Good	Powdery	White	-	-	+	+
13	SHFA 1	Good	Leathery	Pale yellow	-	+	+	+
14	SHFA 2	Good	Rough	Yellowish grey	-	-	+	+
15	SHFA 3	Good	Leathery	Dirty white	-	-	+	+
16	SHFA 3	Good	Leathery	Dirty white	-	-	+	+

(+ - present; - - absent; AMC - aerial mass colour; RSP - reverse side pigment; SP - soluble pigment, AM – Aerial Mycelium, SM – Substrate Mycelium).

Table 2
Screening of fish gut actinobacteria for antibacterial activity against the *Klebsiella pneumonia* ATCC 13882

S.N.	Strain name	Zone of inhibition (mm in diameter)
1	SFA	26±0.4
2	SFA1	-
3	SEFA1	-
4	SEFA2	-
5	SEFA3	-
6	SEFA4	-
7	SEFA5	-
8	SEFA6	-
9	SEFA7	-
10	SEFA8	-
11	SEFA9	-
12	SEFA10	-
13	SHFA 1	-
14	SHFA 2	-
15	SHFA 3	-
16	SHFA 3	

Zone of inhibition summarized as Mean (n=3) ±SD

Fig. 1: Colony and micromorphology of actinobacterial strain SFA

Table 3
Physiological characteristics of potential actinobacterial strain SFA

Factors	Variables	Growth
Sugars	Glucose	Good
	Arabinose	Good
	Sucrose	Poor
	Xylose	Good
	Inositol	Good
	Mannitol	Good
	Fructose	Good
	Rhamnose	Good
	Raffinose	Good
	Cellulose	Good
Amino acid	Asparagine	Good
	Glutamine	Good
	Tyrosine	Poor
pH	5	No Growth
	7	Good
	9	Good
	11	Moderate
Temperature (^{0}C)	20	Moderate
	30	Good
	40	Moderate
	50	No Growth
NaCl %	0	Good
	1	Good
	2.5	Good
	5	Moderate
	7.5	Poor
	10	No Growth

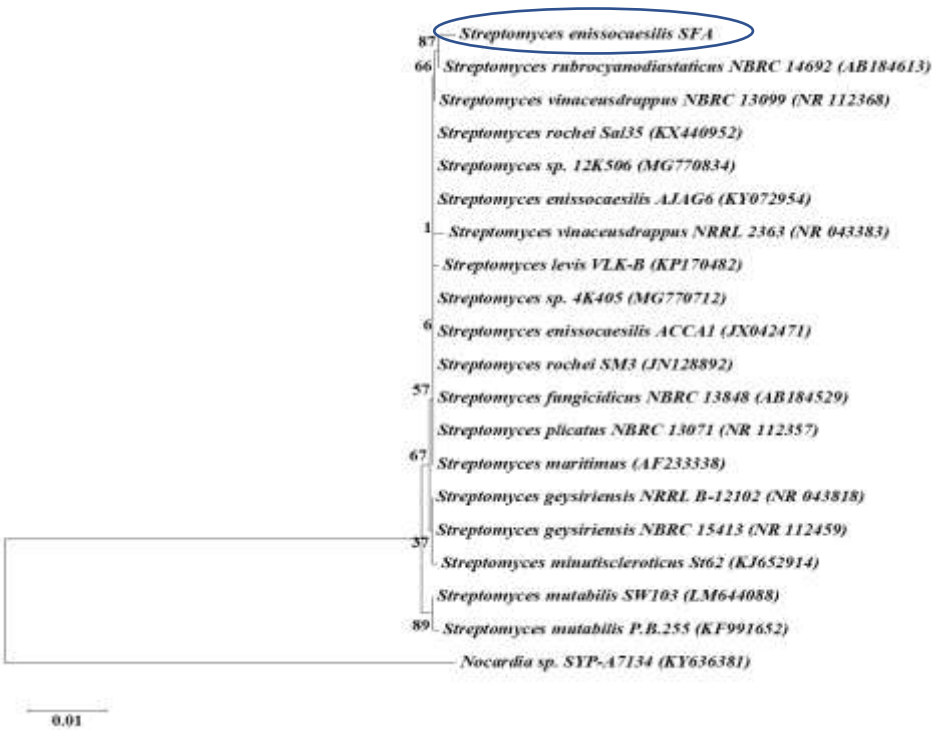

Fig. 2: Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences shows the strain SFA to be a sister taxa of *Streptomyces rubrocyanodiastaticus* with in the family Streptomycetaceae. *Nocardia*sp. SYP-A7134 was used as the out-group.

L-asparaginase enzyme production was reported by Sirisha et al[28] and larvicidal ovicidal activity was reported by Ganesan et al[29] reporting the application of *Streptomyces enissocaesilis isolated* from different ecosystem.

Determination of antitubercular activity of strain SFA: In luciferase reporter phage assay, the actinobacterial crude extract showed 59.87±0.20% and 70.41±0.72% reduction in RLU at 250 and 500µg/ml concentration respectively (Table 4) The method showed that fish gut associated actinobacteria *Streptomyces sp* SFA was active against *M. tuberculosis* H37Rv.The similar study was reported by Radhakrishnan et al[30] that marine *Streptomyces sp* and *Micromonospora* showed antitubucular activity 90% RLU reduction analyzed by using Luciferase reporter phage assay.

Anticancer activity of SFA: As clearly shown in table 5, the effect of SFA crude extract on MCF7 cell line exhibited a degree of anticancer activity causing almost 75.78% inhibition at 1000 µg/ml concentration (Fig. 3). Using 300 and 100 µg/ml concentration of crude extract of SFA showed 57.08 µg/ml and 43.32 µg/ml inhibition respectively by using the MTT assay.

Abd-elnaby et al[31] isolated the *Sterptomyces sp* from sediment in the sea shore of Suez Bay, Egypt having anticancer activity against human liver cancer cell line (HepG2), mouse lymphoma cell line (EI-4), breast cancer cell line (MCF-7) and human colon cancer cell line (Caco-2).

Table 4
Anti TB activity of SFA

S.N.	Concentration (µg/ml)	RLU reduction (%)		
		Isoniazid (H)	Rifampicin (R)	SFA
1	2	97.23±0.63	98.33±0.11	2.39±1.25
2	250	99.36±0.32	99.56±0.79	59.87±0.20
3	500	99.21±0.11	99.53±0.56	70.41±0.72

Table 5
Percentage of growth inhibition and cell viability of *Streptomyces* strain SFA against MCF7 cell line using the MTT assay.

Concentration (µg/ml)	% inhibition	% cell viability
1000	79.78±0.35	1.21±0.23
300	57.08±0.67	2.91±0.24
100	43.32±1.00	6.67±0.10
30	33.33±1.44	6.66±0.56
10	32.05±6.41	7.94±0.57
3	11.60±0.23	8.39±0.34
1	2.15±1.90	7.84±0.32

Values are represent as (n=3) Mean±SD

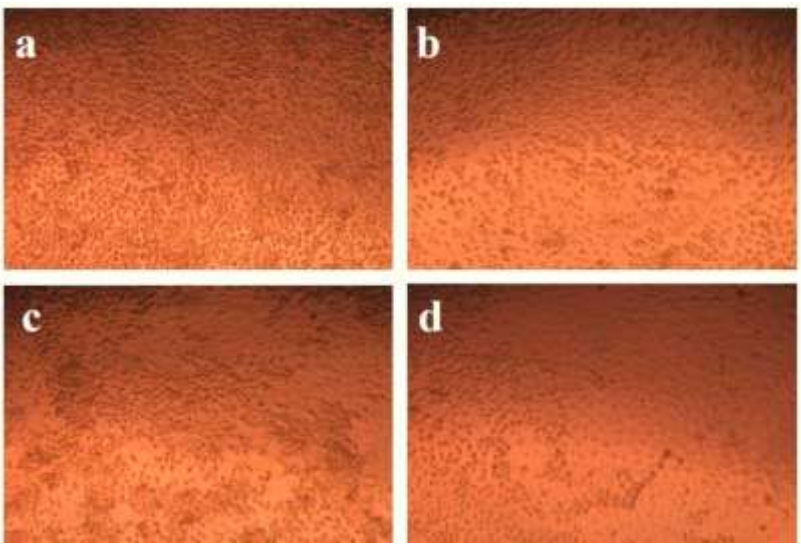

Fig. 3: Growth inhibition effect of the tested Streptomyces strain SFA on MCF7 cell line at different concentrations. (a) Comparison of control, (b) 100 µg/ml, (c) 300 µg/ml and (d) 1000 µg/ml concentration of SFA.

TLC and bioautography: TLC profiling of ethyl acetate extract of SFA provided the presence of six individual compounds with the solvent system of chloroform: methanol (7:3).This partially separated bioactive compound was found to have an Rf value of 0.76 and showed clear zone of inhibition against *Klebsiella pneumonia* by bioautography (data not shown). Selvameenal et al[32] evaluated the crude bioactive compound from *Streptomyces sp* by thin layer of chromatography with the solvent system of chloroform: methanol (70:30) and active spots were identified using bioautography against ESBL *Klebsiella pneumonia* ATCC 13882.

Conclusion

Fish gut is a promising priceless natural resource for bioactive actinobacteria isolation. The secondary metabolites from SFA showed antibacterial activity against ESBL *Klebsiella pneumonia* ATCC 13882 and *Mycobacterium tuberculosis* also active against breast cancer cells. Based on the conventional and molecular identification strain, SFA was identified as *Streptomyces enissoceasilis*. Thus, our study leads to future development of promising therapeutic drugs. Further research including preparative HPLC purification, chemical characterization and structure elucidation is yet to prove its potential.

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