

***Streptomyces castaneoglobisporus* AJ9, A haloalkaliphilic actinomycetes isolated from solar salt works in southern India and its pharmacological properties**

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Five strains of haloalkaliphilic *Streptomyces* sp. were isolated and identified. Among the five strains, the *Streptomyces* sp. AJ9 which isolated Kovalam solar salt works had the potential ability of protease, gelatinase enzymes production and highly antagonistic against the pathogenic bacteria including *Aeromonas hydrophila*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*. The *Streptomyces* sp. AJ9 was identified by genomic level through 16S rRNA sequencing and confirmed as *Streptomyces castaneoglobisporus* AJ9 (GenBank KC603900.1). Further the secondary metabolites were extracted with ethyl acetate, purified through silica column chromatography and screened the fractions (FI to FVII) against pathogenic bacteria, fungi, the shrimp killer virus, White Spot Syndrome Virus (WSSV). Among the fractions FIV to FVI had highest bioactivities and the pooled fractions were structurally characterized by GC-MS analysis and confirmed as the benzoic acid derivatives of 2-hydroxy-4-methoxy-6-methyl-, methyl ester. The pooled fraction (FIV to FVI) were significantly ($P < 0.001$) controlled the bacterial, fungal and viral pathogens by *in vitro* levels and able to kill the L 929 cancer cells at the rate of 75.58 % $\mu\text{g/ml}$.

[Keywords: Antimicrobials, Anticancer, Haloalkaliphilic, *Streptomyces castaneoglobisporus*]

Introduction

Microbes found in the extreme environments have been attracted a great deal of attention in recent years due to the production of various biotechnologically important natural compounds and their specialized mechanisms for adaptation to extreme environment¹. Extreme environments are populated by groups of organisms that are specifically adapted to these particular conditions and these types of extreme micro-organisms are usually referred to as alkaliphiles, halophiles, thermophiles and acidophiles, reflecting the particular type of extreme environment which they

inhabit². Halophilic microorganisms are categorized as extreme halophiles (2.5–5.2 M NaCl), borderline extreme halophiles (1.5–4.0 M NaCl), moderate halophiles (0.5–2.5 M NaCl), and halotolerants that do not show an absolute requirement to salt for growth but grow well up to often very high salt concentrations³ and tolerate 100 g/l salt (equivalent to 1.7 M NaCl) at least⁴.

Majority of the studies on extremophilic organisms, however, have been confined to extremophilic bacteria and actinomycetes are relatively less explored group⁵. Study of

extremophilic actinomycetes and identification of their metabolic properties are most important tasks in biotechnology⁶. Actinomycetes are an important group of microorganisms due to their ability to produce a wide array of secondary metabolites. Especially, the genus *Streptomyces* were the largest antibiotic-producing actinomycetes^{7&8}. Several actinomycetes, found to be proficient to produce antimicrobial compounds and halotolerant enzymes, have been reported from the coastal solar salterns^{5&9}.

Drug discovery from natural products has been traditionally focused on empirical exploitation of the most prolific microbial groups including actinomycetes and filamentous fungi. The literature vividly displays that actinomycetes have been the origin of the largest number of new antibiotic drug candidates and also lead molecules with applications in many other therapeutic areas^{10&11}. It is accepted that halophilic actinomycetes will provide a valuable resource for novel products of industrial interest, including antimicrobial, cytotoxic, neurotoxic, antimitotic, antiviral and antineoplastic activities¹². The present study intends to isolate, identify and characterize the pharmacologically important haloalkaliphilic *Streptomyces* sp. from different solar salt works and to determine their antimicrobial and anticancer activities.

Materials and Methods

Sediment samples of condenser ponds in Kovalam (8° 05' 04.35" N 77° 31' 17.07" E), Thamaraiikulam (8° 07' 06.64" N 77° 28' 59.26" E) and Puthalum (8° 06' 8.77" N 77° 28' 36.13" E) solar salt works of India were collected for isolating the *Streptomyces* sp. The physicochemical parameters of the sampling site were done following the protocols described by Bhaskaran¹³ (Table 1).

About ten grams of sediment mud samples were suspended in 100 ml of sterile saline water and 0.1 ml of suspension from this was spread over 10 % NaCl concentration on Knight's agar media (pH 7.2) and incubated at 28° C for 2-3 weeks. Based on the colony colour and morphology five different colonies were selected and sub-cultured in the Knight's agar slants at 4 °C for future use.

Table 1-Physicochemical parameters of the sampling site where the *Streptomyces* strains isolated

Physicochemical parameters	Sampling area for <i>Streptomyces</i> strains isolation		
	Kovalam	Thamaraiikulam	Puthalum
Nature of Sample	Mud sediment	Mud sediment	Mud sediment
Depth (cm)	5.0±0.0	5.0 ±0.0	5.0±0.0
Salinity (‰)	182.0±0.0	207.0±0.0	196.0±0.0
pH	8.25±0.71	8.88±0.34	8.15±0.50
Dissolved Oxygen (mg/l)	4.92±0.31	4.13±0.80	4.52±0.35
Chloride (mg/l)	158.18±1.3	189.01±1.09	162.14±0.5
Magnesium (mg/l)	14.8±0.33	19.1±0.60	15.3±0.15
Major identical colonies isolated	23±1.5	11.0±0.70	9.5±0.46

The pure culture of the actinomycetes colony was identified by morphological, biochemical and physiological tests following Bergey's Manual of Systematic Bacteriology and the International *Streptomyces* Project (ISP) procedures¹⁴. The cultural characteristics including growth, soluble pigment, aerial mycelium and substrate mycelium was examined using different culture media followed the ISP procedures¹⁵. Cell morphology was observed under a light microscope and by scanning electron microscopy (SEM). For SEM, the agar blocks containing the organisms (cultivated on YS agar medium supplemented with 5% NaCl for 14 days at 28°C) were fixed with the vapor of 1% osmium tetroxide and dried samples were sputter coated and viewed with scanning electron microscope¹⁶.

The extra cellular enzymes including amylases¹⁷, proteases¹⁸, lipases¹⁹ and gelatinases²⁰ assays were carried out. After respective incubation times for each activity, the clearance zone around the colony were measured and tabulated.

Matured culture of *Streptomyces* strains including KJ1, KJ2, AJ9, TJ1 and PJ1 were spot inoculated on starch casein agar medium and were incubated at 28° C for 4 days. Colonies were then covered with a 0.6 % of nutrient broth previously

seeded with the bacterial pathogens including *Aeromonas hydrophila*, *Escherichia coli*, *Salmonella typhi*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*. The plates were incubated at 37° C for 24 hours and the zone of inhibition (mm) was measured²¹.

Based on the antagonistic activities, the potential *Streptomyces* sp. AJ9 was identified by genomic level. One hundred nanogram of genomic DNA was isolated from *Streptomyces* sp. AJ9 and amplified by PCR using 16S rRNA universal primers (Forward: 5' CAGGCCTAACACATGCAAGTC 3'; Reverse: 5' GGGCGGWTGTACAAGGC 3'). The PCR product was purified and sequenced using the M13⁺ and M13⁻ universal primers. Sequences were compared with other 16S rRNAs obtained from GenBank using the BLAST program. Phylogenetic tree was constructed by Geneious 5.4.6 software and evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood method²² and are in the units of the number of base substitutions per site.

S. castaneoglobisporus AJ9 was inoculated into starch casein broth and incubated at 28 °C on a shaker (200-250 rpm) for seven days. After seven days incubation, the culture was spun at 5000 g for 30 min and the supernatant filtered through a 0.22 µm membrane filter. The filtrates were extracted with the equal volume of ethyl acetate. Extraction process was repeated for four times and then the extract was concentrated in a rotary evaporator and lyophilized. Lyophilized metabolites were purified through preparative silica column chromatography (50–80 µm particle size; 30 cm column length) with a flow rate of 0.5 ml elution per minute. Different proportions of the mobile phases such as hexane/ethyl acetate and ethyl acetate/methanol were used for eluting the active compounds. Finally the fractions were collected, concentrated in a rotary evaporator and stored at 4°C.

The eluted fractions of the secondary metabolites were spotted on TLC (Merck) sheets and run with CHCl₃/CH₃OH/H₂O (65:15:1) as mobile phase. The chromatogram was developed under short UV light and calculated the R_f value as per the standard database (Janek *et al.* 2010). Gas Chromatography-Mass Spectrometry (GC-MS) analysis was used to identify the active compounds from the active fractions. Based on the better

antimicrobial activity of the fractions, FIV to FVI were pooled and structurally characterized by Agilent GC–MS 5975 Inert XL MSD (United States) gas chromatography equipped with J and W 122–5532G DB-5 ms 30 × 0.25 mm × 0.25 µm and mass detector (EM with replaceable horn) that operated in EMV mode.

In vitro antibacterial activity was performed by using different fractions (FI to FVII) against few pathogenic bacteria by agar diffusion method. For antifungal activity, known quantity of fractions was poured into a well made in the centre of potato dextrose agar (PDA) plates and fungal spores (approximately 10 spores) were inoculated onto the plates and incubated at 35°C for 48 hr. The zone of inhibition was recorded by Baur *et al.*²³. Antiviral activity was performed against White Spot Syndrome Virus (WSSV) following the method of Balasubramanian *et al.*²⁴. Metabolite fractions incubated WSSV suspensions (29°C for 3 h) were injected with intramuscularly to the Indian white shrimp, *Fenneropenaeus indicus*. Hemolymph was bled from the shrimps after the 3rd day of injection, and extracted the genomic DNA following the method of Namita *et al.*²⁵. Double step diagnostic PCR were performed from the genomic DNA template using the WSSV VP28 primer designed by²⁶ and standard PCR protocols were followed Takahashi *et al.*²⁷. Anticancer activity was performed in HCT-118 colon cancer cell lines with the lyophilized fractions following the method of Freshney²⁸ and the activity was monitored after 48 hrs.

In order to get the higher yield of antimicrobial compounds, solid state fermentation culture (SSFC) was supplemented with 5 % agro-industrial waste including rice bran, wheat bran, ground nut oil cake and oil seed cake. The sterilized solid substrate was inoculated with 2.5 ml of the *S. castaneoglobisporus* AJ9 spore inoculums containing 1.7×10⁵ spores. The contents were mixed properly and incubated at 30°C for 7 days. Culture was harvested from the SSFC, extracted the secondary metabolites and performed the antibacterial and antifungal activities followed the same protocol mentioned in the earlier section.

One way Analysis of Variance (ANOVA) was carried out using the software SPSS 14.0 package and the means were compared at 0.001 % level.

Results

The phenotypic identification protocols including morphology, biochemical and physiological confirmations of the *Streptomyces* sp. isolates isolated from the solar salt works were given in the table 2. Isolates were mostly white, grey and ash colours and mostly non motile except the

Streptomyces sp. KJ1. All the strains were nitrite and TSI positive and urease negative except the *Streptomyces* PJ1 isolated from Puthalum solar salt works. The *Streptomyces* sp. AJ9 was effectively ferments the carbohydrates including glucose, fructose, sucrose, sorbitol, maltose and manitol.

Table 2-Phenotypic identification *Streptomyces* strains isolated from solar salt works for biosurfactant screening

Sl. No	Confirmative tests	<i>Streptomyces</i> strains isolated from solar salt works				
		KJ1*	KJ2*	AJ9*	TJ1**	PJ1***
1	Gram staining	+	+	+	+	+
2	Colony colour	White	White	Ash	Grey	White
3	Motility	motile	non motile	non motile	non motile	non motile
4	Methyl Red	+	-	+	+	+
5	Voges-Proskauer	-	-	-	+	-
6	Indole	-	-	-	-	+
7	Nitrate	+	+	+	+	+
8	Citrate	-	-	+	+	+
9	Urease	-	-	-	-	+
10	TSI	+	+	+	+	+
11	Catalase	+	+	-	-	-
12	Oxidase	+	+	+	-	-
13	Starch hydrolysis	+	-	+	-	-
14	Casein hydrolysis	-	-	-	+	-
15	Gelatin hydrolysis	-	-	-	-	-
16	Haemolysis	+	-	-	+	+
17	Carbohydrate fermentation	Glucose	+	+	+	+
		Fructose	+	+	+	-
		Sucrose	+	+	+	-
		Lactose	+	-	+	-
		Sorbitol	+	+	+	+
		Maltose	+	+	+	-
		Manitol	+	-	+	-

*: KJ1, KJ2 and AJ9: Kovalam isolates; **TJ1: Thamaraiikulam isolate and ***PJ1: Puthalum isolate

The cultural characteristics of the *Streptomyces* sp. were given in the table 3 and all the isolates were grown well in the culture media of actinomycetes isolation agar and starch casein agar media compared to the other media. The *Streptomyces* sp. AJ9 was well grown in all the media except Glycerol -Aspergin Agar (ISP-5) and had the substrate and aerial mycelium of ash, cream and white colour.

All the *Streptomyces* isolates exhibited positive for protease, gelatinase and amylase enzymes. *Streptomyces* sp-KJ1, *Streptomyces* sp-KJ2 and *Streptomyces* sp-TJ1 were absence of lipase activities were shown table 4. The *Streptomyces* sp AJ9 had higher protease and gelatinase activities compared to the others isolates.

Table 3-Cultural characteristics of the *Streptomyces* strains isolated from solar salt works for biosurfactant screening

Growth Media	Morphology	Streptomyces strains isolated from solar salt works				
		*KJ1	*KJ2	*AJ9	**TJ1	***PJ1
Actinomycetes Isolation Agar	Growth	Abundant	Abundant	Good	Good	Fair
	Aerial mycelium	Pale cream	White	Cream	Pale cream	Pale cream
	Substrate mycelium	White	Pale cream	White	Pale cream	Pale cream
Glycerol -Aspergin Agar (ISP-5)	Growth	Fair	Moderate	Moderate	Moderate	Abundant
	Aerial mycelium	Cream	White	White	Cream	Cream
	Substrate mycelium	Yellowish White	White	cream	Pale cream	cream
Starch Casein Agar	Growth	Abundant	Good	Abundant	Abundant	Fair
	Aerial mycelium	Cream	White	Cream	Pale cream	White
	Substrate mycelium	Yellowish White	White	Pale cream	cream	White
Knight's Agar	Growth	Good	Good	Good	Abundant	Fair
	Aerial mycelium	White	White	Ash	Grey	White
	Substrate mycelium	Pale cream	White	White	cream	cream

*: KJ1, KJ2 and AJ9: Kovalam isolates; **TJ1: Thamaraiikulam isolate and ***PJ1: Puthalum isolate

Table 4- Extracellular Enzyme screening from the *Streptomyces* sp which isolated from solar salt work sediments

Streptomyces strains	Enzymes screened			
	Amylase	Protease	Lipase	Gelatinase
<i>Streptomyces</i> sp-KJ1	++	++	-	++
<i>Streptomyces</i> sp-KJ2	+	+	-	++
<i>Streptomyces</i> sp-AJ9	+	+++	+	+++
<i>Streptomyces</i> sp-TJ1	+	++	-	++
<i>Streptomyces</i> sp-PJ1	+	++	+	+

-: No activity; +: Low activity; ++: Medium activity and +++: Higher activity

The antagonistic activity of the five isolates of *Streptomyces* sp-KJ1, *Streptomyces* sp-KJ2, *Streptomyces* sp-AJ9, *Streptomyces* sp-TJ1 and *Streptomyces* sp-PJ1 against the pathogenic bacteria were shown in table 5. Highest zone of inhibition was shown by the *Streptomyces* sp-AJ9 against all of the pathogens. It showed the antagonistic activity of 19.23, 16.04, 14.88, 11.6 and 11.56 mm of zone of inhibition against *V. parahaemolyticus*, *A. hydrophila*, *S. typhi*, *S. aureus* and *E. coli* respectively. The other isolates had moderate and less antagonistic activities

compared to the *Streptomyces* sp-AJ9 against the tested bacterial pathogens.

The blast search of the M13⁺ and M13⁻ sequence of the 16S rRNA PCR product was confirmed as *Streptomyces castaneoglobisporus*. Phylogenetic and evolutionary analysis revealed that, *S. castaneoglobisporus* shared more than 90% similarity to *S. castaneoglobisporus* NBRCI 13669, *S. globosus* CGMCC 4.320 and *Streptomyces* sp.

Table 5-In vitro antagonistic activity of the *Streptomyces* strains isolated from solar salt works sediments against pathogenic bacteria

Bacterial pathogens	Zone of inhibition (mm) formed against <i>Streptomyces</i> strains				
	KJ1*	KJ2*	AJ9*	TJ1**	PJ1***
<i>Aeromonas hydrophila</i>	5.67	9.32	16.04	5.31	4.47
	±	±	±	±	±
	0.23	0.9	1.08	0.23	0.37
<i>Escherichia coli</i>	8.26	6.61	9.78	6.65	5.10
	±	±	±	±	±
	0.76	0.6	0.87	0.33	0.02
<i>Salmonella typhi</i>	7.07	10.25	14.88	3.89	8.83
	±	±	±	±	±
	0.34	0.97	0.89	0.01	0.60
<i>Staphylococcus aureus</i>	6.12	4.32	11.56	4.60	6.15
	±	±	±	±	±
	0.55	0.01	1.01	0.23	0.72
<i>Vibrio parahaemolyticus</i>	7.12	8.50	19.23	8.21	11.57
	±	±	±	±	±
	0.08	0.02	1.43	0.02	0.89

*: KJ1, KJ2 and AJ9: Kovalam isolates; **TJ1: Thamaraiikulam isolate and ***PJ1: Puthalum isolate

R14-4 from the NCBI database. The sequence was deposited in NCBI database and strain name and GenBank accession number were *S. castaneoglobisporus* AJ9; KC603900.1 (Fig. 1). Thin layer chromatography result revealed that, there is a single spot was separated band separated from the pooled fraction of FIV- VI and had the Rf

value of the active compound was 0.68 which shown in Figure 2. The GC-MS analysis spectral peaks (29.28) confirmed that, the compound, Benzoic acid, 2-hydroxy-4-methoxy-6-methyl-, methyl ester belonging to the benzoic derivatives had the molecular formula: C₁₀H₁₂O₄ and molecular weight: 196.1999 (Fig. 3).

The antibacterial and antifungal activities performed against pathogenic bacteria and fungi using the purified pooled fractions (FIV- VI) were given in the table 6. Among the different fractions (FI to FVII) screened against the bacteria and fungi, the fractions FIV to VI were effectively suppressed the bacteria and fungi by *in vitro* level

antimicrobial activity. Antibacterial activity found of 17.6, 20.3, and 19.2 mm of zone of inhibition in FIV, FV and FVI respectively against *V. parahaemolyticus*. The same fractions also controlled the fungi, *A. niger* and *Fusarium* sp at the range of 7.3 to 8.2 mm of zone of inhibition.

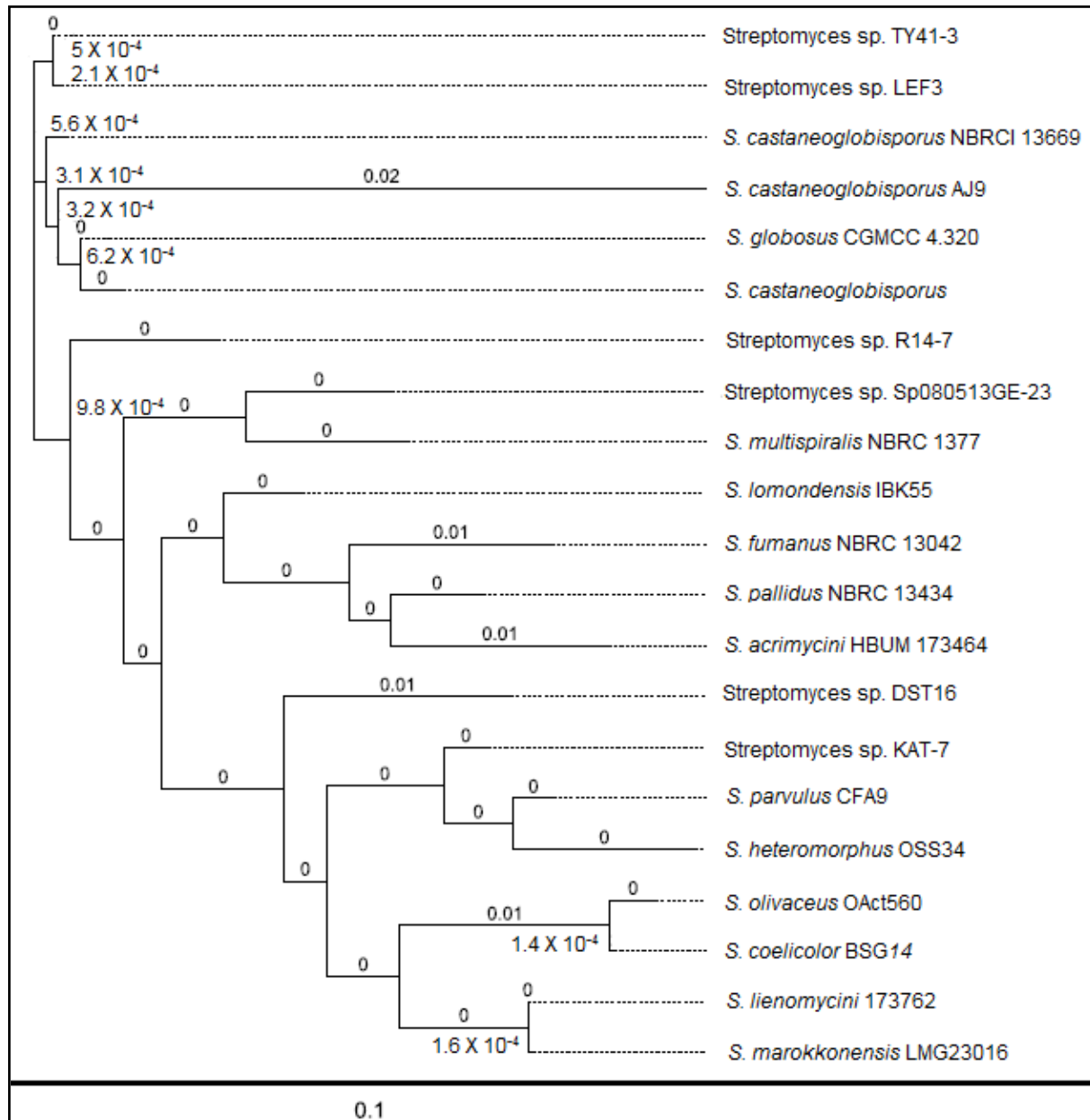


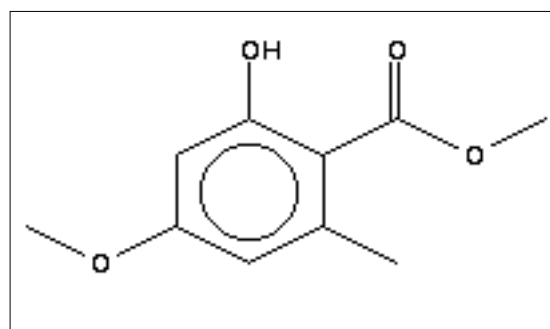
Fig. 1- Graphical phylogenetic tree analysis of *Streptomyces castaneoglobisporus* AJ9 based on 16S rRNA gene sequence data compare with other sp.

Table 6- Antimicrobial activities of *S. castaneoglobisporus* AJ9's purified fractions after column purification against bacterial and fungal pathogens

Fractions	Mobile Phase	Antimicrobial activities					
		Antibacterial (mm of zone of inhibition)				Antifungal activity	
		<i>A. hydrophila</i>	<i>S. aureus</i>	<i>S. typhi</i>	<i>V. parahaemolyticus</i>	<i>A. niger</i>	Fusarium sp.
FI	H 100: EA 0	3.7 ± 0.1 ^a	1.9 ± 0.02 ^a	5.6 ± 0.51 ^a	4.3 ± 0.06 ^a	1.8 ± 0.05 ^a	2.6 ± 0.01 ^a
FII	H 80: EA 20	4.3 ± 0.05 ^b	3.1 ± 0.3 ^b	6.5 ± 0.32 ^b	5.7 ± 0.26 ^b	2.5 ± 0.02 ^b	3.3 ± 0.02 ^b
FIII	H 60 EA 40	7.6 ± 0.33 ^c	4.9 ± 0.18 ^c	8.1 ± 0.03 ^c	9.23 ± 0.81 ^c	2.8 ± 0.13 ^b	3.8 ± 0.31 ^c
FIV	H 40: EA 60	14.1 ± 1.03 ^d	10.6 ± 0.92 ^d	15.2 ± 0.7 ^d	17.6 ± 0.34 ^d	7.6 ± 0.5 ^c	6.3 ± 0.04 ^d
FV	H 20 EA 80	17.3 ± 1.2 ^e	13.1 ± 0.7 ^e	16.1 ± 1.3 ^e	20.3 ± 1.6 ^e	7.3 ± 0.12 ^c	8.2 ± 0.5 ^e
FVI	H 0 EA 100	15.2 ± 0.70 ^f	12.4 ± 1.3 ^f	15.9 ± 0.5 ^e	19.2 ± 1.1 ^f	8.1 ± 0.33 ^a	7.6 ± 0.32 ^f
FVII	EA 80 M 20	8.11 ± 0.64 ^g	6.3 ± 0.12 ^g	7.6 ± 0.4 ^f	9.3 ± 0.8 ^c	3.3 ± 0.26 ^d	3.3 ± 0.12 ^a



Fig. 2-Qualitative analysis of the pooled antimicrobial secondary metabolite active fraction (FIV- VI) after purification

Fig. 3-Benzoic acid, 2-hydroxy-4-methoxy-6-methyl-, methyl ester (molecular formula: C₁₀H₁₂O₄ and molecular weight: 196.1999) a benzoic acid derivative identified from the pooled fractions (FIV- VI) by GCMS analysis (Retention time 29.28; quality 48).

The first, second and overall PCR detection results revealed that, the pooled fractions (FIV-FVI) had effectively suppressed the WSSV multiplication. There was 100% PCR positive signals observed after overall detection in the control and FI treated groups. PCR signals were significantly ($P \leq 0.001$) decreased to 50, 28 and 23 % in the FIV, FV and FVI fractions incubated WSSV injected shrimps (Fig. 4).

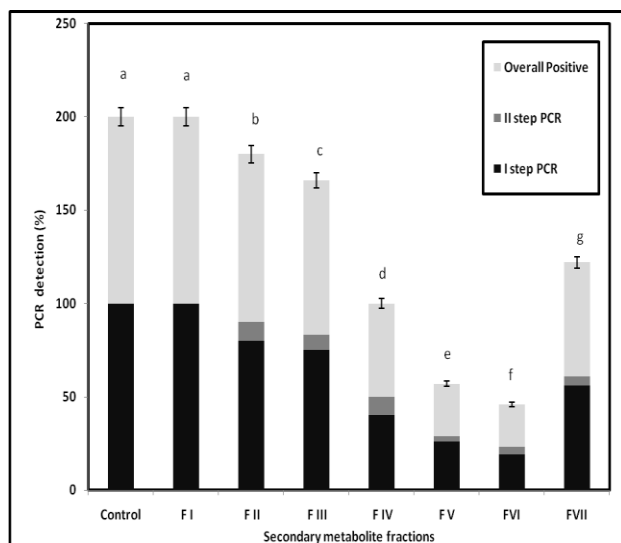


Fig. 4-Antiviral activity performed against WSSV viral particles by treating with *S. castaneoglobisporus* AJ9 secondary metabolites pooled fractions (FIV-FVI). Means with the same superscript (a-g) do not differ from each other ($P < 0.001$) – One Way ANOVA

In vitro antiproliferative activity performed by MTT cell viability assay in L 929 cell lines by treating with *S. castaneoglobisporus* AJ9 secondary metabolites' fractions and it showed inhibitory activity (Fig. 5). There was 100 % cell viability observed in the control cells and it was significantly ($P \leq 0.001$) decreased to 80.15, 61.43, 49.64, 38.56 and 24.42 % in 20, 40, 60, 80 and 100 $\mu\text{g/ml}$ of the pooled fractions respectively.

The better production of antimicrobial secondary metabolites was optimized by using different agricultural waste substrates in the culture media were given in the Figure 6. Among the different substrate used for the better production, the ground nut oil cake (GOC) influenced to higher antimicrobial production and it reflected in the higher zone of inhibition of 21.19, 22.16, 13.98 and 12.55 against *A. hydrophila*, *V. parahaemolyticus*, *A. niger* and *Fusarium* sp. respectively. Next to GOC the better antimicrobial activity given by OSC, RB and WB etc and the values were significantly ($P \leq 0.001$) varied among the different substrates.

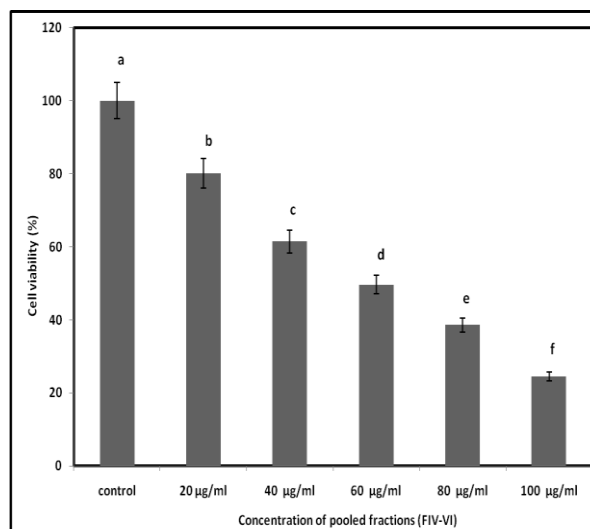


Fig. 5- *In vitro* antiproliferative activity performed by MTT cell viability assay in L 929 cell lines by treating with *S. castaneoglobisporus* AJ9 secondary metabolites pooled fractions (FIV-FVI). Means with the same superscript (a-f) do not differ from each other ($P < 0.001$) – One Way ANOVA

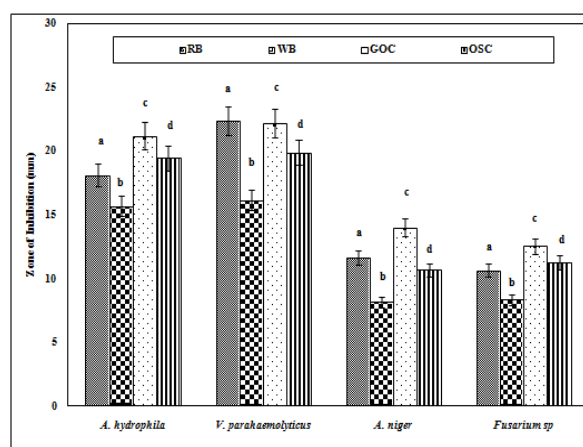


Fig. 6-Antimicrobial activity of *S. castaneoglobisporus* AJ9 secondary metabolites extracted from solid state fermentation culture against bacterial and fungal pathogens. Means with the same superscript (a-f) do not differ from each other ($P < 0.001$) – One Way ANOVA

Discussion

Streptomyces sp-AJ8, isolated from solar salt works also ferment glucose, sucrose, mannitol, starch and sorbitol etc²⁹. Generally actinomycetes are gram positive and filamentous in nature³⁰ and

they should offer a higher probability of discovering novel compounds. Alexander³¹ reported that about 20-45% of marine actinomycetes exhibited antimicrobial activity. Based on this hypothesis, our all isolates, *Streptomyces* sp. KJ1, KJ2, AJ9, TJ1 and PJ1 isolated from different solar salt had the antagonistic ranged between 5 to 16 mm of zone of inhibition against different pathogenic bacteria.

Our earlier study by Jenifer *et al.*²⁹, the *Streptomyces* sp. AJ7 and AJ10 isolated from solar salt works also were well grown in starch casein agar, Tryptone yeast extract agar (ISP7) and Knight's agar and produced the mycelium of light ash to white colour. *Streptomyces* have been reported to grow well on starch casein agar by earlier workers in this field³². The different culture media are also responsible for the morphological variations of diffusible pigments and the aerial and substrate mycelia etc³³. In our study, the different media also influenced to change the aerial and mycelial color of white, cream and ash in *Streptomyces* sp. AJ9.

Several actinomycetes, found to be proficient to produce halotolerant enzymes, have been reported from the coastal solar salterns. The combined broad range of hydrolytic enzymes found in the actinomycetes, which may be the results from the natural selection of the microorganisms in order to survive in a competing environment³⁴. *Streptomyces* sp. also secretes a wide range of extracellular hydrolytic enzymes, which enable them to degrade lignocellulosic materials in soil, and many of these enzymes also have commercial interest³⁵. The highest protease and gelatinase activities found in the *Streptomyces* sp. AJ9 also help to better antimicrobial activities which was help to degraded the bacterial and fungal cell walls leading to better antimicrobial activities.

Williams and Goodfellow³⁶ observed that compared to other Actinomycetes, *Streptomyces* sp. showed efficient antagonistic activity. In our studies, it was also similar to that; our entire tested *Streptomyces* sp. has moderate and good antagonistic activities against pathogenic bacteria. The haloalkaliphilic nature of the *Streptomyces* and other actinomycetes, their antimicrobial products were highly stable in even extreme conditions also. *Streptomyces* sp. SBU1 isolated from the saltpan regions of Cuddalore, Tamilnadu, India showed most promising antagonistic activity against

Escherichia coli, *Pseudomonas aeruginosa* and *Staphylococcus aureus*³⁷

The 16S rRNA sequencing has been widely used as a molecular clock to estimate relationships among the microbes, but more recently it has also become important as a means to identify unknown microbes up to the species level³⁸. Sacchi *et al.*³⁹ isolated the salt tolerant *Streptomyces* sp. C-2012 from the Iranian soil, identified by taxonomic level using 16S rRNA gene sequencing and it revealed that, closely related to *S. rimosus* JCM 4667T. Sequencing and database searches also helps to find the conserved sequences among the *Streptomyces* sp. in the NCBI database and help to confirm the genus and species levels.

Generally the benzoic derivatives characterized from the microbial and plant sources are very good antibacterial, antifungal, antiviral, antioxidant and anticancer properties. A novel 2-(substituted sulfonamido) benzoic acid derivatives were effectively suppressed the Gram positive and negative bacterial pathogens including *Plesiomonas shigelloides* and *E.coli*⁴⁰. Mannich bases 2-(3-Phenylaminopropionyloxy)-benzoic acid possessed significant antifungal activity against *Candida albicans*, *Rhizopus stolonifer*, *Aspergillus niger* and *Penicillium notatum* etc⁴¹. Benzoic acid derivative, 4-[(5-bromo-4-{[2,4-dioxo-3-(2-oxo-2-phenylethyl)-1,3-thiazolidin-5-ylidene] methyl}-2-ethoxyphenoxy) methyl]benzoic acid (D77) had antiretroviral activity against HIV-1⁴². The 2-aminobenzimidazole exhibited cytotoxic activity against different human cancer cell lines, including SW707, HCV29T, A549 and T47D⁴³. In our present study, the 2-hydroxy-4-methoxy-6-methyl-, methyl ester characterized from the *Streptomyces* sp. had potent antimicrobial and anticancer activities.

Streptomyces sp. showed significant antimicrobial activity against *S. aureus*, *P. aeruginosa* and *V. cholera*⁴⁴. A moderately halophilic *Streptomyces* sp. (JAJ06) producing an antimicrobial compound of polyketide type was isolated from saltpan soil collected at Tuticorin, India⁴⁵. The presence of benzoic derivatives in the pooled fractions may inhibit the bacterial cell wall synthesis and block the transcription and translation leading to arrest the bacterial multiplication.

Many species of actinomycetes have the capacity to inhibit pathogenic fungi⁴⁴. *S. violaceusniger* G10 produced antifungal

metabolites that effectively inhibited the growth of *F. oxysporum*⁴⁶. Antifungal secondary metabolites have been isolated from marine *Streptomyces* sp. DPTB16 that able to degrade the fungal pathogen cell wall due to its hydrolytic enzymes^{47&48}. Due to the presence of hydrolytic enzyme activities in the *Streptomyces* sp. AJ9 strain, it may be involved in the fungal cell wall degradation and leading to fungal death.

Antiviral activities of marine actinomycetes have been extensively studied^{49&50} characterized a novel proteinaceous protease inhibitor from *S. Chromofuscus* 34-1 (SS34-1) demonstrated a specific and selective anti-influenza virus effect. Fattiviracin A1, a novel antiviral compound isolated from *S. microflavus* 2445 effectively controlled the herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), influenza A virus and human immunodeficiency virus type 1(HIV-1). The benzoic derivatives may be interacted with the outer and nucleocapsid proteins of the WSSV and affect the transcription and leading to WSSV multiplication.

Pyrocoll, an antitumour compound was recently detected in novel alkaliphilic *Streptomyces* strain⁵¹. Lomaiviticins A and B had the dimeric diazobenzofluorene glycoside structures are potent cytotoxic metabolites produced by the halophilic actinomycetes *Micromonospora lomaivitiensis* LL-371366⁵². Benzoic derivatives may be interacted with the apoptotic pathway and induce the cell death.

The potential development of SSF has been used in production of secondary metabolic components due to several production advances like higher product yield, often shorter time, higher product stability, lower energy requirements and the use of agro industrial waste as low cost carbon and nitrogen sources when they are used as a substrate⁵³. Agro wastes are highly influenced to increase the production of enzymes, antibiotics and other secondary metabolites. The sweet potato residue and corncob were highly influenced to increase the production of tetracycline from *S. viridifaciens* and *S. rimosus* through SSF^{54&55}. Itrulin A production was optimized in *S. marinensis* SSF with wheat bran, rice bran and wheat rawa^{56&57}. Ezejitor *et al.*⁵⁸ optimized the production of oxytetracycline using cocoyam peels (household kitchen wastes of agricultural produce) through SSF in *S. speibonae* OXS1 and achieved

the peak production at third day. The present view, *S. castaneoglobisporus* AJ9 cultured in the GOC and OSC enriched media responsible for higher antimicrobial secondary metabolites than the others. This may be due to the higher protein and oil content in the substrate. *S. castaneoglobisporus* AJ9 may be utilize the oils from the media and produce more secondary metabolites. Also the antimicrobial biosurfactants, lipopeptides and rhamnolipid production was optimized with soybean flour and mahua oil cake through SSF in *Bacillus amyloliquefaciens* XZ-173 and *Serratia rubidaea* SNAU02 respectively^{59&60}. The agro waste enriched SSF had higher yield of biosurfactants with better antimicrobial activity against plant pathogens. Other point of view, during fermentation the ingredients may induce secretion of enzymes including protease and gelatinase and it may involved in the higher antimicrobial activities. The benzoic acid derivatives characterized from the haloalkaliphilic *S. castaneoglobisporus* AJ9 had wide range of pharmacological activities including antibacterial, antifungal, antiviral and anticancer. Also the highest production was optimized by utilizing the agro industrial wastes including ground nut cake and oil seed cake. Based on the present data, this molecule will be useful for developing novel drug molecules.

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