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Research Article

QUALITATIVE ANALYSIS OF ANTIMICROBIAL COMPOUND BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY METHOD

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ABSTRACT

Objective: To perform qualitative analysis of antimicrobial compound by High performance thin layer chromatography method.
Methods: *Streptomyces* sp. 2011 (JF751041) had been isolated from the marine coastal soil of Marina beach, Chennai. *Streptomyces* sp. 2011 was found to be the most potent isolate and the solvent ethyl acetate was used for extracting the bioactive compounds. The bioactive compounds were separated by solvent extraction and phase separation method. The ethyl acetate solvent containing the extract was subjected to High Performance Thin Layer Chromatography to find out the presence of ester, quinone, macrolide and terpenoids.
Results: Ten bacterial and two fungal pathogens were challenged against the crude extract. *Salmonella paratyphi, Pseudomonas aeruginosa, Bacillus subtilis* were resistant to the extract where as *Escherichia coli* and *Lactobacillus acidophilus* were less sensitive. All the other five bacteria and two fungi were showing excellent inhibition diameter. Clotrimazole, Ampicillin and Tetracycline were used as positive controls where as ethyl acetate solvent were used as negative control. The results of High Performance Thin Layer Chromatography proved the presence of ester, macrolide and terpenoids.
Conclusion: The results of the antimicrobial activity of *Streptomyces* sp. 2011 and the presence of bioactive compounds like ester, terpenoid and macrolide clearly proved that the marine soil of Marina beach is a good harbor for isolating novel varieties of antagonistic *Streptomyces* sp. and for producing broad spectrum antibiotics.

Keywords:*Streptomyces* sp. 2011 (JF751041), HPTLC, Antibiotics, Macrolide, Terpenoid, Quinone, Ester, Antimicrobial

INTRODUCTION

Microbial natural products still seem to be the most promising source for the future antibiotics [1]. Mathematical models propose that the number of antibiotics still to be discovered from actinomycetes could be above 105 [2]. Actinomycetes are the most economically and biotechnologically valuable prokaryotes and they are responsible for the production of about half of the discovered bioactive secondary metabolites [3] and most importantly antibiotics [3,4]. *Streptomyces* sp. capable of producing different types of secondary metabolites belongs to the order Actinomycetales, which are gram positive bacteria that grow extensively in soils [5, 6, 7, 8]. The *Streptomyces* species produce about 75% of commercially and medically useful antibiotics [9]. The present study was designed to evaluate the antimicrobial efficacy of the ethyl acetate extract of *Streptomyces* sp. 2011 which was isolated from the marine soil of marina beach, Chennai, India and to perform high performance thin layer chromatography to identify the bioactive compounds present in the crude extract.

PROCEDURE

Isolation of *Streptomyces* sp. 2011

The actinomycete strain *Streptomyces* sp. 2011 was isolated from the marine soil of Marina beach, Chennai, Tamil Nadu, India (latitude $13^0$03'20.09''N and longitude $80^0$17'01.32''E). The soil was collected in sterile container at 15cm depth. The actinomycete strain *Streptomyces* sp. 2011 was isolated by serial dilution plating method in Starch Casein agar medium supplemented with 10µg/ml amphotericin and 25µg/ml streptomycin (Himedia, Mumbai) to inhibit fungal and bacterial contamination respectively. These plates were incubated for 20 days at 28^0C.

Antimicrobial activity of the *Streptomyces* sp. 2011 by solvent extraction and phase separation method

Streptomyces sp. 2011 was mass cultivated in Actinomyces broth and incubated at 30^0C in a water bath shaker at 200 to 250 rpm for 10 to 15 days. After incubation, culture filtrate was subjected for solvent extraction method using Ethyl acetate in the ratio 1:1 (v/v). The phase (organic phase) having the antimicrobial property was poured in centrifuge tubes and the tubes were centrifuged at 5000 rpm for 10 minutes to extract the antimicrobial compound [10]. The supernatants were tested for their activity against the test pathogens by agar well diffusion method. The test organisms used for in the study were obtained from MTCC Chandigarh. *Streptococcus mutans* (MTCC 890), *Lactobacillus acidophilus* (MTCC 447), *Escherichia coli* (MTCC 390), *Salmonella paratyphi* (MTCC 735), *Shigellasonnei* (MTCC 2957), *Klebsiella pneumoniae* (MTCC 3384), *Staphylococcus aureus*(MTCC 3160), *Streptococcus* sp. (MTCC 889), *Bacillus subtilis* (MTCC 121) *Pseudomonas aeruginosa* (MTCC 4676) *Candida albicans* (MTCC 183) and *Aspergillus flavus* (MTCC 277).

HPTLC analysis of *Streptomyces* sp. 2011 extract for ester profile

The crude extract of *Streptomyces* sp. 2011 was dissolved in 200 µl of ethyl acetate. 2µl of standard solution (Cholesterin) and 1µl of crude extract were loaded as 6mm band length in the 3 x 10cm Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The crude extract loaded plate was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with cyclohexane and ethyl acetate as a mobile phase in the ratio 1:2. The plate was developed in the mobile phase up to 90mm and it was then dried by hot air to evaporate solvents from the plate.

HPTLC analysis of *Streptomyces* sp. 2011 extract for macrolide profile

The crude extract of *Streptomyces* sp. 2011 was dissolved in 200 µl of ethyl acetate. 5µl of standard solution (Erythromycin) and 1µl of crude extract were loaded as 6mm band length in the 3 x 10cm Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The plate loaded with crude extract was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with chloroform and methanol as a mobile phase in the ratio 9.5:0.5. The plate was developed in the mobile phase up to 90 mm and it was then dried by hot air to evaporate solvents from the plate.

HPTLC analysis of *Streptomyces* sp. 2011 extract for quinone profile:

The crude extract of *Streptomyces* sp. 2011 was dissolved in 200 µl of ethyl acetate. 3µl of standard solution (Plumbagin) and 1µl of crude extract were loaded as 6mm band length in the 3 x 10cm Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The TLC plate loaded with crude extract was kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with Toluene and Formic acid as a mobile phase in the ratio 9.9:0.1. The plate was developed in the mobile phase up to 90mm and it was then dried by hot air to evaporate solvents from the plate.

HPTLC analysis of *Streptomyces* sp. 2011 extract for terpenoid profile

The crude extract of *Streptomyces*sp. 2011 was dissolved in 200 µl of ethyl acetate. 5µl of standard solution (Solanesol) and 1µl of crude extract were loaded as 6mm band length in the 3 x 10cm Silica gel 60F254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The TLC plate loaded with samples were kept in TLC twin trough developing chamber (after saturated with Solvent vapor) with n-hexane and ethyl acetate as a mobile phase in the ratio 7.2:2.9. The plate was developed in the mobile phase up to 90mm and it was then dried by hot air to evaporate solvents from the plate.

Photo documentation

The TLC plate was then kept in photo documentation chamber and the images were taken at white light, UV 254nm and UV 366nm. The developed TLC plate was sprayed with anisaldehyde sulphuric acid reagent for ester and terpenoid, 5% Methanolic Potassium hydroxide reagent for quinone and 10% Sulphuric acid reagent for macrolide and the TLC plates were dried at 100^{0}C in Hot air oven. The TLC plate was again photo captured in day light mode and UV 366nm mode using photo documentation chamber. After derivatization, the TLC plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at 366nm. The peak table and peak densitogram were noted.

RESULT AND DISCUSSION

Antimicrobial activity of the *Streptomyces* sp. 2011 by solvent extraction and phase separation method:

Table 1: Antimicrobial activity of *Streptomyces* sp. 2011 by solvent extraction and phase separation method of isolation of antimetabolites

Test organisms	Ethyl acetate (mm)
Aspergillus flavus	14
Shigellasonnei	24
Salmonella paratyphi	0
Klebsiella pneumoniae	29
Staphylococcus aureus	17
Lactobacillus acidophilus	4
Streptococcus sp.	12
Streptococcus mutans	15
Candida albicans	12
Pseudomonas aeruginosa	0
Bacillus subtilis	0
Escherichia coli	5

The ethyl acetate extract of *Streptomyces* sp. 2011 exhibited good activity over the test organisms except *Salmonella paratyphi, Pseudomonas aeruginosa* and *Bacillus subtilis*. In total, the actinomycetes strain "A" showed good activity over gram negative bacteria than the gram positive bacteria and fungi. The result for the antibiogram pattern of actinomycetes "A" strain by solvent extraction and phase separation method of isolation of antimetabolites were tabulated in Table 1.

The ethyl acetate extract of *Streptomyces* sp. 2011 was controlling *Aspergillus flavus* with a zone of inhibition 14 mm. It was better than commercial antibiotic, clotrimazole (9 mm). The ethyl acetate extract of *Streptomyces* sp. 2011 was controlling *Candida albicans* with a zone of Inhibition 12 mm.

The ethyl acetate extract of *Streptomyces* sp. 2011 was controlling *Shigellasonnei* with a zone of Inhibition 24 mm. It was having activity better than ampicillin (0 mm), and tetracycline (16 mm). The ethyl acetate extract of *Streptomyces* sp. 2011 was controlling *Staphylococcus aureus*, *Lactobacillus acidophilus* and *Streptococcus* sp. with a zone of inhibition 17 mm, 4 mm and 12 mm respectively. The ethyl acetate extract of *Streptomyces* sp. 2011 was better than ampicillin (7 mm) for *Streptococcus* sp.The ethyl acetate extract of *Streptomyces* sp. 2011 was controlling *Streptococcus mutans*with a zone of inhibition 15 mm. It was having activity better than ampicillin (7 mm), ceftazidime (0 mm). The ethyl acetate extract of *Streptomyces* sp. 2011 was controlling *Escherichia coli* with 5 mm of inhibition.

Table 2: Antibiogram pattern of the selected microbes to the commercially available antibiotics

Test organism	Zone of inhibition (mm)		
	Clotrimazole	Tetracycline	Ampicillin
Aspergillus flavus	9	NA	NA
Candida albicans	19	NA	NA
Shigellasonnei	NA	16	0
Salmonella paratyphi	NA	15	0
Klebsiella pneumoniae	NA	12	0
Staphylococcus aureus	NA	21	20
Lactobacillus acidophilus	NA	21	10
Streptococcus sp.	NA	23	7
Streptococcus mutans	NA	21	0
Pseudomonas aeruginosa	NA	3	0
Bacillus subtilis	NA	24	30
Escherichia coli	NA	7	0

The ethyl acetate extract of *Streptomyces* sp. 2011 was controlling *Klebsiellapneumoniae* with a zone of Inhibition 29 mm. It was having better activity than ampicillin (0 mm), and tetracycline (12 mm).

HPTLC analysis of *Streptomyces* sp. 2011 extract for ester profile

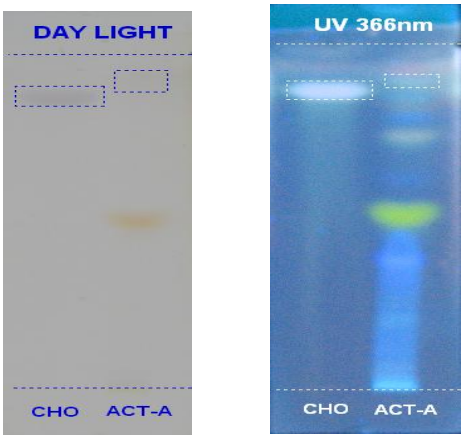


Figure 1: TLC plates showing the presence of ester after derivatization

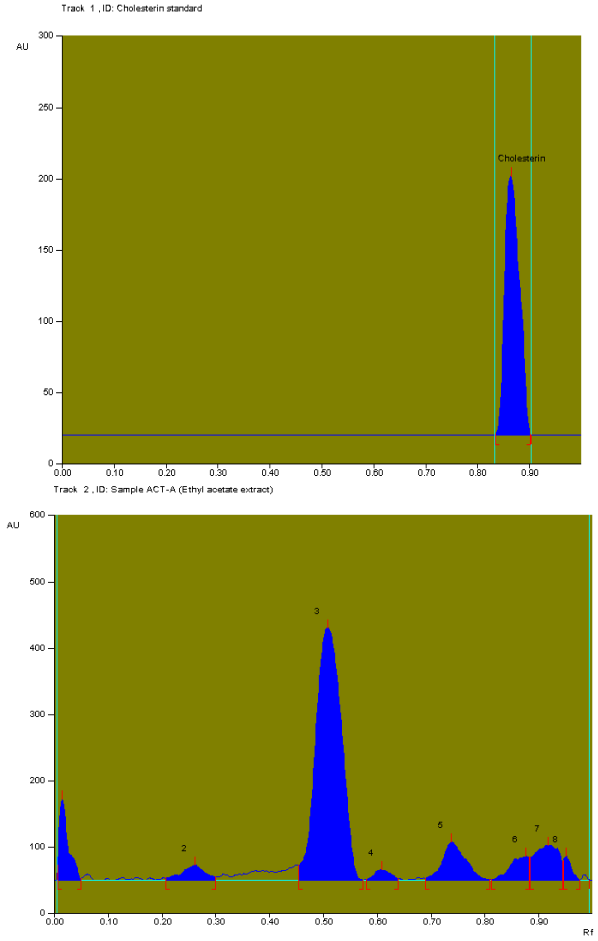
Table 3: Peak table for ester profile

Track	Peak	Rf	Height	Area	Assigned substance
CHO	1	0.86	198.9	5867.8	Cholesterin standard
Sample	1	0.01	122.6	1858.5	Unknown
Sample	2	0.26	22.7	896.1	Unknown
Sample	3	0.51	380.5	16667.2	Unknown
Sample	4	0.61	15.9	450.8	Unknown

Sample	5	0.74	58.1	2279.5	Unknown
Sample	6	0.88	35.9	1174.5	Unknown
Sample	7	0.92	53.1	2206.5	Ester 1
Sample	8	0.95	36.8	480.5	Unknown

Blue-violet colour spots at day light mode in standard and sample track was observed and the chromatogram after derivatization confirmed the presence of esterin the standard and in the sample track.

Figure 2: Cholesterin standard and ethyl acetate extract showing the presence of ester peak densitogram display



HPTLC analysis of *Streptomyces* sp. 2011 extract for macrolide profile

Brown and brownish violet colour spots were observed at day light mode in the standard track and the sample track and after derivatization the chromatogram confirmed the presence of macrolide in the standard track and also in the sample track.

Table 4: Peak table for macrolide profile

Track	Peak	Rf	Height	Area	Assigned substance
ERY	1	0.07	673.4	19888.0	Erythromycin standard
Sample	1	0.01	421.7	5220.9	Unknown
Sample	2	0.05	262.8	5415.7	Macrolide 1
Sample	3	0.22	20.0	417.1	Unknown
Sample	4	0.31	14.0	280.9	Unknown
Sample	5	0.44	42.7	1113.8	Unknown
Sample	6	0.50	12.8	138.4	Unknown
Sample	7	0.61	35.8	964.6	Unknown
Sample	8	0.75	69.5	2345.1	Unknown
Sample	9	0.81	62.1	1367.5	Unknown
Sample	10	0.92	178.1	9782.1	Unknown

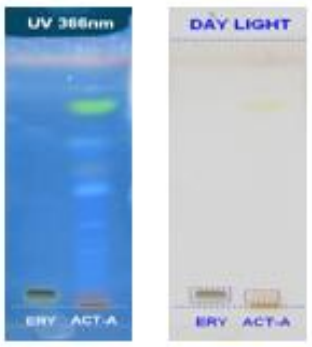


Figure 3: TLC plates showing the presence of macrolides after derivatization

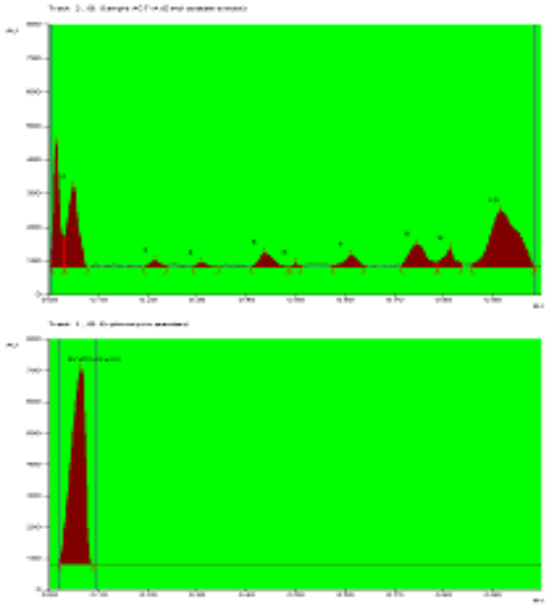


Figure 4: Ethyl acetate extract and erythromycin standardshowing the presence of macrolide in peak densitogram display

HPTLC analysis of *Streptomyces* sp. 2011extract for quinone profile:

Table 5: Peak table for quinone profile

Track	Peak	Rf	Height	Area	Assigned substance
PLU	1	0.60	78.3	3040.4	Plumbagin standard
Sample	1	0.03	531.5	12613.8	Unknown
Sample	2	0.36	23.8	760.1	Unknown
Sample	3	0.47	11.9	321.1	Unknown

A pinkish red colour spot at day light mode was observed in the standard track and after derivatization the chromatogram confirmed the presence of quinonein the standard track but not in the sample track.

HPTLC analysis of *Streptomyces* sp. 2011extract for terpenoid profile

Blue-violet colour spot at day light mode were observed in the standard track and the sample track and after derivatization the chromatogram confirmed the presence of terpenoidin the standard and sample track.

Table 6: Peak table for terpenoid profile

Track	Peak	Rf	Height	Area	Assigned substance
Sample	1	0.01	146.6	1091.0	Unknown
Sample	2	0.07	39.1	675.8	Terpenoid 1
Sample	3	0.15	17.9	386.0	Unknown
Sample	4	0.19	35.6	984.6	Unknown
Sample	5	0.36	175.0	6962.1	Terpenoid 2
Sample	6	0.82	12.7	350.3	Unknown
Sample	7	0.93	30.3	772.2	Unknown
Sample	8	0.96	31.5	765.6	Unknown
SOL	1	0.88	247.5	5744.7	Solanesol standard

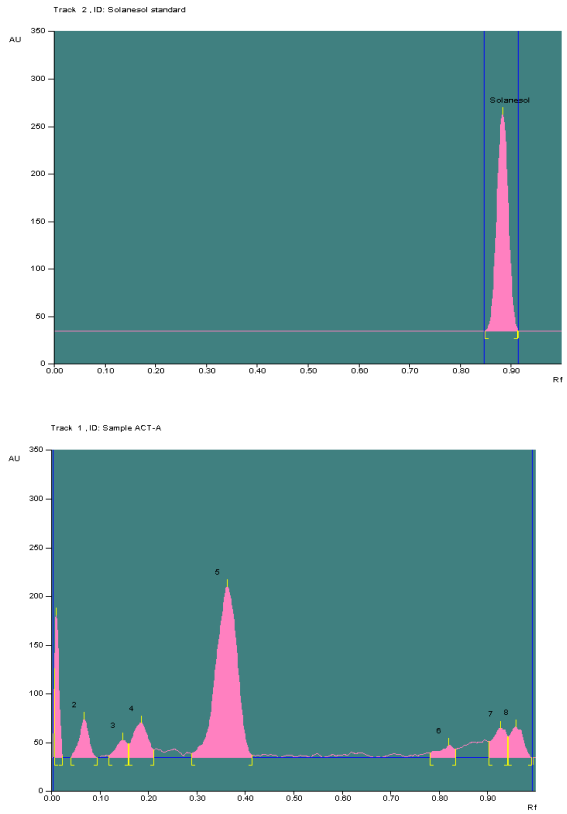


Figure 5: Solanesol standard and ethylacetate extract showing the presence of terpenoids in peak densitogramdisplay

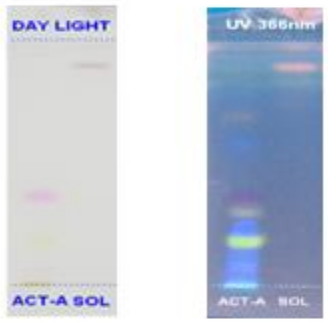


Figure 6: TLC Plates showing the presence of terpenoids after derivatization

CONCLUSION

The present study revealed that *Streptomyces*sp. 2011 (JF751041) was producing bioactive metabolites like macrolides, terpenoids and esters and the strain exhibited wide spectrum of antimicrobial activity against tested microbial pathogens. It could also be inferedthat specific isolation of compounds from *Streptomyces* sp. 2011(JF751041) could provide industrially important bioactive molecules.

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