Research Article

Gas Chromatography Mass Spectrometry Analysis and Cytotoxic Potential of Ethyl Acetate Extract of *Streptomyces* sp. Kod10

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ABSTRACT

The objective of this study was to extract the bioactive compounds from *Streptomyces* sp. KOD10 and to identify the components present in the extract using GC-MS and to find its cytotoxic potential. We had taken an initiative to isolate *Streptomyces* sp. KOD10 and to extract the bioactive compounds by solvent extraction method. The ethyl acetate extract was subjected to preparative high performance thin layer chromatography analysis and the spot was scrapped off and subjected to GC-MS analysis. The partially purified ethyl acetate extract was screened for the cytotoxic potential by brine shrimp lethality assay. The ethyl acetate extract subjected to Preparative high performance thin layer chromatography analysis had spot at R_f value 0.27 and the GC-MS analysis revealed the presence of diisooctyl phthalate, bis (2-ethylhexyl) phthalate, phthalic acid di (2-propylpentyl) ester, hentriacontane, 2-bromotetradecane, decane 2,3,5 trimethyl, pentadecane and tetradecane that provided scientific evidences for the antibacterial activity. 250µl of the partially purified ethyl acetate extract showed 60% of mortality of brine shrimp nauplii. The result of this study suggested that the actinomycetes from the Bay of Bengal coast could provide lead compounds of therapeutic value.

Keywords: PHPTLC, GC-MS, Cytotoxicity, ethyl acetate, *Streptomyces* sp. KOD 10

INTRODUCTION

Bioactive substances were low molecular compounds which exhibited numerous biological activities and microorganisms had been an important source of natural medicinal substances[1,2] and remained to be the most promising source of antibiotics.[3] Marine actinomycetes were prospective organisms for producing novel natural products and they had a exclusive metabolic diversity and excellent prospective in producing new compounds[4]. Over the past fifty years, marine organisms had provided many important structures and compounds that substantiated their potential for the development of industries as cosmetics, nutritional supplements, fine chemicals, agro chemicals and pharmaceutical agents for various diseases.[5,6] During the past thirty years, thousands of novel compounds with different biological activities ranging from anticancer to antiviral, had been isolated from various marine sources and some of them were currently in use.[5-7] According to Antibase 2012 database, there were more than 40,000 natural products reported from microorganisms and higher fungi. Marine derived antibiotics were more efficient at combating microbial infections because bacteria from terrestrial sources had not developed any resistance against them.[8,9] Various antimicrobial substances from actinomycetes had been isolated and characterized including aminoglycosides, anthracyclines, glycopeptides, beta-lactams, macrolides, nucleosides, esters, alkaloids, quinones, terpenes, terpenoids, marinopyrroles, angucyclinone, peptides, phenazine, octaketide, polyenes, polyesters, polyketides, polycyclic xanthones, abyssomicins, bonatin, triandamycins, glaciapyrroles, lactones, anthraquinones, trioxacarcins, tirandamycin, benzoxazolophenanthridines, actinomycins, heptadecaglycosides, and tetracyclines.[10-17] The aim of the present study was to describe shore line soil inhabiting actinomycete exhibited cytotoxicity. The active compounds responsible for antibacterial activity were analyzed by GC-MS analysis.

MATERIALS AND METHODS

Isolation of Actinomycetes

Streptomyces sp. KOD10 was isolated from the shore line soil of Kodiyakarai, Tamil Nadu, India (latitude 10º16'59.55''N and longitude 79º49'56.15''E). The soil was collected in sterile screw cap tube at 15cm depth. The actinomycete was isolated by serial dilution plating method in Starch Casein agar medium supplemented with 50mg/l cyclohexamide. The plate was incubated at 28˚C for 7 days.

Extraction of Bioactive Compounds

Streptomyces sp. KOD10 was inoculated in Yeast extract malt extract broth for 7 days. After the incubation period, equal volume of ethyl acetate was added to the broth containing actinomycetes and bioactive metabolites. It was then centrifuged at 5000rpm for 10 minutes.[18] A transparent layer of supernatant was collected and kept in water bath at 45˚C for evaporation of the solvent. The residue was collected and stored at 4˚C for further analysis.

Preparative High Performance Thin Layer Chromatography (PHPTLC) Analysis of Ethyl Acetate Extract

300µl of crude extract of *Streptomyces* sp. KOD10 were loaded as 25mm x 6 time band length in the 20x10 Silica gel $60F_{254}$ TLC plate using Hamilton syringe. A thin layer

chromatography plate loaded with sample and standard was kept in TLC twin trough developing chamber with chloroform and methanol in the ratio 9.5:0.5 as a mobile phase after saturated with solvent vapour.

The spot was scrapped off and suspended in ethyl acetate for further analysis. Erythromycin was used as a standard.

Gas Chromatography Mass Spectrometry Analysis of Partially Purified Ethyl Acetate Extract

The partially purified ethyl acetate extract was analyzed by gas chromatography mass spectrometry on GCMS5975C agilent at column oven temperature 70˚C, injector temperature 250˚C at split mode ratio 10 with a flow rate 1.51 ml/min.

MS condition with ion source temperature 230˚C, interface temperature 240˚C, Scan range 40 to 700 m/z, solvent cut time 5 minutes, MS start time 5 minutes, MS end time 35 minutes and Ionization EI (-70ev).

Interpretation on mass spectrum of gas chromatography mass spectrometry was done using the database of National Institute Standard and Technology (NIST) having spectra for 2,42,466 chemical compounds. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the ethyl acetate extract were determined.

Cytotoxicity Bioassay of Partially Purified Ethyl Acetate Extract

Brine Shrimp lethality bioassay[19-21] was a modern development in the assay technique of bioactive compounds which showed cytotoxicity as well as various pharmacological activities of the compounds. It was a rapid, inexpensive, in-house, general bioassay which was developed for screening and monitoring, physiologically active natural products.[19]

Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in sterile artificial seawater (Sea salt 38 g/l, pH 8.5) under constant aeration for 48hours.

After hatching, active nauplii were collected and used for assay. Ten nauplii were taken through a glass capillary and kept in separate vial containing 9ml of brine solution. 1ml of the partially purified ethyl acetate extract was added to 9ml of brine solution and maintained at 37˚C for 24 hours.

Experiments were conducted along with control (vehicle treated), different concentrations (250µl/ml to 2000µl/ml) of the test substances in a set of three petriplates per dose.

Vincristine Sulphate was used as the standard (1.562µg/ml to 50µg/ml). After 24 hours, the vials were observed for mortality, if any.

The number of live nauplii in each vial was totalled and the percentage of lethality of the brine shrimp nauplii was calculated from which the LC_{50} of the ethyl acetate extract was determined.

The regression values of partially purified ethyl acetate extract and vincristine sulphate were calculated.

RESULTS AND DISCUSSION

Preparative High Performance Thin Layer Chromatography (PHPTLC) Analysis of Ethyl Acetate Extract

Preparative high performance thin layer chromatography analysis showed that *Streptomyces* sp. KOD10 ethyl acetate extract had spot at R_f value 0.27 and the spot was scrapped off and weighed as 101.6 mg.

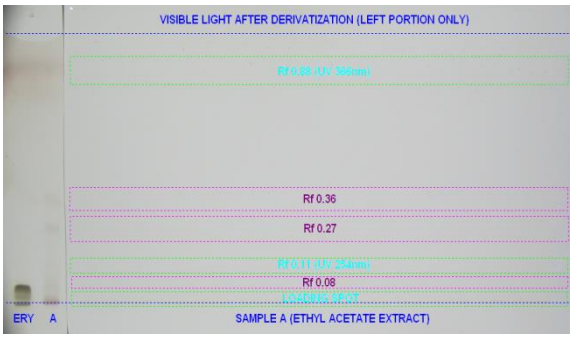

Figure 1: Chromatogram of Preparative TLC Plates after Derivatization in Visible Light

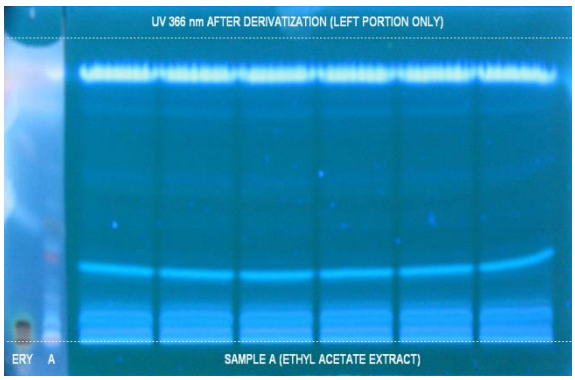

Figure 2: Chromatogram of Preparative TLC Plates after Derivatization in UV Light

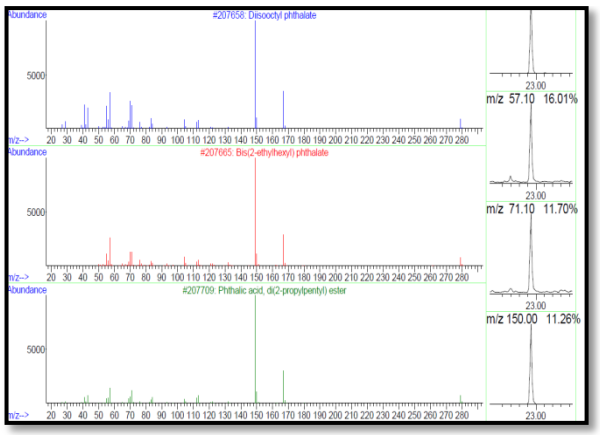

Figure 3: Gas chromatography mass spectrometry data of peak number 35 of *Streptomyces* sp. KOD10 partially purified ethyl acetate extract

Gas Chromatography Mass Spectrometry Analysis of Partially Purified Ethyl Acetate Extract

The partially purified ethyl acetate extract of *Streptomyces* sp. KOD10 was subjected to gas chromatography mass spectrometry analysis.

The identification of compounds was based on the peak area, molecular weight and molecular formula.

The area was directly proportional to quantity of the compound present in the extract.

The compounds showing high percentage peak area were Diisooctyl phthalate, Bis (2-ethylhexyl) phthalate, Phthalic acid di (2-propylpentyl) ester, Hentriacontane, 2-Bromotetradecane, Decane 2,3,5 trimethyl, Pentadecane and Tetradecane.

GCMS analysis revealed that the compounds responsible for antibacterial activity were present in the filtrate. Bis (ethyl hexyl) phthalate reported from *Streptomyces bangladeshiensis* showed antimicrobial activity against gram positive bacteria and some pathogenic fungi.[9,22,23]

1,2-Benzenedi carboxylic acid isolated from a marine alga, *Sargassum weightii* found to have antibacterial effect on a number of bacteria.[24]

Diisooctyl phthalate isolated from *Nigella glandulifera* was identified as inhibiting melanogenesis.[25]

Cytotoxicity Bioassay of Partially Purified Ethyl Acetate Extract

Table 1: GCMS Peaks of Ethyl Acetate Extract of Partially Purified Ethyl Acetate Extract of *Streptomyces* sp. KOD10

Compound	Molecular Formula	Molecular Weight	Retention Time	Peak Area
1,2-Benzenedi carboxylic acid, bis (4-methylpentyl) ester	$C_{20}H_{30}O_4$	334.45	17.92	2.05
Diisooctyl phthalate	$C_{24}H_{38}O_4$	390.56	22.94	12.35
Bis (2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.56	22.94	12.35
Phthalic acid, di(2-propylpentyl) ester	$C_{24}H_{38}O_4$	390.56	22.94	12.35

Table 2: Cytotoxic activity of partially purified ethyl acetate extract of *Streptomyces* sp. KOD10 on Brine shrimps nauplii

Concentration (µl/ml)	Log C	% Mortality	Log C_{50} (µl/ml)
2000	3.301	100	1.8211
1500	3.176	100	
1000	3.000	100	
500	2.699	100	
250	2.398	60	

Table 3: Cytotoxic Activity of Vincristine Sulphate on Brine Shrimps Nauplii

Concentration (µg/mL)	Log C	% Mortality	Log C_{50} (µg/mL)
50	1.699	100	0.9463
25	1.398	40	
12.5	1.097	30	
6.25	0.796	30	
3.125	0.495	20	
1.562	0.193	10	

Table 4: Regression Value of Partially Purified Ethyl Acetate Extract of *Streptomyces* sp. KOD10 and Vincristine Sulphate on Brine Shrimps Nauplii

Sample	LC 50 based on log C	Regression Equation	R^2
Extract	1.8211 (µl/ml)	Y=38.403x-19.938	0.6202
Vincristine sulphate	0.9463 (µg/ml)	Y=66.407x-12.843	0.8748

The organism mortality (in %) was made related to logarithm of concentration of tested compound. Using the values on died individuals in given concentrations the percent of mortality was determined using the formula.

$$Mm_{ct} = NM_n/N_0 * 100$$

Where, Mm_{ct}= mortality of individuals in time t [%]

NM_n= average number of died individuals

N_0 = initial number of living individuals put into every concentration at the test start

The LC_{50} values were assessed using non-linear regression, where mortality was related to decimal logarithm of concentration. Individual LC values were determined for each replicate separated and then the average values were determined. LC_{50} values cannot differ more than 30%. The results were compared with control and it had shown that 0% mortality rate after 24 hours of the experiment. In the present study, the partially purified ethyl acetate extract of *Streptomyces* sp. KOD10 showed 100% mortality at 500µl concentration and above. LC_{50} value was 1.8211µl/ml for the partially purified ethyl acetate extract. In the standard set, all the shrimp died in 50µg concentration. LC_{50} value was 0.9463µg/ml for Vincristine sulphate[9] reported that, if the brine shrimp lethality assay displayed LC_{50}<1000µg/ml of the extract was known to contain physiologically active principles. According to this report it was proved that the partially purified ethyl acetate extract had physiologically active principles. On the other hand almost all the bioactive compounds were always toxic in high doses.

Thus *in vivo* lethality in a simple zoological organism could be used for screening and fractionation in the discovery and monitoring bioactive compounds.[26]

The potential cytotoxicity of the compounds in this study, suggested that they had the potential to exhibit antibacterial, antitumor or anticancer activity.

CONCLUSION

The GC-MS analysis was the first step towards understanding the nature of active principles in the partially purified ethyl acetate extract of *Streptomyces* sp. KOD10 and this type of study would be helpful for further detailed study.

Based on the results of our investigation, the extract from the isolated strain revealed the presence of some of the important constituents that provides scientific evidences for the antibacterial activity.

Further investigations on the pharmacological importance and detailed chemistry have to be done in the future.

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