International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.12 No.04, pp 108-116, 2019

Anticancer activity of *Streptomyces cacaoi* subsp*cacaoi*.M20 against Breast Cancer (MCF-7) Cell Lines

T. Janaki

Department of Botany, K. M. Centre for P. G. Studies (Autonomous), Puducherry-8, India

Abstract : Increased use of chemotherapeutic drugs and their undesirable side effects on humans is an adverse impact in the medical world. This initiated the increased demand for novel antitumor drugs that are active against untreatable tumors with fewer side effects, and with the greater therapeutic efficiency. Mangrove actinomycetes have attracted great attention and offer the prospective to produce compounds with antitumor and other pharmacological activities. The actinomycete isolate M20 was isolated by dry heat (70 ºC) pre-treatment method on Starch casein agar media from the soil sample that was collected nearer to the root region of the mangrove *Avicennia marina* from the back water area, Ariyankuppam, Puducherry (UT).Among the 25 isolates, the isolate M20 had broad spectrum antimicrobial activity against tested pathogens. Physiological, biochemical and molecular characterization was done for M20. It was identified as *Streptomyces cacaoi* subsp. *cacaoi*M20 by the 16S RNA sequencing technique. The presence of anticancer property of partially purified compound fraction of isolate M20 was tested against breast cancer (MCF-7) cell lines using MTT assay. Partially purified compound fraction had ability to control the breast cancer cell lines moderately at the concentration of compound 200µg/ml (IC_{50}value) and showed 50.1 % inhibition of MCF-7 cell lines with 49.9% cell viability. This shows that the isolate M20-*Streptomyces cacaoi* subsp. *Cacaoi* has anticancerous activity against breast cancer (MCF-7) cell lines.

Keywords : breast cancer (MCF-7) cell lines, *Streptomyces cacaoi*subsp. *cacaoi*, Mangrove actinomycetes.

Introduction

The active secondary metabolites produced by actinomycetes are reported to be around 10,000. Among actinomycetes, nearly 7,600 bioactive compounds are produced by *Streptomyces* species[1]. Most of these secondary metabolites are potent antibiotics. As a result of which streptomycetes have become the primary antibiotic-producing organisms exploited by the pharmaceutical industry[1].

Members of this group are producers of clinically useful antitumor drugs such as anthracyclines, aureolic acids, enediynes, antimetabolites, peptides, carzinophilin, mitomycins, etc[2,3].

DOI= http://dx.doi.org/10.20902/IJCTR.2019.120415

The rapid development of chemotherapeutic drugs and their undesirable side effects has increased demand for novel antitumor drugs that are active against fewer side effects with untreatable tumors, and with the greater therapeutic efficiency [4]. Recently more attention has been paid on drug discovery from actinomycetes by using emerging techniques in compound extraction[5]. Marine and mangrove microorganisms encompass a complex and diverse assemblage of microscopic life forms, of which it is estimated that only 1% has been cultured or identified[6]. Mangrove actinomycetes have attracted great attention since they have developed unique metabolic and physiological capabilities and also offer the prospective to produce compounds with anticancer and other interesting pharmacological activities that would not be observed in land microorganisms[7,8,9,10,11]. The mangrove ecosystem is a largely unexplored source for actinomycetes with the potential to produce biologically active secondary metabolites. Over 2,000 actinomycetes were isolated and of these approximately 20%, 5%, and 10% inhibited the growth of Human Colon Tumor 116 cells[12]. Members of *Streptomyces*, *Micromonospora*, *Sacharomonospora*, *Actinomadura*and *Nocardiopsis*from mangrove sediments in Zhangzhou, Fujian, China, showed antitumor activities for BEL7402, A549 and HL60 tumor cells[13].

Streptocarbazoles, novel staurosporine analogs with unprecedented cyclic N-glycosidic linkages between 1,3-carbon atoms of the glycosyl moiety and two indole nitrogen atoms of the indolocarbazole core were other compounds isolated from mangrove actinomycetes. Streptocarbazoles A and B were obtained[14] from *Streptomyces sp.* In vitro anticancer activity was carried out against breast (MCF-7 and MDA-MB-231) cancer cell lines[15]. The ethyl acetate crude extracts of the *Streptomyces globisporous* strain SU7 was subjected to MTT assay. The crude extract exhibited anticancer activity towards MCF-7 and HeLa cell lines with the IC50 of 19.95 and 25.1 µg/ml respectively[16].

The anticancer activity of the genus streptomyces is reported by many researchers.However, the search for novel efficient drugs from novel source is still a main focused goal for cancer therapy. Hence, this study is aimed at developing better anti cancerous drugs from mangrove actinomycetes.

Materials and Methods

Isolation and screening of mangrove actinomycetes

Soil samplewas collected near the root region of the mangrove plant, *Avicennia marina (Forsk). Vierh – (Avicenniaceae)* in Ariyankuppam back water area, Puducherry. The soil samplewas subjected to dryheat(70^{o}C for 15 min)[17,18] pretreatment. After pretreatment, one gram soil was mixed and serially diluted in sterile water blanks. 0.1 ml of last two dilutions ($10^{-5}$and 10^{-6}) was inoculated by pour plate method usingStarch casein agar[19] supplemented with Fluconazole 80µg/ml and Nalidixlic acid 75µg/ml. Plates were incubated at 30 ±ºC for up to 30 days. Plates were periodically examined for actinomycetes colonies. Selected colonies were transferred to Yeast Malt extract agar slants and maintained in the same medium.The isolated actinomycetes were tested for antimicrobial activity[20,21].

Physiological, biochemical and molecular characterization of isolate M20

For physiological characterization, growth and activity of M20 in different pH (6, 7, 7.5, 8, 9, 10, 11, 12) , temperatures (25ºC, 30ºC, 37ºC, and 45ºC), concentrations of sodium chloride (0%, 2%, 4%, 6%, 8%, 10%, 12% and 14%) was tested. The isolate M20 was tested against amylase[22], catalase[23], gelatinase[24], lipase[22], protease[24], urease[25], phosphatase[26], citrate utilization, MR-VP, nitrate reduction, ammonia production, hydrogen sulphide production, tyrosine utilization, HCN production and cellulase[27] for biochemical characterization. For the 16sRNA sequencing analysis of M20, the purified PCR products of approximately 1,400bp were sequenced by using 2 universal primers: 518F 5'CCAGCAGCCGCGGTAATACG 3', 800R 5' TACCAGGGTATCTAATCC 3'. The isolate M20 was identified and phylogenetic tree was constructed.

Extraction of the active compound in organic solvents

To 20 ml portions of culture filtrate (12 days growth in modified nutrient glucose broth, pH 7.5) equal volumes of eight different solvents- methanol, acetone, n-butanol, chloroform, ethyl acetate, benzene, ethyl methyl ketone, petroleum ether were added and extracted for 6 hrs. Then the organic fraction was transferred to 11 cm dia. clean petriplates and allowed to dry. The dry residue was dissolved in 1ml of the respective solvents.

Fermentation of M20in modified nutrient glucose broth for active compound production and purification

Eight 250 ml of portions of modified nutrient glucose broth, pH 7.5 was prepared and sterilized. Each flask was inoculated with 2 ml of spore suspension of isolate M20 and incubated under static conditions at 30ºC for 12 days. The culture broth was filtered through the sterilized Whatmann No.1 filter papers and centrifuged at 10,000 rpm for 15 minutes. To the clean supernatant (180 ml), equal volume of methanol was added and extracted overnight in an orbital shaker at 150 rpm. Then the entire content was dried in an oven at 40ºC by transferring 100 ml portions at a time to five 25 cm dia. petriplates. Finally, 6.0 g brown oily residue was collected from the plates. The methanol active compound of isolate M20 was stored in glass vials for future use.

Compound separation

a. Thin layer Chromatography

A 100 mg dry residue was dissolved in 5 ml methanol. 10 µl methanol extract was spotted in pre-coated TLC sheets ALUGRAM® Xtra SIL G/UV_{254} using different combinations ofchloroform: methanol: acetic acid as mobile phase. Then the TLC sheet was placed in a glass jar and incubated at room temperature for 30 min. The developed spots were detected by UV light and their R*f* values were recorded.

b. Column Chromatography

The methanol fraction after drying produced six gram of dark brown oily residue. The 3g dark brown oily residue was only dissolved in 25 ml of methanol, mixed with 80-120 mesh type silica gel and the slurry was air dried. A glass column (5cm diameter x 80cm height) was used for fractionating the metabolites from the air dried slurry. The silica gel (230–400 mesh) was dried at 80ºC for 5 hours for activation then packed in the glass column using chloroform without any air bubble and air crack upto 40 cm height by gently tapping the wall of column with rubber paper weight. Then the slurry with oily residue was loaded on to the top of the column to a height of 2 cm and eluted initially with 100 % chloroform followed by chloroform: methanol: acetic acid in the ratio 95:5:0.5, 90:10:1, 85:15:2, 80:20:2, 70:30:2, 50:50:2 and finally with 100% of methanol. The active fractions obtained from the silica gel column chromatography were checked for anticancer activityusing MCF-7 cell linesand further compound was examined by using UV-Vis spectral analysis and GC-MS.

Anticancer activity of partially purified compound fraction of *Streptomyces cacaoi*subsp.*cacaoi.*M20

Cell viability test

Viability of cells was assessed by MTT assay[28] using breast cancer MCF-7 cell lines.

Reagents

1. **MTT (3-[4, 5-dimethylthiazol-2-yl] 2,5-diphenyl tetrazolium bromide):** 0.5 mg MTT/ml of serum-free DMEM medium.
2. **Solubilizing agent:** Dimethyl sulfoxide
3. **Phosphate buffered saline (PBS) (pH 7.4):** As described under cell culture reagents.

Procedure

The cells were plated separately in 96 well plates at a concentration of 1×10^5 cells/well. After 24 hrs, cells were washed twice with 100 µl of serum-free medium and starved for an hour at 37ºC. After starvation, cells were treated with the partially purified compound at different concentration for 24 hrs. At the end of the treatment period the medium was aspirated and serum free medium containing MTT (0.5 mg/ml) was added and incubated for 4 hrs at 37ºC in CO_2 incubator.

The MTT containing medium was then discarded and the cells were washed with PBS (200 µl). The crystals were then dissolved by adding 100 µl of DMSO and this was mixed properly by pipetting up and down.

Absorbance of the purple blue formazan dye was measured in a microplate reader at 570 nm (Biorad 680). Cytotoxicity was determined using Graph pad prism5 software.

Dual staining

Fluorescence Microscopic Analysis of apoptotic cell death

Acridine orange/ethidium bromide (AO/EB) double staining assay was usedin this study,

Dye preparation and drug preparation

A 200 µL of dye mixture (100µL/mg AO and 100µL/mg EB in distilled water) was mixed with 2 mL cell suspension (30,000 cells/mL) in a 6-well plate. The suspension was immediately examined and viewed under Olympus inverted fluorescence microscope (Ti-Eclipse) at 200× and 400 x magnification. Untreated cells served as controls and cells treated with drug treatment of different concentration for 24 hrs of exposure were observed for activity. A minimum of 100 cells were counted in each sample.

Drug treatments

Cells were seeded in a 24-well plate (50,000 cells per well). After 24 hrs of incubation, the medium was replaced with 100 µL medium containing various doses of drug (25µg, 50µg, 100µg and200µg) for 24 hrs, untreated cells served as the control. After 24 hrs, aspirated the medium and treated with dye and observed under the fluorescence microscope.

Ultra Violet- Visible Spectrum analysis

UV-Vis spectral analysis of partially purified methanol extract of M20 was carried out by usingInstrument: Hitachi (U-2010) Spectrophotometer. The spectrum was recorded between the wavelength range: 200 nm to 800 nm.

Analysis of partially purified compound fraction through GC-MS

Partially purified compound fraction was analyzed through Gas chromatography- mass spectrometry in chemistry department, the south India textile research association, Coimbatore - 641 014. The partially purified compound fraction (sample) was dissolved in methanol and 1 µl volume of sample was injected in the GC-MS equipment: THERMO GC - TRACE ULTRA VER: 5.0, THERMO MS DSQ II with column : ZB 5 - MS Capillary standard non -polar column and its dimension : 30 Mts, ID : 0.25 mm, Film : 0.25 µm using the helium as the carrier gas. The carrier gas flew 1.0 ml/min. The oven temperature programme was initially 70ºC and this was raised to 260 ºC at rate of 6ºC/min. The instrument was set to analysis the compounds in the sample from Low Mass (m/z): 50 to High Mass (m/z): 650, the total run time for the analysis of sample from isolate M20 was 37.50 min.

Results and Discussion

Isolation and screening of actinomycetes

The pH of mangrove soil sample collected from *Avicennia marina*was 7.5. Totally 25 actinomycetes were isolated from soil sample of *Avicennia marina* by dry heat (70^{o}C for 15 min) pretreatment method. Dry heat method yielded activeactinomycetes. Out of 25 actinomycetes, the isolate M20 has shown very good antimicrobial activity towards the tested pathogens. So, it was characterized further for identification[20,21].

Physiological, Biochemical and molecular characterizationof M20

Growth and activity of M20 in pH 7, 7.5, 8, 9 and 10 was observed, with maximum activity in pH 7.5. Maximum growth and activity was observed in 30ºC. Growth and activity was noticed from 0%-10% sodium chloride, maximum activity observed in 6 & 8% of sodium chloride. Isolate M20 was positive for amylase, catalase, gelatinase, lipase, protease, urease, phosphatase, citrate utilization, MR-VP, nitrate reduction and ammonia production, weakly positive for hydrogen sulphide production and tyrosine utilization and negative for HCN production and cellulose. The 16S rRNA gene sequence was submitted to Gene Bank with the

accession No.KP872910. The isolate M20 branched along with *Streptomyces cacaoi* subsp*cacaoi* (NRBC 12748(T)-AB184115) in the analysis.

Extraction of active compound in organic solvents

Among the eight solvent fractions tested, Methanolic solvent extract showed maximum inhibitory activity.

Fermentation of M20in modified nutrient glucose broth for active compound production and purification

The methanol extract was selected for further study of active compound from isolate M20. The cell free fermented broth 2 litres were extracted with equal volume of methanol.

Compound separation

Thin layer chromatography

The mobile phase for studying the metabolite profile as active compound from methanol crude extract of isolate M20 was Chloroform: methanol: acetic acid (8.5:1.5: 0.2 ml). R*f*-value 0.76 was determined for yellow compound fraction and 0.9 for pink compound fraction in TLC.

Column chromatography

The solvent ratio system which contained 85% chloroform: 5% methanol: 5 ml of acetic acid resulted in better separation of compounds through silica gel column chromatography. There were two colour bands were separated in this solvent system. The solvent colour fractions were eluted out and tested for anticancer and anticandidal activity[29] (Janaki et al, 2016g). This yellow compound fraction was selected for further study in metabolite profile.

Anticancer activity of compound fraction of *Streptomyces cacaoi*subsp*cacaoi.*M20

The viability of cells was assessed by MTT assay using MCF-7 (Breast cancer) cell lines. The reduction of soluble yellow tetrazolium salt to insoluble purple formazan crystals by metabolically active cells was observed. Only live cells were able to take up the tetrazolium salt. The enzyme (mitochondrial dehydrogenase) present in the mitochondria of the live cells was able to convert internalized tetrazolium salt to formazan crystals, which were purple in colour.

Table 1:Anticancer activity of partially purified compound fraction

M20 (µg)	**Optical density**		**% of cell viability**	
Control	1.39	1.39	100	100
25	1.14	1.12	82.01439	80.57554
50	1.01	1.05	72.66187	75.53957
100	0.871	0.843	62.66187	60.64748
200	0.694	0.701	49.92806	50.43165

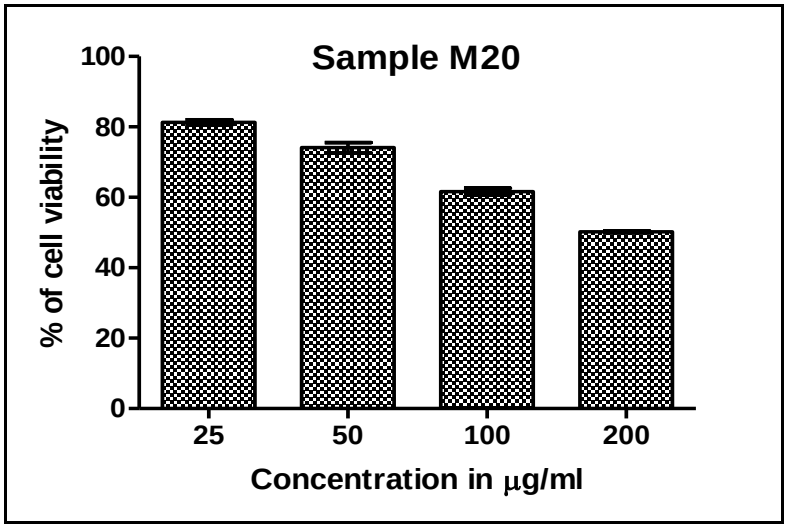
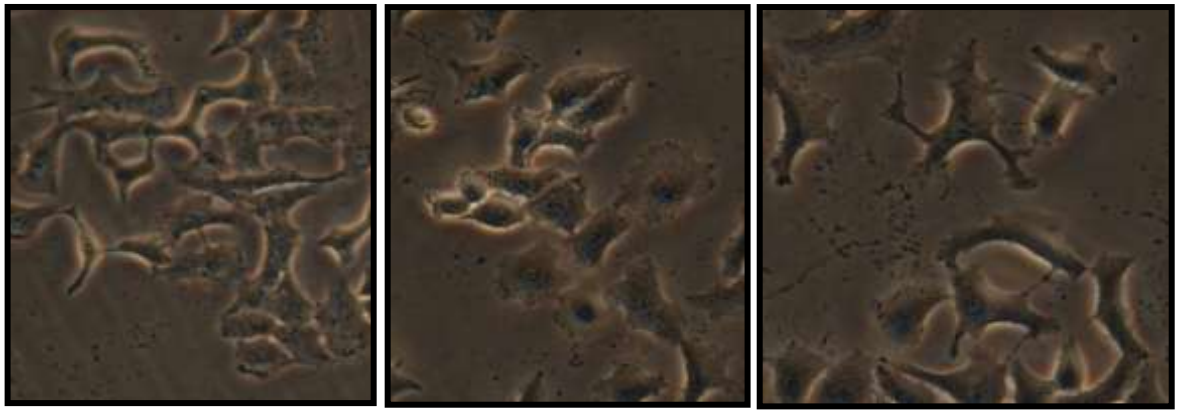


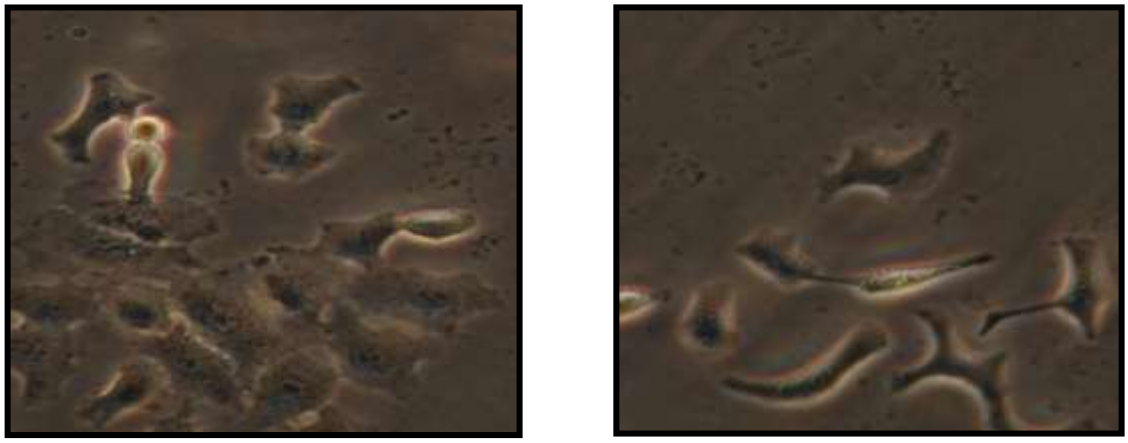
Figure 1:Histogram shows the percentage of cell viability of MCF-7 cell lines in anticancer activity

It was clear that the spectrophotometrical absorbance of the purple blue formazan dye was in cells were decreased when the concentration of the compound fraction increased from 25µg to 200µg and also it was noted that the cell viability was reduced when the concentration of the compound was increased from 25µg to 200µg. The 50% MCF-7 cells were reduced at the 200µg concentration of partially purified compound fraction; this concentration was standardized as the IC50 value for this study. On treatment ofMCF-7 cells with partially purified compound fraction, distinct morphological changes were observed with the less number of viable cells.

Induction of apotopsis by partially purified compound fraction of *Streptomyces cacaoi*subsp*cacaoi.* M20

Acridine orange was taken up by both viable and nonviable cells and emitted green fluorescence with double stranded nucleic acid (DNA), red fluorescence with single stranded nucleic acid (RNA). Ethidium bromide was taken up only by nonviable cells and emitted red fluorescence by intercalation into DNA.The types of cells were distinguished according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei. Viable cells had uniform bright green nuclei with organized structure. Apoptotic cells had orange to red nuclei with condensed or fragmented chromatin. Necrotic cells had a uniformly orange to red nuclei with condensed structure.





Control Sample M20: 25µg treated50 µg treated 100 µg treated200 µg treated

Plate 1: Effect of partially purified compound of *Streptomyces cacaoi* subsp*cacaoi.* M20 on MCF-7 cell lines

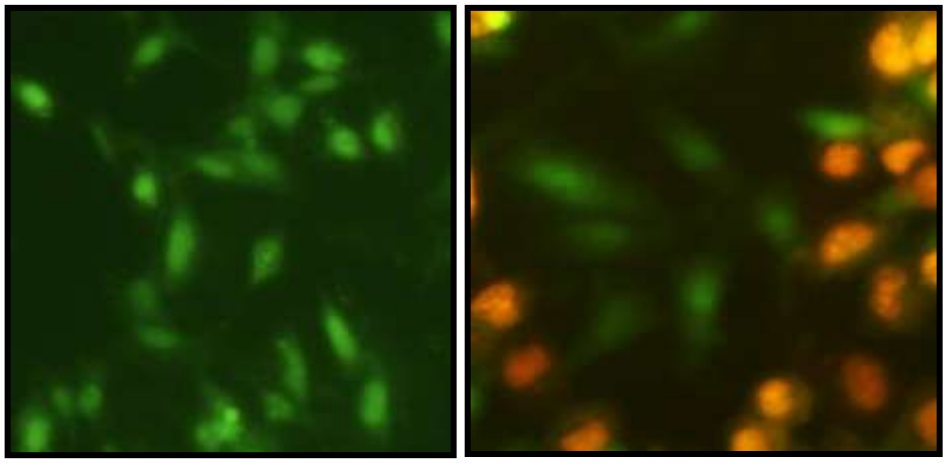


Plate 2: Dual staining: Fluorescence Microscopic analysis of apoptotic cell death

Control M20 (Treated): IC50

Partially purified compound fraction had ability to control the breast cancer cell lines moderately at the concentration of compound 200µg/ml (IC50)and showed 50.1 % inhibition of MCF-7 cell lines with 49.9% cell viability. Hence the partially purified compound fraction was found to possess the anticancer property by inducing apotosis , thereby reducing cancer cell viability. It revealed clearly that the partially purified compound fraction had moderate anticancer (cytotoxicity)to the MCF-7 cell lines.

Partially purified compound fraction had ability to control the breast cancer cell lines moderately at the concentration of compound 200µg/ml (IC_{50}value) and showed 50.1 % inhibition of MCF-7 cell lines with 49.9% cell viability. No data are available regarding inhibition of MCF-7 cell lines with the help of compound from *Streptomyces cacaoi* subsp. *cacaoi*. Relevant research works regarding anticancer activity of actinomycetes from marine sediment using MCF-7 cell lines was reported [15]. Antibreast cancer activity using mangrove *Streptomyces sp* HBO 84 was reported [30]; anticancer activity against MCF-7, Hela, Vero cell lines using *Streptomyces globisporus*strain SU7 was reported[16].

Ultra Violet- Visible Spectrum of partially purified yellow compound fraction

The maximum absorbance of partially purified yellow compound fraction was occurred ($\lambda_{max)}$ at 387.0, 282.0 and 239.0 nm.

Detection of compounds through Gas Chromatography- Mass Spectrometry.

There were 4 compounds were detected from GC-MS analysis with the retention times 6.71, 26.05, 32.40 and 37.61.

Table 2: Compounds detected through GC-MS analysis from partially purified compound fraction

S.no	Retention Time	Compound Name	Molecular Formula	Molecular Weight
1	6.71	7-Aminoheptamide, N-methyl-N-[4-(pyrrolidinyl)-2-butynyl]-	$C_{16}H_{29}N_{30}$	279
2	26.05	Nonadecane	$C_{19}H_{40}$	268
3	32.40	Hentriacontane	$C_{31}H_{64}$	436
4	37.61	Tetratetracontane	$C_{44}H_{90}$	618

Alkanes like nonadecane, hentriacontane and tetratetracontane were detected with high peak height and area. 7-Aminoheptamide, N-methyl-N-[4-(pyrrolidinyl)-2-butynyl]- was detected with low peak area. These compounds have antifungal, antibacterial, anticancer and insecticidal properties.

Conclusions

Mangrove ecosystem is enriched with diverse forms of microorganisms, among this; actinomycetes are prominent source for extracting novel compounds for antimicrobial, antitumour, anticancer, antihelmintic, insecticidal properties. The isolate M20-*Streptomyces cacaoi*subsp*cacaoi* has showed broad spectrum antimicrobial activity and showed high potency in enzymatic reactions. The metabolite profile of partially purified compound fraction of *Streptomyces cacaoi* subsp*cacaoi* showed the presence of 7-Aminoheptamide, N-methyl-N-[4-(pyrrolidinyl)-2-butynyl and alkanes. These compounds have antifungal, antibacterial, anticancer and insecticidal properties.So far, no data is available for supporting the anti cancerous study from *Streptomyces cacaoi subspcacaoi*, it is an approach to find anticancerous property from it. Further work is needed for finding better drug from the same. It is an initial work to show the property of anticancerous substance in *Streptomyces cacaoi* subsp*cacaoi* from mangrove habitat.

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