Marine Isolates *Streptomyces* sp. MCCB 267 and *Pseudonocardia* sp. MCCB 268 as source of Potential Anticancer Molecules Ikarugamycin Type Polyketides and 1-Acetyl-β-Carboline: Isolation, Purification and *in vitro* Evaluation of Anticancer Activity

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By

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Certificate

This is to certify that the research work presented in this thesis entitled "Marine isolates *Streptomyces* sp. MCCB 267 and *Pseudonocardia* sp. MCCB 268 as source of potential anticancer molecules Ikarugamycin type polyketides and 1-acetyl-β-carboline: Isolation, purification and *in vitro* evaluation of anticancer activity" is based on the original work done by Ms. Dhaneesha M (Reg. 4404) under my supervision and guidance, at National Centre for Aquatic Animal Health, School of Environmental Studies, Cochin University of Science and Technology in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition. All the relevant corrections and modifications suggested by the audience and recommended by the doctoral committee of the candidate during the presynopsis seminar have been incorporated in the thesis.

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Declaration

I hereby do declare that the work presented in this thesis entitled **"Marine isolates *Streptomyces* sp. MCCB 267 and *Pseudonocardia* sp. MCCB 268 as source of potential anticancer molecules Ikarugamycin type polyketides and 1-acetyl-β-carboline: Isolation, purification and *in vitro* evaluation of anticancer activity"** is based on the original work done by me under the guidance of Dr. Sajeevan T P, Assistant Professor, National Centre for Aquatic Animal Health, Cochin University of Science and Technology, Cochin-682 016, and that no part of this work has previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition.

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Dhaneesha M

Dedicated to

My beloved Family

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Dhaneesha M

Contents

Chapter 1

GENERAL INTRODUCTION

Contents

1.1. Bioprospecting

Natural products (NP) contribute significantly to the history and landscape of modern drug development, especially as antibacterial and anticancer agents and account for more than one-third of all FDA-approved drugs (Patridge et al. 2016). The serendipitous discovery of penicillin in 1928 from fungus *Penicillium notatum* by Scottish scientist Alexander Fleming, and later clinical success of penicillin lead to the continuous search for anti-infective natural product from environmental samples

(Abraham et al. 1941; Hersbach 1983). Later, discovery of streptomycin derived from *Streptomyces griseus* made to realize that bacteria, not only fungi, are sources of important natural products (Zaffiri et al. 2012). This ultimately lead to the discovery and development of impressive antibacterial and antifungal agents from microbes including Amphotericin B, Daptomycin, Chloramphenicol and Tetracycline (from *Streptomyces* sp.), the Rifamycins (from *Amycolatopsis rifamycinica*) and the Polymyxins (from *Paenibacillus polymyxa*) (Procópio et al. 2012; Saxena et al. 2014; Cochrane and Vederas 2016)

Morphine marketed by Merck in 1826 is the first commercial pure natural product introduced for therapeutical use from plants and followed by first semi-synthetic plant derived pure drug aspirin introduced by Bayer in 1899, based on a natural product salicin isolated from plant *Salix alba.* Other well known plant derived drugs are Paclitaxel from *Taxus brevifolia* for lung, ovarian and breast cancer, Artemisinin from traditional Chinese plant *Artemisia annua* to combat multidrug resistant malaria, Silymarin extracted from the seeds of *Silybum marianum* for the treatment of liver diseases etc. (Veeresham 2012). Animals, in particular venomous animals, such as spiders, snakes, caterpillars, scorpions, bees, wasps, centipedes, ants, toads, and frogs etc. represents sources of bioactive natural products and have attracted much attention (Dossey 2010). Venom constituents (enzymes, peptides, nucleotides, etc.) have very specific interactions with a macromolecular target in the body (α-bungarotoxin from cobras), and making them a promising drug. For example, a peptide Teprotide, derived from the venom of the Brazilian pit viper *Bothrops jararaca*, was a lead compound in the development of the antihypertensive agents, Cilazapril and Captopril (Bunag 2007).

Bioprospecting is referred as the process of systematic search for and development of new sources of chemical compounds, genes, micro-organisms, macro-organisms, and other valuable products from various natural sources (Beattie et al. 2011). It also includes the discovery of new natural products based on traditional knowledge. By and large bioprospecting can be considered as a process of discovery and commercialization of new products based on biological resources (Müller et al. 2016). So, in short, bioprospecting means looking for ways to commercialize biodiversity (Purkayastha 2016). Cox and King (2013) described bioprospecting into four phases which are tightly interconnected with each other. These are:

- Phase 1: on-site collection of biological samples.
- Phase 2: isolation and characterization of lead compounds of interest.
- Phase 3: screening for potential uses, such as pharmaceutical, nutraceutical or other uses.
- Phase 4: product development and commercialization, including patenting, trials, sales etc.

1.2. Marine bioprospecting

Oceans constitute more than 70% of Earth's surface. It encompasses most of the biological activities on planet earth. In certain marine ecosystems (e.g. coral reefs, the deep sea floor etc), the biological diversity is even greater than in tropical rain forest (Ebada et al. 2008). The high diversity of the marine environment offers enormous scope for the discovery of novel compounds, several of which are potential candidates for biomedical development. Among the many phyla found in the ocean, rich sources of pharmacologically active natural products were reported from

bacteria (including cyanobacteria), fungi, certain algae, sponges, soft corals and gorgonians, sea hares and nudibranchs, bryozoans and tunicates. These organisms have the ability to produce secondary metabolites, which may have activity against other microorganisms or active against certain physiological conditions (Bhatnagar and Kim 2010a).

The intense concentration of species coexisting in these limited extent habitats in ocean inevitably makes them very competitive and complex. Nutrient, light, water current and temperature represent additional growth limiting components, further fueling competition. This finally leads to the production of useful secondary metabolites such as alkaloids, terpenoids, peptides, polyketides, shikimic acid derivatives, sugars, steroids, and a multitude of mixed biogenesis metabolites. These metabolites can function as defenses against predators, settlement cues for larvae, inhibition of fouling and overgrowth by competitors, and pheromones in mate-searching behaviour, which ultimately help to enhance the vitality of organisms. In addition, over millions of years of high pressure and high concentrations of marine environment have been optimized by the complex and highly chiral structural compounds produced by marine organisms, which make them the potential to produce valuable therapeutic entities. Here comes the importance of marine bioprospecting. Marine bioprospecting is concerned with the exploration and exploitation of the rich biological and chemical diversity found in marine environment. It was in the early 1960s that researchers began to study and concentrated on marine environment as a novel and unexplored source of potentially useful bioactive natural products. In recent years, a renaissance has occurred in marine pharmacology for the discovery of novel marine drugs.

1.3. Marine derived drugs

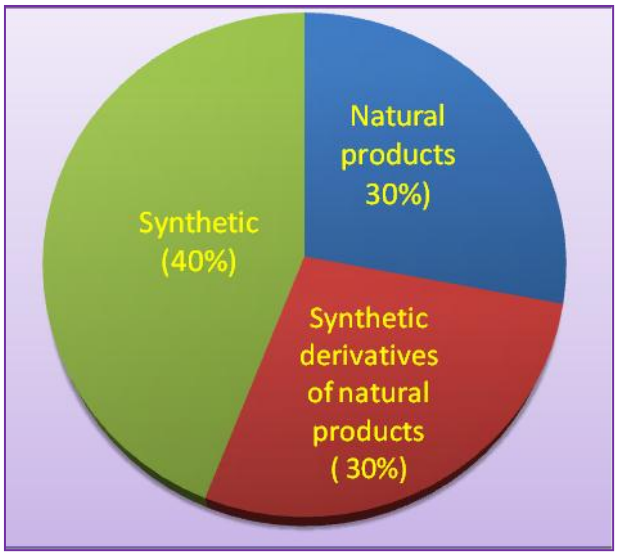
Present marine pharmaceutical pipeline includes seven Food and Drug Administration (FDA) approved marine derived drugs: for cancer, Cytarabine (Cytosar-U®, Depocyt®, FDA-approved in 1969), Eribulin mesylate (Halaven®, FDA-approved in 2010), Brentuximab Vedotin (Adcetris®, FDA-approved in 2011), Trabectedin (Yondelis®, FDA-approved in 2015), for pain, Ziconotide (Prialt®, FDA-approved in 2004); for hypertriglyceridemia, omega-3-acid ethyl esters (Lovaza®, FDA-approved in 2004); for antiviral,Vidarabine (Ara-A) (Vira-A®, FDA-approved in 1976). Further to this many marine natural products are in different stages of clinical trials and a large number of marine compounds in the preclinical pipeline (Mayer et al. 2017). As of August 2017, there are 23 marine-derived compounds in different stages of clinical trials including the 5 compounds in Phase III trials, 9 compounds in Phase II, and 9 in Phase I clinical trials for various disease conditions (http://marinepharmacology. midwestern.edu/clinPipeline.htm).

1.4. Marine bioprospecting for anticancer compounds

Cancer is still considered as one of the most serious human diseases in world, characterized by the uncontrolled cell division (Anibou et al. 2008). Its occurrence is increasing day by day with changing life style, nutrition and many other undefined factors (Ornish et al. 2005). Many cancers are curable, still some cancer treatments do not have effective medicine as the available drugs are causing side effect in many instances (Goldie and Coldman 1984).

Medicines derived from both synthetic and natural source origin have gained significant attention in the treatment of cancer. Analysis of available chemotherapeutic drugs for cancer indicates that approximately 60% of available drugs are derived from natural compounds and they are either natural products or its synthetic analogues (Cragg et al. 1997; Newman et al. 2003). Emerging evidences suggests that the marine sources are far more expected to yield anticancer drugs than the terrestrial sources, especially the secondary metabolites from marine organisms. Most of these compounds are presently in preclinical stage or in early clinical development but a few have reached to the market (Schwartsmann et al. 2001). Fig. 1.1 illustrates the contribution of drugs natural and synthetic sources.

Fig. 1.1: Illustration of contribution of drugs by natural and synthetic source (Sithranga and Kathiresan 2010)



The diversity of organisms in the marine environment has inspired researchers for many years, and this reflects in the enormous quantum of work on this subject over the past several years (Mayer et al. 2010).

Currently, there are four Food and Drug Administration (FDA) approved drugs for cancer therapy and 20 marine natural products in various stages of clinical trials against cancer alone. However, the approval for many marine derived natural products has come after several years of research, mainly by the academic community and sporadic involvement of major pharmaceutical companies (Glaser and Mayer 2009). This situation was about to change, as the National Cancer Institute discovered that bioassays with marine organism extracts were far more likely to yield anticancer drugs than terrestrial sources. This realization resulted in significant financial support to the academic community for the discovery of anticancer drugs.

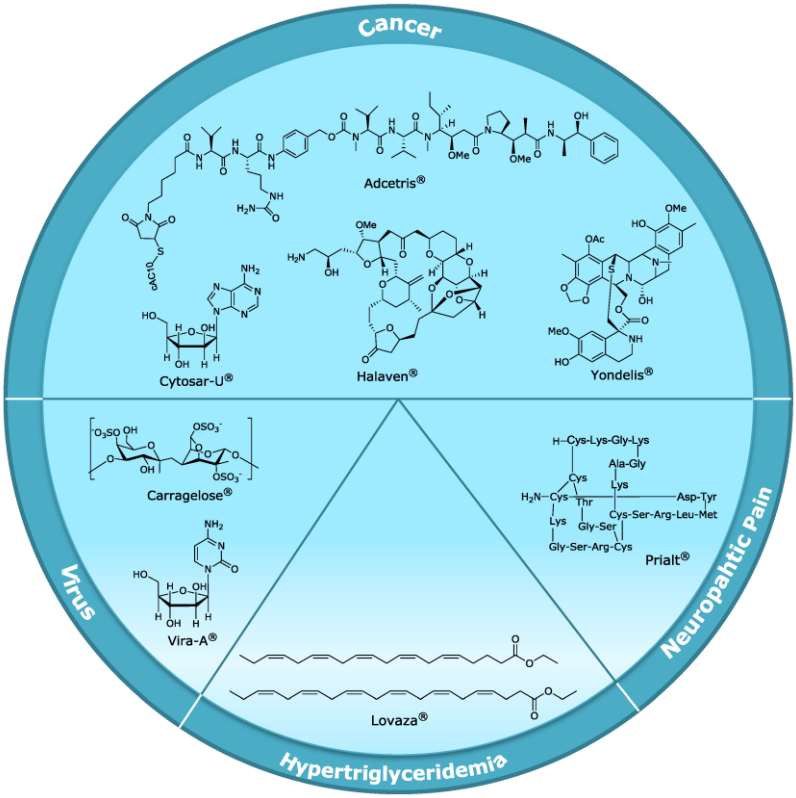


Fig. 1.2: FDA approved marine drugs (Martins et al. 2014)

1.4.1. FDA approved marine drugs for cancer

It was in 1969 that the first marine derived natural product, as well as first marine anticancer drug Cytarabine (Ara-C) got FDA approval for the treatment of Leukemia, and became an important part of marine pharmaceutical pipeline. It took over 40 years for another marine-derived anticancer natural product, Eribulin Mesylate (E7389) (2010), to gain approval and become part of the pharmacopeia for the treatment of metastatic breast cancer. This was soon followed by Brentuximab vedotin (SGN-35) (2011) for the treatment of anaplastic large T-cell systemic maglinant lymphoma, Hodgkin's disease and Trabectedin (ET-743) (2015) for soft tissue sarcoma and ovarian cancer.

Cytarabine [Cytosar-U®, arabinosyl cytosine or cytosine arabinoside, Ara-C] (Fig. 1.2) is a synthetic pyrimidine nucleoside which was developed from spongothymidine, a nucleoside originally isolated from the Caribbean sponge *Tethya crypta* (Newman and Cragg 2007). It is an S-phase specific antimetabolite cytotoxic agent, which when transformed intracellularly into cytosine arabinoside triphosphate competes with the physiologic substrate deoxycytidine triphosphate, thus resulting in both inhibition of DNA polymerase and DNA synthesis. Cytarabine is used for treatment of acute lymphocytic leukemia, acute myelocytic leukemia and blast crisis phase of chronic myelogenous leukemia, and meningeal leukemia (Absalon and Smith 2009; Thomas 2009).

Trabectedin (Yondelis®, ET-743) (Fig. 1.2) is a marine derived natural product isolated from *Ecteinascidia turbinata*, a tunicate found in the Caribbean and Mediterranean Sea (Wright et al. 1990). Trabectedin is a tetrahydroisoquinoline alkaloid and is the first marine anticancer agent

accepted in the European Union for patients with soft tissue sarcoma (STS) (Verweij 2009) and patients with relapsed platinum-sensitive ovarian cancer (Yap et al. 2009). The mechanism of action of Trabectedin is not yet fully elucidated, but it is well known that trabectedin binds by a covalent reversible bond to the DNA minor groove (Zewail-Foote and Hurley 2001) and interacts with different binding proteins of the Nucleotide Excision Repair (NER) system (Takebayashi et al. 2001).

Eribulin Mesylate (Halaven®, E7389) (Fig.1.2), is a poly-ether macrolide originally isolated from a marine sponge *Halichondria okadai* (Jackson et al. 2009). It is a tubulin-targeting agent, like the widely used taxane and vinca alkaloid chemotherapeutics though it inhibits microtubule dynamics through a unique mechanism of action distinct from those of the taxanes and vincas (Jordan 2005). E7389 exerts very potent and irreversible anti-mitotic effects against cancerous cells, which ultimately leads to cell death by apoptosis (Kuznetsov et al. 2004). In all phase trials, the compound showed very promising anticancer activities (Vahdat et al. 2009; Cortes et al. 2010).

Brentuximab vedotin (Adcetris®, SGN-35) (Fig. 1.2), is an antibody-drug conjugate (ADC) with a "warhead" derived from dolastatin 10, monomethylauristatin E. It is used to treat Hodgkin lymphoma (HL) and systemic anaplastic large cell lymphoma (ALCL). It selectively targets and acts on tumor cells expressing the CD30 antigen, a defining marker of Hodgkin lymphoma and ALCL. Promising anti cancer activity has also been seen in other lymphomas that express CD30 (Ansell 2014). Dolastatin10 is a highly potent anticancer compound initially isolated from the sea hare *Dolabella auricularia* (Pettit et al. 1987). Due to the very low concentration

of the active principle (approximately 1.0 mg/100 kg of collected organism), the structure elucidation of dolastatin 10 took nearly 15 years to complete (Pettit et al. 1987). The very low concentrations of dolastatin 10 in sea hares implicates a cyanobacterial diet as the origin of this bioactive secondary metabolite (Harrigan et al. 1998), and this was later confirmed by direct isolation of dolastatin 10 from field collections of the marine cyanobacterium *Symploca* (Luesch et al. 2001).

1.4.2. Marine derived anticancer compounds in different clinical trials

The marine derived compounds in Phase III clinical trials for the treatment of cancer include Plinabulin (NPI-2358), Plitidepsin (Aplidin®) and Glembatumumab Vedotin (CDX-011). **Plinabulin** (NPI-2358) is a fully synthetic analog of the diketopiperazine phenylahistin (halimide) from marine and terrestrial *Aspergillus sp*. (Kanoh et al. 1997). Plinabulin binds at a boundary region between a- and b-tubulin near the colchicine binding site and inhibits tubulin polymerization, leading to destabilization of tumor vascular endothelial architecture. Thus, plinabulin functions as a Vascular Disrupting Agent (VDA) that induces selective collapse of established tumor vascular, in addition to its direct apoptotic effect on tumor cells (Nicholson et al. 2006). **Plitidepsin** (Aplidin®) is a depsipeptide initially known as dehydrodidemnin B, isolated from the Mediterranean tunicate *Aplidium albicans* (Urdiales et al. 1996; Danu et al. 2013). The preclinical findings for the compound recommended potentially high anticancer activity against various, rapidly proliferating tumour cell types, as a result of interference with cell-cycle progression at G1 phase of cell division (Urdiales et al. 1996). **Glembatumumab Vedotin** (CDX-011) is an antibody-drug conjugate (ADC) that targets cancer cells expressing

transmembrane glycoprotein non-metastatic B (GPNMB) (Roth et al. 2016). The drug has derived from dolastatin 10, monomethylauristatin isolated initially from sea hare *Dolabella auricularia* (Pettit et al. 1987). It is a fully human IgG2 monoclonal antibody glembatumumab (CR011), which is linked to monomethyl auristatin E (MMAE) that targets glycoprotein NMB (gpNMB). gpNMB is a protein over expressed by multiple tumor types, including breast cancer and melanoma, and it shows very promising results against metastatic breast cancer, metastatic melanoma and triple negative breast cancer (Keir and Vahdat 2012).

The marine derived compounds in Phase II clinical trials for cancer include Depatuxizumab mafodotin (ABT-414), Denintuzumab mafodotin (SGN-CD19A), Lurbinectedin (PM01183), AGS-16C3F, Polatuzumab vedotin (DCDS-4501A), PM184, Tisotumab Vedotin and Enfortumab Vedotin ASG-22ME. Depatuxizumab mafodotin (ABT-414) is an antibody-drug conjugate composed of an EGFR IGg1 monoclonal antibody (depatuxizumab) conjugated to the tubulin inhibitor monomethyl auristatin F via a stable maleimidocaproyl link (van den Bent et al. 2017). It is in phase II clinical trial for the treatment of glioblastoma, pediatric brain tumors. It targets epidermal growth factor receptor (EGFR) & microtubule and inhibits cancer cell growth (Yoshitaka et al. 2016). Similarly, Denintuzumab mafodotin (SGN-CD19A) is another anti-CD19 monoclonal antibody linked to a synthetic cytotoxic cell-killing agent, monomethyl auristatin F (MMAF) targeting CD19, a protein expressed broadly on B-cell malignancies (Van Epps et al. 2016). It is in clinical trials for the treatment of relapsed/refractory and frontline Diffuse large B cell lymphoma (DLBCL) (Moskowitz et al. 2015). Lurbinectedin (PM01183) is an alkaloid isolated from marine tunicate, which acts on RNA polymerase II. It is in

clinical trials for the treatment of ovarian cancer, breast cancer, and small cell lung cancer (Vidal et al. 2012; Elez et al. 2014). PM184 is a polyketide tubulin inhibitor derived from the marine sponge *Lithoplocamia lithistoides.* It is in clinical trials for the treatment of solid tumors (Martínez-Díez et al. 2014). AGS-16C3F, Polatuzumab vedotin (DCDS-4501A), Tisotumab Vedotin and Enfortumab Vedotin ASG-22ME are antibody-drug conjugates (ADC). All the ADC contain one primary antibody along with monomethyl auristatin which targets different tumor types (Palanca-Wessels et al. 2015; Thompson et al. 2015; Challita-Eid et al. 2016; Chenard-Poirier et al. 2017).

Phase I clinical trials mainly comprise Bryostatin, Marizomib (Salinosporamide A; NPI-0052) and 7 different ADC containing monomethyl auristatin as drug component, and each ADC target different cancer tissue type. **Bryostatin** is a marine derived macrocyclic lactone isolated from the bryozoan, *Bugula neritina* (Pettit et al. 1983). It has shown antitumour activity in phase I trials in patients with melanoma, renal cell cancer, lymphoma, pancreatic cancer, fallopian tube cancer, gastric cancer, prostate cancer, kidney cancer, and lung cancer (Kortmansky and Schwartz 2003; El-Rayes et al. 2006; Roberts et al. 2006). Feling et al. (2003) reported a novel β-lactone- γ-lactam**,** Salinosporamide A, isolated from a fermentation broth of a new obligate marine actinomycete, *Salinispora tropica*. Salinosporamide A is an orally active proteasome inhibitor that induces apoptosis in multiple myeloma cells with mechanisms distinct from the commercial proteasome inhibitor anticancer drug, Bortezomib (Chauhan et al. 2005). **Salinosporamide A (Marizomib, NPI-0052**) is being developed by Nereus Pharmaceuticals, Inc. and has entered phase I clinical trial studies for treatment of cancer. Table 1.1 shows the current status of marine natural products in anticancer preclinical or clinical trials.

Table 1.1: Current status of marine natural products in anticancer clinical trials

Clinical Status	Compound Name	Marine Organism	Molecular Target	Reference
FDA-Approved	Trabectedin (ET-743)	Tunicate	Minor groove of DNA	(Verweij 2009)
	Brentuximab vedotin (SGN-35)	Mollusk/ cyanobacterium	CD30 & microtubules	(Ansell 2014)
	Eribulin Mesylate (E7389)	Sponge	Microtubules	(Cortes et al. 2010)
	Cytarabine (Ara-C)	Sponge	DNA polymerase	(Absalon and Smith 2009)
Phase III	Plinabulin (NPI-2358)	Fungus	Microtubules	(Nicholson et al. 2006)
	Plitidepsin	Tunicate	Rac1 & JNK activation	(Danu et al. 2013)
	Glembatumumab Vedotin (CDX-011)	Mollusk/	GPNMB & microtubules	(Roth et al. 2016)
Phase II	ABT-414 Depatuxizumab mafodotin	Mollusk/ cyanobacterium	EGFR & microtubules	(van den Bent et al. 2017)
	Denintuzumab mafodotin (SGN-CD19A)	Mollusk/ Cyanobacterium	CD19 & microtubules	(Van Epps et al. 2016)
	Lurbinectedin (PM01183)	Tunicate	RNA Polymerase II	(Vidal et al. 2012)
	AGS-16C3F	Mollusk/ Cyanobacterium	ENPP3 & microtubules	(Thompson et al. 2015)
	Polatuzumab vedotin (DCDS-4501A)	Mollusk/ Cyanobacterium	CD79b & microtubules	(Palanca-Wessels et al. 2015)
	PM184	Sponge	Minor groove of DNA	(Martínez-Díez et al. 2014)
	Tisotumab Vedotin	Mollusk/ Cyanobacterium	Tissue Factor & microtubules	(Chenard-Poirier et al. 2017)

Cont… Table1.1

Clinical Status	Compound Name	Marine Organism	Molecular Target	Reference
Phase II	Enfortumab Vedotin (ASG-22ME)	Mollusk/ cyanobacterium	Nectin-4 & microtubules	(Challita et al. 2016)
Phase I	GSK2857916	Mollusk/ cyanobacterium	BCMA	(Tai et al. 2014)
	ABBV-085	Mollusk/ cyanobacterium	LRRC15	(Purcell et al. 2016)
	ABBV-399	Mollusk/ cyanobacterium	c-Met	(Wang et al. 2017)
	ABBV-221	Mollusk/ cyanobacterium	EGFR & microtubules	(Emiliano et al. 2017)
	ASG-67E	Mollusk/ cyanobacterium	CD37 & microtubules	(Nilan and Ramasamy 2016)
	ASG-15ME	Mollusk/ cyanobacterium	SLITRK6 & microtubules	(Morrison et al. 2016)
	Bryostatin	Bryozoan	Protein kinase C	(El-Rayes et al. 2006)
	Marizomib (Salinosporamide A; NPI-0052)	Bacterium	20S proteasome	(Harrison et al. 2016)
	SGN-LIV1A	Mollusk/ cyanobacterium	LIV-1 & microtubules	(Sussman et al. 2014)

1.5. Antitumor compounds from marine actinomycetes

Microorganisms are the key sources of many chemotherapeutics used in cancer treatment; and among them actinomycetes are the most promising candidates. Clinically useful anticancer agents like Bleomycin, Actinomycin D, Anthracyclines, Mithramycine and Mitomycine C were originally isolated from actinomycetes (Newman and Cragg 2007).

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. Actinomycetes sources account for about 45% of all microbial bioactive secondary metabolites with approximately 80% compounds being produced by *Streptomyces* alone (Bérdy 2005). Despite this impressive bioactive compound production potential, studies indicate that only about 10% of the total number of bioactive natural products that can be synthesized by these organisms have been discovered (Watve et al. 2001). Because of the outstanding track record of actinomycetes for bioactive compound production potential, present studies have been mainly focused on successful isolation of novel actinomycetes. Though the rate of discovery of new compounds from terrestrial actinomycetes has decreased, the rate of re-isolation of known compounds has increased (Fenical et al. 1999) and a great deal of interest has been shown on new groups of actinomycetes from unexplored or underexplored habitats to be pursued as sources of novel bioactive compounds. As a result, marine actinomycetes have received great attention during this period (Lam 2006).

One of the major problems associated while working with marine actinomycetes is the difficulty faced in its isolation and culturing due to their specific growth requirements as well as its slow growth rate. There are a number of reports on techniques and approaches for isolating previously uncultured soil actinomycetes and the biosynthesis gene clusters they harbor (Donadio et al. 2002). In the case of marine actinomycetes, these studies are only beginning, but there have already been quite a few attempts to optimize their isolation and growth from several sources (Bull and Stach 2007; Abd-Elnaby et al. 2016) as well as the development of tools to facilitate the

genetic manipulation of the isolated biosynthesis gene clusters (Hou et al. 2008).

During the last decade, marine actinomycetes have been a great source of new compounds and their isolation all around the globe, from shallow costal sediments to the deepest sediments, demonstrates that actinomycetes are ubiquitous in marine sediments (Maldonado et al. 2005). These marine actinomycetes have got great attention since they have developed unique physiological and metabolic capabilities that not only help them survive in extreme habitats, but also make them the potential producers of compounds with anticancer and other interesting pharmacological activities. In addition to the production of antibiotics, actinomycetes produces clinically useful antitumor drugs such as peptides (bleomycin and actinomycin D), anthracyclines (aclarubicin, daunomycin and doxorubicin), aureolic acids (mithramycin), enediynes (neocarzinostatin), antimetabolites (pentostatin), carzinophilin, mitomycins etc. (Newman and Cragg 2007). However, the search for novel drugs is still a priority goal for cancer therapy, due to the rapid development of resistance to multiple chemotherapeutic drugs.

Recently Lai et al. (2017) isolated three new angucycline glycosides, designated **Grincamycin** I, J ,and K along with known congener A-7884 from marine-derived actinomycete *Streptomyces lusitanus* SCSIO LR32. Among these compounds, grincamycin J and congener A-7884 showed antitumor activity against human cancer cells NCI-H460, MDA-MB-435, MDA-MB-231, HCT-116 and HepG2 with very low IC_{50} value. In another study, Naine et al (2016), isolated and studied binding and molecular dynamic of a sesquiterpenes (**2R-acetoxymethyl-1, 3, 3-trimethyl-4t-(3-**

methyl-2-buten-1-yl)-1t-cyclohexanol) isolated from the marine *Streptomyces* sp. VITJS8 as potential anticancer agent.

Feling et al. (2003) reported a novel β-lactone- γ-lactam**,** Salinosporamide A, isolated from a new obligate marine actinomycete, *Salinispora tropica.* Salinosporamide A is an orally active proteasome inhibitor that induces apoptosis in multiple myeloma cells with mechanisms distinct from the commercial proteasome inhibitor anticancer drug**,** Bortezomib (Chauhan et al. 2005). **Salinosporamide A (Marizomib , NPI-0052**) is being developed by Nereus Pharmaceuticals, Inc. and has entered phase I clinical trial studies for treatment of cancer (Millward et al. 2012; Harrison et al. 2016).

Myhren et al. (2013) isolated a marine actinomycetes bacterium *Streptosporangium sp.* which produces bioactive compound **Iodinin** (1, 6-dihydroxyphenazine 5, 10-dioxide). This compound has the ability to induce cell death in a range of cell types and it showed selective toxicity to acute myeloid leukemia (AML) and acute promyelocytic (APL) leukemia cells. Molecular modeling suggested that iodinin intercalate between bases in the DNA, causing DNA-strand breaks. The low cytotoxicity towards normal cell types suggests that iodinin and related compounds represent promising candidates in the development of anti-cancer therapy (Myhren et al. 2013).

Zhou et al. (2012) reported a new sequential tristhiazole-thiazoline containing cyclic peptide**, Marthiapeptide A**, which was isolated from a deep South China Sea-derived strain *Marinactinospora thermotolerans* SCSIO 00652. Marthiapeptide A exhibited strong cytotoxic activity against a panel of human cancer cell lines (Zhou et al. 2012). Hu et al. (2012) reported new anthraquinone analogues, **Galvaquinones A−C** from a

marine-derived *Streptomyces spinoverrucosus*. Galvaquinone B was found to show epigenetic modulatory activity at 1.0 μM and exhibited moderate cytotoxicity against non-small-cell lung cancer (NSCLC) cell lines Calu-3 and H2887.

Wang et al. (2011) isolated three new γ-pyrones named **Marinactinones A–C** from marine- actinomycete *Marinactinospora thermotolerans* SCSIO 00606. All the three new compounds were evaluated for cytotoxic effects on six cancer cell lines and inhibitory activities of DNA topoisomerase II. All the compounds exhibited moderate cytotoxicity against SW1990, HepG2 and SMCC-7721 cell lines, and compound marinactinones B showed weak DNA topoisomerase II inhibition activity. This is the first report on the chemical constituents and their biological activities from *Marinactinospora*, a novel genus of marine actinomycetes.

Abdelfattah et al (2011) reported three new glycosylated phenazine derivatives, **Izuminosides A–C** from *Streptomyces sp.* IFM 11260. Compounds were evaluated for their activity in overcoming tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) resistance in human gastric adenocarcinoma cells. Compounds izuminoside B in combination with TRAIL showed synergistic activity in sensitizing TRAIL-resistant AGS cells (Abdelfattah et al. 2011). Similarly, Fu et al. (2011) reported five new bipyridine alkaloids and a new phenylpyridine alkaloid, which they named **Caerulomycins F-K** isolated from the marine actinomycete *Actinoalloteichus cyanogriseus* WH1-2216-6. Compounds showed cytotoxicity against the HL-60, K562, KB, and A549 cell lines (Fu et al. 2011)

Murphy et al. (2010) reported a **Saliniquinones A–F**, new members of highly cytotoxic anthraquinone- γ-Pyrones from the marine actinomycete *Salinispora arenicola* (strain CNS-325). Saliniquinone A exhibited potent inhibition of the human colon adenocarcinoma cell line HCT-116. A 26-membered polyunsaturated macrolactones, **Arenicolides** produced by a different strain of marine actinomycete *Salinispora arenicola* (strain CNR-005) was isolated from marine sediment from the coastal water around the Island of Guam. **Arenicolide A** was found to exhibit moderate cytotoxicity toward the human colon adenocarcinoma cell line HCT-116 (Williams et al. 2007b). Two bicyclic polyketides, **Saliniketal A and B** were also isolated from the same strain of *S. arenicola*. Using cultures of human bladder carcinoma T24 cells in conjunction with terephthalic acid, a potent tumor promoter that induces ornithine decarboxylase (ODC), saliniketals were found to inhibit ODC induction (Williams et al. 2007a). Several macrolactones have been reported in addition to the arenicolides.

Igarashi et al. (2010) reported a compound **Rakicidin D**, an inhibitor of tumor metastasis from marine-derived *Streptomyces* sp. (Igarashi et al. 2010). Tumor metastasis is the process by which a tumor cell leaves the primary tumor, disseminates to a distant site through the circulatory system and establishes a secondary tumor. Again, in 2012, they reported another inhibitor of tumor cell invasion **Catechoserine**. Catechoserine, is a new catecholate-type inhibitor of tumor cell invasion from *Streptomyces* sp. (Igarashi et al. 2012).

IB-96212, a 26-membered macrolide containing a spiroketal lactone structure, is produced *by Micromonospora* sp. L-25-ES25-008 isolated from a sponge collected from the Indian Ocean (Cañedo et al. 2000). This

compound showed cytotoxic activity against mouse leukemia P-388 and human lung non-small cell A-549, colon adenocarcinoma HT-29 and melanoma MEL-28 cell lines. **Manumycin A and chinikomycins A and B** were isolated from marine *Streptomyces* sp. M045 and showed moderate antitumor activity. Chinicomycin A selectively inhibited proliferation in cell lines of mammary cancer MAXF 401NL, melanoma MEXF 462NL, and renal cancer RXF 944L. Chinikomycin B exhibited selective antitumor activity against the mammary cancer cell line MAXF 401NL (Li et al. 2005). **Nonactin**, a cyclic polyether also known as macrotetrolide, has been isolated from cultures of marine *Streptomyces sp*. KORDI-3238. Nonactin has been shown to be an effective inhibitor against the multidrug-resistant human erythroleukemia cell line K-562 (Borrel et al. 1994).

Two novel anthracycline compounds, **Komodoquinone A**, and **Komodoquinone B**, were isolated from marine *Streptomyces* sp. KS3 (Itoh et al. 2003). Another anthracycline related compound**, Chartreusin**, has been isolated from cultures of *Streptomyces* sp.QD518 isolated from the Jiaozhou Bay of Quindao, China (Wu et al. 2006). Four new antitumor pyranones **PM050511, PM050463, PM060054, and PM060431** were isolated from marine actinomycetes *Streptomyces albus* POR-04-15-053. Compounds PM050511 and PM060431 showed strong cytotoxicity against three human tumor cell lines and could be developed as potential antitumor agents (Schleissner et al. 2011).

In India, a few dedicated scientists have been conducting investigation on marine natural product isolation at Central Drug Research Institute (CDRI) (Bhakuni 1995; Lakshmi et al. 2006). Isolation and culturing of marine actinomycetes for the production of secondary metabolites had also

been reported recently (Gandhimathi et al. 2008). However, most of these works have been focused on antibacterial or antiviral activities of these secondary metabolites produced by marine actinomycetes. Very few reports are available regarding antitumor property of marine actinomycetes from Indian context.

Dasari et al. (2012) isolated a novel a cytotoxic compound, which was identified as **1(10-aminodecyl) Pyridinium** salt antibiotic from *Amycolatopsis alba* var. nov. DVR D4. *Amycolatopsis alba* var. nov. DVR D4 was isolated from marine sediment samples from Visakhapatnam coast of Bay of Bengal, India. The compound exhibited promising cytotoxic activity against cancer cell lines of cervix (HeLa), breast (MCF-7), and brain (U87MG) *in vitro* and also showed antibacterial activities against gram-positive and gram-negative bacteria.

Sateesh and Rathod (2011) isolated 28 marine isolates of actinomycetes from Karwar, west coast of India and found that the metabolite from *Streptomyces* sp. KR-5 had an inhibitory activity against the growth of human breast cancer cell line. Suthindhiran and Kannabiran (2009) isolated and studied the cytotoxic and antimicrobial potential of actinomycete species *Saccharopolyspora salina* VITSDK4 isolated from marine sediment samples from Bay of Bengal coast of India and the compounds showed cytotoxic activity on HeLa cells.

Adinarayana et al. (2006) isolated marine actinobacteria *Strepomyces corchorusii* AUBN1/7 from marine sediment samples collected from Bay of Bengal, India. Two cytotoxic compounds, identified as **Resistomycin**, a quinone-related antibiotic**,** and **Tetracenomycin D,** an anthraquinone

antibiotic, were isolated which demonstrated in vitro potent cytotoxic activity against cell lines HMO2 and HePG2.

Gorajana et al. (2005) isolated a *Streptomyces chinaensis* AUBN1/7 from marine sediment samples of Bay of Bengal, which yielded **1-hydroxy-1-norresistomycin** and **Resistoflavin**. These compounds showed potent cytotoxic activities against cell lines like HMO2 (gastric adenocarcinoma) and HePG2 (hepatic carcinoma).

Apart from these few reports, the effort to isolate antitumor compounds from marine actinomycetes isolated from Indian Ocean is almost scanty. In this backdrop, the present work aims to explore antitumor compound production potential of marine actinomycetes isolates from Indian Ocean. Table 1.2 shows antitumor compounds identified from marine actinomycetes.

Table 1.2: Potential anticancer compounds produced by marine actinomycetes

Compound	Structural type	Organism	Reference
Grincamycin	Angucycline glycosides	*Streptomyces lusitanus* SCSIO LR32	(Lai et al. 2017)
2R-acetoxymethyl-1,3,3-trimethyl-4t-(3-methyl-2-buten-1-yl)-1t-cyclohexanol)	Sesquiterpenes	*Streptomyces* sp. VITJS8	(Naine et al. 2016)
Iodinin	Phenazine	*Actinomadura* sp. M048 *Streptosporangium* sp	(Myhren et al. 2013)
Marthiapeptide A	Cyclic peptide	*Marinactinospora thermotolerans* SCSIO 00652	(Zhou et al. 2012)

Cont… Table 1.2

Compound	Structural type	Organism	Reference
Marinactinones A–C	Pyrones	*Marinactinospora thermotolerans* SCSIO 00606	(Wang et al. 2011)
Galvaquinones A−C	Anthraquinone	*Streptomyces spinoverrucosus*	(Hu et al. 2012)
Izuminosides A–C	Phenazine	*Streptomyces* sp. IFM 11260	(Abdelfattah et al. 2011)
Caerulomycins F-K	Alkaloids	*Actinoalloteichus cyano-griseus* WH1-2216-6	(Fu et al. 2011)
Saliniquinones A–F	Anthraquinone-γ-Pyrones	*Salinispora arenicola* (strain CNS-325)	(Murphy et al. 2010)
Arenicolides	Macrolactones	*Salinispora arenicola* (strain CNR-005)	(Williams et al. 2007b)
Saliniketal A and B	Polyketides	*S. arenicola.*	(Williams et al. 2007a)
Proximicins	Non -ribosomal peptide	*Verrucosispora* strain MG-37	(Schneider et al. 2008)
Manumycin A	Quinine	*Streptomyces* sp. M045	(Li et al. 2005)
Chinikomycins A and B	Quinine	*Streptomyces* sp. M045	(Li et al. 2005)
IB-96212	Macrolide	*Micromonospora* sp.	(Cañedo et al. 2000)
Salinosporamide A	β-lactone- γ-lactam	*Salinispora tropica*	(Feling et al. 2003)
Marmycins A and B	Quinines	*Streptomyces*	(Martin et al. 2007)
Chartreusin	Anthracycline	*Streptomyces* sp.QD518	(Butler 2008)

Cont… Table1.2

Compound	Structural type	Organism	Reference
Nonactin	Polyether	*Streptomyces* sp. KORDI-3238	(Borrel et al. 1994)
Lucentamycins A-D	Peptides	*Nocardiopsis lucentensis* (strain CNR-712).	(Cho et al. 2007)
Nataxazole	Benzoxazole	*Streptomyces* sp.	(Sommer et al. 2008)
Komodoquinone A	Anthracycline	*Streptomyces* sp. KS3	(Itoh et al. 2003)
PM050511	Pyranones	*Streptomyces albus* POR-04-15-053	(Schleissner et al. 2011)
MFTZ-1	Macrolide	*Streptomyces* sp.	(Xie et al. 2007)
Marinomycins	Macrodiolide	*Marinispora sp.* CNQ-140	(Kwon et al. 2006)
Tartrolon D	Tartrolon	*Streptomyces* sp. MDG-04-17069	(Pérez et al. 2009)
Arenamides	Peptide	*S. arenicola* CNT-088	(Asolkar et al. 2009)
Piperazimycins	Peptide	*Streptomyces* sp. strain CNQ593	(Miller et al. 2007)
Lajollamycin	Non -ribosomal peptide	*S. nodosus*	(Manam et al. 2005)
Altemicidin	Alkaloid	*S. sioyaensis* SA1758	(Takahashi et al. 1989)
IB-00208	Xanthone	*Actinomadura* sp BL-42-PO13-046	(Malet-Cascon et al. 2003)
K252c	Indolocarbazole	*Actinomycete* Z2039-2	(Liu et al. 2007)
Marineosins	Polypyrrole	*Streptomyces* strain CNQ617	(Boonlarppradab et al. 2008)
Streptopyrrolidine	Tetrahydropyrrole	*Streptomyces* sp. KORDI-3973	(Shin et al. 2008)

Cont… Table1.2

Compound	Structural type	Organism	Reference
Marinones	Isoprenoid	*Actinomycete* isolate CNH-099	(Hardt et al. 2000)
Ammosamides	Pyrroloiminoquinone	*Streptomyces* strain CNR-698	(Hughes et al. 2009)
Caboxamycin	Benzoxazole	*Streptomyces* sp. NTK 937	(Hohmann et al. 2009)
3,6-disubstituted indoles	Indole	*Streptomyces* strain BL-49-58-005	(Sánchez López et al. 2003)
Streptochlorin	Methylpyridine	*Streptomyces* sp. KORDI-3238	(Jeong et al. 2006)
1-hydroxy-1-norresistomycin	Polyketide	*Streptomyces chinaensis* AUBN1/7	(Gorajana et al. 2005)
Resistoflavin	Polyketide	*Streptomyces chinaensis* AUBN1/7	(Gorajana et al. 2005)
Resistomycin	Quinine	*Strepomyces corchorusii*. AUBN1/7	(Adinarayana et al. 2006)
Tetracenomycin D	Anthraquinone	*Strepomyces corchorusii*. AUBN1/7	(Adinarayana et al. 2006)
1(10-aminodecyl) Pyridinium	alkylpyridinium	*Amycolatopsis alba* var. nov. DVR D4	(Dasari et al. 2012)

1.6. Type of marine actinomycetes derived antitumor compounds based on chemical structure

Based on the chemical nature the antitumor compounds can be classified as below.

1.6.1. Polyketides

Polyketides are a large family of natural products produced by step-wise decarboxylative claisen- type condensation of acyl-CoA precursors,

reactions catalyzed by polyketide synthases (PKSs) (Rix et al. 2002). The carbon skeleton of polyketides may be further reduced and modified by different domains present in PKSs with ketoreductase, dehydratase and enoylreductase activities (Ayuso-Sacido and Genilloud 2005). Three types of PKSs are known to date: type I PKSs are multifunctional enzymes organized into modules, type II PKSs are multienzyme complexes carrying out a single set of activities and type III PKSs, also known as chalcone synthase-like PKSs, are iteratively acting condensing enzymes (Gokhale et al. 2007). Numerous type I polyketide derived compounds with antitumor activity have been isolated from marine actinomycetes. Arenicolide A and Saliniketal A and B produced by *S. arenicola* CNR-005 is such an example for marine derived polyketide (Williams et al. 2007a). Aromatic polyketides are synthesized by type II PKS and are further divided into different structural classes such as angucyclines, anthracyclines and tetracyclines, among others.

1.6.2. Non-ribosomal peptides

This class of natural products comprises peptides synthesized by non-ribosomal peptide synthetases (NRPS) (Ayuso-Sacido and Genilloud 2005). The amino acid monomers incorporated by NRPS assembly lines are aminoacyl-AMP mixed anhydrides that follow the same chemical logic as PKSs for chain elongation and are then modified based on the program encoded by different domains present in NRPS modules, which can include epimerization, methyltransferase, reductase or oxidase activities (Du and Lou 2010). Quite often non-ribosomal peptides also contain some unique structural features such as heterocyclic elements and deoxysugars (Walsh

2004). Proximicins and lucentamycins are typical examples of NPRS (Fiedler et al. 2008).

1.6.3. Mixed polyketide- non ribosomal peptides

NRPSs and type I PKSs are multifunctional proteins that are organized into modules and use a similar strategy for the assembly of these two distinct classes of natural products (Boddy 2014). Mixed Polyketide-Non ribosomal Peptides compounds are derived from amino acids and short-chain carboxylic acids (Ansari et al. 2004). The well known compound of this family, salinosporamide A, is currently in phase I clinical trials for the treatment of cancer (Feling et al. 2003).

1.6.4. Isoprenoids

Isoprenoids, are one of the biggest families of natural compounds similar to terpenes. These groups of compounds are derived from five-carbon isoprene units assembled and modified in different ways (Chandran et al. 2011). Isoprenoids are classified into several groups based on the number of C5 units, which form part of their structure. They are classified as monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20) (Dairi 2013). Altemicidin are the typical examples of isoprenoids (Takahashi et al. 1989).

1.6.5. Indolocarbazoles

Indolocarbazoles family of compounds contain a characteristic indolo[2,3-a] pyrrolo [3,4c] carbazole core with sugars attached. These compounds comprise antitumor drugs with several mechanisms of action including DNA-damage by targeting on topoisomerases I and II, and inhibition of protein kinases (Sánchez López et al. 2003; Curiel et al. 2005).

One of the well known indolocarbazole, staurosporine has been isolated from several marine actinomycetes including *Streptomyces sp.* KS3 (Itoh et al. 2003) and *S. staurosporeus* AM-2282 (Omura et al. 1977).

1.7. Screening of anticancer drug from natural prodcuts

US National Cancer Institute (NCI) has started anti-cancer screening support to researchers worldwide in 1955. Until 1985, the NCI screening program depended predominantly on the *in vivo* L1210 and P388 murine leukemia's and certain other transplantable tumor models for the selection of compounds for further preclinical and clinical development (Boyd 1997). During the time period 1975-1985, primary anticancer screening was performed exclusively using the *in vivo* P388 mouse leukemia. As a result, most of the available clinical anticancer agents are active in the P388 system as well as it reflects in the successful treatment of hematological malignancies such as leukemia's and lymphomas. In 1984, introduction of a so-called "disease oriented *in vitro* primary anticancer drug screen" as a promising replacement to the P388 *in vivo* primary screen, was introduced. This strategy was implemented fully in 1990 (Body and Paull 1995)

NCI *in vitro* anticancer drug discovery screening was distinctive and unique in several respects because it has changed the approach from a compound-oriented drug discovery to a disease-panel oriented discovery. It uses human tumor cells derived from solid tumors and established an *in vitro* screen that would avoid the use of animals, and save on the amount of compound required for the initial screening (Shoemaker 2006). In order to establish a new *in vitro* primary screen they mainly focused on three main fronts: investigation to find best assays for in vitro drug sensitivity (Alley et

al. 1988); development of the cell line panel (Monks et al. 1991); and information technology (Paull et al. 1989).

1.7.1. Cell line panel and end point assay

The *in vitro* screening panel contains a total of 60 different human tumor cell lines derived from nine cancer types: Lung, Colon, Melanoma, Renal, Ovarian, Brain (CNS), Leukemia, Breast and Prostate (Body and Paull 1995). Table 1.3 shows 60 cell line panel used in NCI 60 *in vitro* anticancer drug screen.

Table 1.3: NCI 60 cell line panel (Body and Paull 1995)

Tissue of origin	Cell lines
Lung	NCI-H23, NCI-H522, A549-ATCC, EKVX, NCI-H226, NCI-H332M, H460, H0P62, HOP92
Colon	HT29, HCC-2998, HCT116, SW620, COLO205, HCT15, KM12
Breast	MCF7, MCF7ADRr, MDAMB231, HS578T, MDAMB435, MDN, BT549, T47D
Ovarian	OVCAR3, OVCAR4, OVCAR5, OVCAR8, IGROV1, SKOV3
Leukemia	CCRFCEM, K562, MOLT4, HL60, RPMI8266, SR
Renal	UO31, SN12C, A498, CAKI1, RXF393, 7860, ACHN, TK10
Melanoma	LOXIMVI, MALME3M, SKMEL2, SKMEL5, SKMEL28, M14, UACC62, UACC257
Prostate	PC3, DU145
CNS	SNB19, SNB75, U251, SF268, SF295, SM539

Three assays for cell viability for possible use in the primary screen were extensively evaluated, which includes MTT, XTT, and Sulforhodamine B (SRB) assays. Of all the three assays tested, non-

tetrazolium assay based on sulforhodamine B (SRB) was chosen as the best possible end point assay. It gave the best combination of signal-to-noise ratio, stain intensity, and linearity with cell number (Boyd and Paull 1995). SRB is a bright pink anionic dye that, in dilute acetic acid, binds electrostatically to the basic amino acids of cells (Vichai and Kirtikara 2006).

1.7.2. NCI 60 screening procedure

NCI 60 antitumor compound screening is a two step process. In the first stage screening compound will evaluate extract against the 60 cell lines at a single high dose of 10^{-4} M. It will be kept for 48h for incubation. In the second stage of screening, compounds exhibiting significant growth inhibition are evaluated against the 60 cell panel at five concentration level. Desired final maximum test concentration and additional four, 10 fold serial dilution are made to provide a total of five drug concentration plus control (10^{-4}, 10^{-5} , 10^{-6} ,10^{-7} and 10^{-8} M) (Alley et al. 1988).

During the course of reviewing data from the NCI cancer screen in early 1995, it became evident that many compounds were completely inactive under the conditions used in the assay. As a result, a protocol for a three cell line pre-screen was developed, which would test for the presence of toxicity at high drug concentration which preserve active agents for multi-dose 60 cell line testing. Computer modelling indicated that approximately 50% of inactive drugs could be eliminated by this pre-screen without a major decrease in ability to identify active agents. The selected cell lines are MCF7 (breast), NCI-H460 (lung) and SF-268 (glioma) (Monga and Sausville 2002). Only the active compound in pre-screen will selected for further detailed screening in 60 cancer cell lines.

1.7.3. Data display and analysis

After NCI-60 screening, each successful test of a compound in the full screen generates 60 dose-response curves. From the data obtained from dose-response curve, we can plot a mean graph, which is a pattern created by plotting positive and negative values, called "deltas" generated from a set of GI_{50} or TGI drug concentrations obtained for a given compound tested against each cell line in the screen (Shoemaker 2006). Mean graph is constructed by plotting the deltas horizontally in reference to a vertical line that represents the calculated mean GI_{50}. The negative deltas representing sensitive cell lines are plotted to the right of the mean reference line. On the other hand, the positive deltas are plotted to the left of the reference line to represent the less sensitive cell lines to the given compound (Body and Paull 1995).

COMPARE software is a computerized, pattern-recognition algorithm which has significant utility in the evaluation of data generated by the NCI 60 cancer screen. Basically, COMPARE is a method of finding and expressing the degree of similarity of mean-graph profiles generated on the same or different compounds (Body and Paull 1995). Profiles of cell line sensitivity of the compound will provide information about mechanisms of action and cell killing. Compounds that function in a similar manner will produce similar patterns of activity (Zaharevitz et al. 2002). Halichondrin B is the first natural product to which the mechanism of action of the compound identified using the COMPARE program (Bai et al. 1991).

1.8. Problems in marine antitumor drug research

Although there are many antitumor compounds reported from marine sources, only very few natural products could find their way to the market. There are ample reasons which may be affecting this short fall, including lack of sufficient amount of natural product, difficulties in accessing the source of the samples, problems associated with harvesting of the product, troubles in synthesizing the necessary amounts of the compound, difficulties in isolation and purification procedures, high toxicity of the active compound, ecological considerations, government policies, lack of infrastructure and insufficient capital investment (Bhatnagar and Kim 2010a).

Microorganisms produce specific metabolites at specific phases of their growth and development. Sometimes slight change in the culture parameters and condition may eventually lead to changes in the concentration and type of metabolite being produced by the microorganism, which ultimately leads to insufficient supply of the active compound. Apart from that many marine microorganisms are very difficult to culture laboratory conditions and lack of information on the isolation procedures and standardized culture conditions makes the situation even bad.

In the case of marine actinomycetes, as a result of the lack of efforts spent in exploring them, they remain as underexploited source for the discovery of novel secondary metabolites, when compare with terrestrial counterparts (Lam 2006). Additionally, the fact that the terrestrial actinomycetes produce very resistant spores which get transported from land to oceans, where it can remain available but dormant for many years, has made researchers more suspicious for the presence of indigenous

populations of marine actinomycetes (Bull et al. 2000). Therefore, the myth prevails that marine actinomycetes are originally terrestrial and this lack of knowledge prevents discovery of the huge potential of marine actinomycetes.

Certain other issues that pose an obstacle to the marine antitumor drug research include lack of funding and infrastructure resources and experience in the biotechnology firms to perform the extensive late-stage clinical development programs that are needed for regulatory approval of a drug. Prevailing threats to global marine biodiversity including habitat loss, overfishing, invasive species and pollution, rising water temperatures and ocean acidification are further making marine antitumor drug research increasingly difficult (Bhatnagar and Kim 2010b)

1.9. Objectives of the study

Considering all these aspects, the present study was undertaken with the following objectives:

1. Isolation of marine actinomycetes and screening for anticancer activity.
2. Evaluation of biosynthetic potential of actinomycetes for secondary metabolite production.
3. Isolation, purification and characterization of the anticancer molecules.
4. Elucidation of mode of action of anticancer lead compounds.

The thesis comprised of 7 chapters and a conclusion at the end. First chapter present a general introduction of the topic. Isolation and Screening of marine actinomycetes for their cytotoxic activity is presented in

Chapter 2. Chapter 3 deals with identification and characterization of marine actinomycetes with potential anticancer activity. Chapter 4 describes three aspects with respect to anticancer potential of the actinomycetes isolates such as (i) confirmation of anticancer activity of selected actinomycetes (ii) Chemical de-replication of crude actinomycetes extract, and (iii) screening for biosynthetic gene(s) involved in secondary metabolite production in selected actinomycetes. Isolation and structural elucidation of cytotoxic compounds from *Streptomyces* sp. MCCB 267 is presented in Chapter 5. In Chapter 6 details of *in vitro* anti-cancer evaluation of ikarugamycin and its analogues isolated from *Streptomyces* sp. MCCB 267 and their ability to bind DNA by molecular docking and molecular dynamic simulation studies were reported. Chapter 7 describe the details of the isolation, purification and characterization of active anticancer compound from *Pseudonocardia* sp. MCCB 268 and it's *in vitro* anticancer activity evaluation. This is followed by summary, list of references and appendices

Chapter 2

ISOLATION OF MARINE ACTINOMYCETES AND *IN VITRO* SCREENING FOR CYTOTOXICITY

Contents

2.1 Introduction

Marine environment is biologically very diverse and this rich diversity of marine environment offers the isolation of unique microorganisms, which are potential producers of novel bioactive compounds. Discovery of such compounds derived from microbes with biomedical importance is becoming increasingly successful. Among microorganisms, actinomycetes account for about 45% of all microbial bioactive secondary metabolites with majority (80%) being produced by *Streptomyces* species alone (Bérdy 2005). Because of intensive isolation and screening of actinomycetes from terrestrial environment over the last few decades, the rate of discovery of new isolates with novel bioactive compounds has decreased from terrestrial environment. Hence, a great deal of interest is being paid to the

bioprospecting for novel bioactive metabolites from actinomycetes from unexplored habitats (Bredholt et al. 2008). Deep sea environment and polar oceans are such two underexploited marine environments where it is believed to harbor unique microorganisms with potential for the production of unique secondary metabolites (Pathom-aree et al. 2006). Apart from microorganisms, marine sponges are also considered to be one of the richest producers of novel bioactive metabolites in marine environment with potential bioactivities, and accounts for 40% of all known marine natural products (Lee et al. 2001). Many bioactive compounds originally isolated from sponges were subsequently reported from its associated microorganisms which reinforce the speculations that associated microbial flora are the true producers of these metabolites (Thakur and Muller 2004; Taylor et al. 2007).

In the present work, marine actinomycetes were isolated from diverse marine environments including sediments collected from Indian Ocean, Arctic Ocean and also microorganisms associated with marine sponges collected from Indian Ocean. The isolated actinomycetes were tested for their potential for the production of anticancer metabolites by screening their cytotoxic activity on non-small cell lung cancer NCI-H460 cell line.

2.2 Materials and Methods

2.2.1 Collection of marine sediments and sponges

Marine sediments were collected from Indian Ocean and Arctic Ocean and sponges were collected from South East Coast of Indian Ocean.

1. Marine sediment samples collected during the cruise No - 310 of Fisheries and Oceanographic Research Vessel (FORV) Sagar Sampada

of Ministry of the Earth Sciences, Govt. of India, along the south west coast of Arabian Sea from off Cape Comorin to off Cochin during 17-11-2012 to 25-11-2012. Sediment samples from 15 stations were collected from different depths by using van Veen Grab operated on-board (Fig. 2.1). For microbiological isolation the sediments were sub-sampled aseptically and stored under -20^{0}C onboard and later transported to Microbiology lab, National Centre for aquatic Animal Health (NCAAH) under cold condition and processed immediately on arrival.

2. Marine Sponges were collected from Rameswaram coast, South East coast of India from the intertidal zone during low tide and used for isolation of associated endophytic actinomycetes as described in the section 2.2.2

3. Sediment samples collected from Kongsfjorden, Arctic Fjord, (79ºN, and 12ºE) at Ny-Ålesund, Svalbard during July 2014 (Fig. 2.2) were obtained through National Centre for Antarctic and Ocean Research (NCAOR), Goa. Sediments were collected by using van Veen grab and were sub sampled into a sterile polythene cover and stored under -20°C till transportation to NCAAH through cold chain.

2.2.2 Pretreatment of samples

1. Sediment Sample

For enrichment and selective isolation of actinomycetes, sediment samples were subjected to initial pretreatment. For this, air dried sediment sample (1g) was mixed with $CaCO_3$ (0.1 g) and incubated at 26º C for a week in humidity controlled environmental chamber.

However, considering the thermal sensitivity of arctic microorganisms, Arctic sediment samples were directly plated on agar plates without any pretreatment, after serial dilution in ice-cold water.

2. Sponge sample

Sponge specimens were rinsed three times with sterile seawater in order to remove loosely attached bacteria and cut aseptically into small pieces and thoroughly homogenized in a sterile mortar and pestle with sterile seawater. The homogenate was used as inoculum for isolation of actinomycetes.

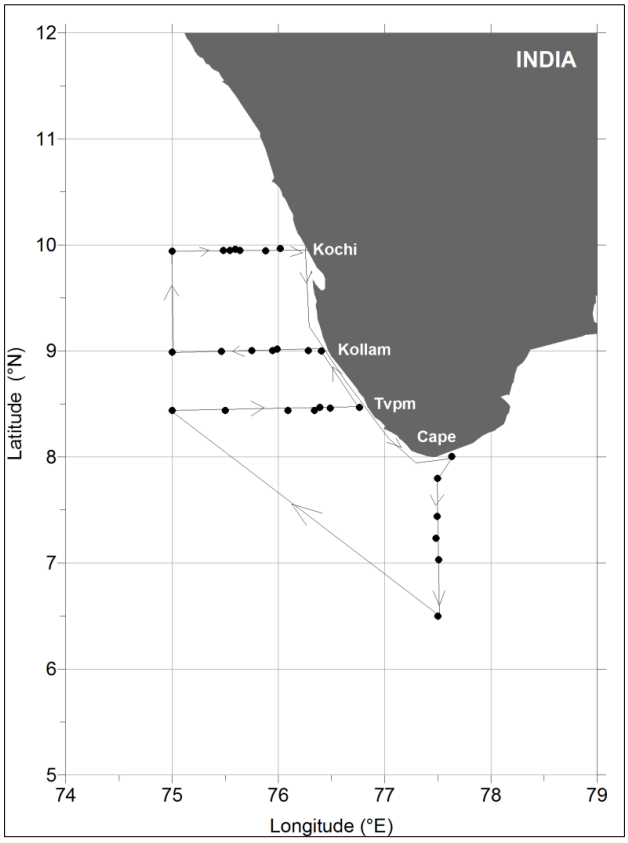


Fig. 2.1: Map showing locations of sampling stations of Cruise no 310 FORV Sagar Sampada (Cape Comorin - Cochin)

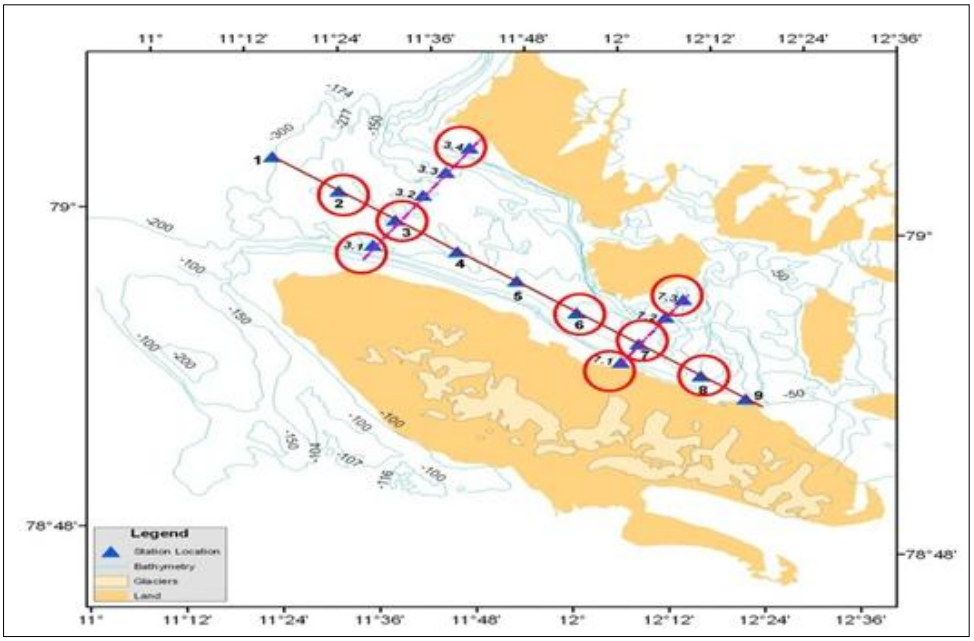


Fig. 2.2: Map showing locations of sampling stations at Arctic Fjord Kongsfjorden

2.2.3 Isolation of actinomycetes

2.2.3.1 Media used for the study

To increase the chance of recovering the actinomycetes from the marine samples, five different media viz., Casein-Starch–Peptone- Yeast extract-Malt Extract Agar (CSPYME) Agar, Starch Casein (SCA) Agar, Starch Glycerol Inorganic Salt (SGI) Agar, Actinomycetes Isolation Agar (AIA), and Zobell Marine Agar were used for isolation purpose.

The composition of media is given below:

1. Casein-Starch–Peptone- Yeast extract-Malt Extract Agar (CSPYME) Agar

Casein	3.0g
Maize Starch	10.0g

Yeast Extract	1.0g
Malt Extract	10.0g
K_2HPO_4	0.5g
Sea water	1.0L
Agar	20.0g
pH	7.5

2. Starch Casein (SCA) Agar

Starch	10.0g
Vitamin free casamino acid	0.3g
$CaCO_3$	0.02g
$Fe_2 (SO4)_3.7H_2O$	0.01g
KNO_3	2.0g
$MgSO_4.7H_2O$	0.05g
Seawater	1.0L
Agar	20.0g
pH	7.2

3. Starch Glycerol Inorganic Salt (SGI) Agar

Starch	10.0g
Glycerol	10.0mL
$(NH_4)_2 SO_4$	2.0g
K_2HPO_4	1.0g
$MgSO_4.7H_2O$	1.0g
$CaCO_3$	2.0g
Seawater	1.0L
Agar	20.0g
pH	7.5

4. Actinomycetes Isolation Agar (AIA)

Sodium caseinate	2.0g
L-Asparagine	0.1g
Sodium propionate	4.0g
Dipotassium phosphate	0.5g
Magnesium sulphate	0.1g
Ferrous sulphate	0.001g
Seawater	1.0L
Agar	15.0g
pH	8.1

5. Zobell Marine Agar

Peptone	5.0g
Ferric phosphate	0.1g
Yeast extract	1.0g
Seawater	1.0L
Agar	20.0g
pH	7.5

All media were prepared and sterilized at 121ºC for 15 minutes in an autoclave. When temperature dropped to 45ºC, the media were supplemented with Gentamycin (0.2 mg L^{-1}), Cycloheximide (0.25 mg L^{-1}) and Amphotericin B (0.1 mg L^{-1}). For isolation of sponge associated actinomycetes, all media were supplemented with 1% sponge extract as described by Zhang et al. (2006). Briefly, sponge extract was prepared by grinding 20g of sponge tissue with 20mL of sea water. The homogenate was centrifuged at 8000rpm for 5 min and supernatant was saved and autoclaved at 121ºC for 15 min, and stored at -20^{0}C till use.

2.2.3.2 Isolation and purification

Spread plate technique was employed to isolate the microorganisms and then incubated at 28ºC for a period of 4 weeks. Isolates with characteristic colonies (tough leathery) were picked and purified by quadrant streak method on nutrient agar plates. Gram staining was performed for all isolates and colonies with Gram-positive and fine filaments were segregated for further study.

2.2.4 Fermentation and crude extract preparation

Pure isolates of actinomycetes were mass cultured for the production of secondary metabolites. Briefly, a loopfull of actinomycete spores was inoculated into a seawater-based seed medium (Beef extract 3.0gL^{-1}, peptone 5.0gL^{-1}). The flasks were incubated at 20 ºC for 48 h on a rotary shaker set at 150 rpm. Subsequently, these were transferred to seawater based fermentation medium (Yang et al. 2013) (Soybean meal 3.0gL^{-1}, Yeast Extract 3.0gL^{-1}, Proline 1.0gL^{-1}, Beef Extract 3.0gL^{-1}, Glycerol 6mL L^{-1}, K_2HPO_4 0.5gL^{-1}, $MgSO_4.7H_2O$ 0.5gL^{-1}, $FeSO_4.7H_2O$ 0.5gL^{-1}, $CaCO_3$ 2.0gL^{-1}, pH 7.4) and incubated at 20 ºC for 10 days on a rotary shaker at 150 rpm. At the end of fermentation the liquid culture was centrifuged at 6500 x *g* for 5 min., and the supernatant was extracted thrice with an equal volume of ethyl acetate (EtOAc) and mycelium cake was extracted separately with ethyl acetate. The extracts were concentrated under reduced vacuum on a rotary evaporator at 35 ºC, and the residue was dissolved in DMSO to give a concentration of 10 mg/mL, and used for screening for cytotoxic activity on the NCI-H460 non-small cell human lung cancer (NSCLC) cell line.

2.2.5 Screening for potential cytotoxic activity using NCI-H460 cells

Evaluation of *in vitro* cytotoxic activity of crude extracts was done using Sulforhodamine-B (SRB) (Sigma, USA) colorimetric assay on 96-well culture plates as described by Skehan et al. (1990) with modification. Briefly, NCI-H460 cell line, obtained from National Centre for Cell Science (NCCS, Pune, India) was maintained in RPMI-1640 (Himedia, India) supplemented with 10% fetal bovine serum (FBS) (Himedia, India) at 37 ºC in CO_2 incubator with 5% carbon dioxide. Aliquots of 190 µL cell suspension with a density of 1.9 x 10^4 cells were pipetted into 96 well micro titer plates. The crude extracts were diluted to 1 mg/mL with sterile deionized water (1 mg/mL in 10% DMSO). Subsequently, 10 µL of the crude extracts from the isolates were added to each well so as to get a final concentration of 50 µg/mL of crude extract in each well and 0.5% DMSO. Control wells were composed of 190 µL cell suspension plus 10 µ L of 10% DMSO. All assays were performed in triplicate. The plates were incubated at 37 ºC in a CO_2 (5%) incubator for 72 h, and fixed with 100 µL ice-cold 30% trichloroacetic acid (TCA) and incubated at 4ºC for another 1 h. The plates were gently washed four times and air-dried at room temperature. To each well, a total of 100 µL of 0.057% (w/v) SRB prepared in 1% acetic acid was added and left at room temperature for 30 min. Unbound stain was removed by washing with 1% v/v acetic acid and the plates were air dried. To dissolve the cell bound dye, 200 µL of 10 mM Tri base solution (pH 10.5) was added to each well and the plate was shaken on a gyratory shaker for 10 min, and optical density (OD) was read at 510 nm in a microplate reader (Tecan, Switzerland). Percentage of cell-growth inhibition (GI) was calculated according to the following equation (Vichai and Kirtikara 2006):

Percentage of Growth Inhibition= 100 - Percentage of control cell growth Percentage of control cell growth = (Mean OD $_{Sample}$ – Mean OD $_{Day\ 0}$)/(Mean OD $_{Negative\ control}$ – Mean OD $_{Day\ 0}$)] ×100

Based on the results of a primary screening, potent actinomycete isolates were selected for further studies.

2.3 Results

2.3.1 Isolation of marine actinomycetes

Marine actinomycetes colonies started developing on various isolation media after two weeks of incubation (Fig. 2.3a). Actinomycetes colonies with characteristic appearance were picked up and purified (Fig.2.3b).

A total of 62 morphologically distinct marine actinomycetes were isolated from different samples used in the study. Of these, 29 isolates were obtained from marine sediment samples collected along the southwest coast of India (Cruise No – 310). Three actinomycetes were isolated from the marine sponge (Fig. 2.4) collected along the West coast of India. The sponge was identified as *Mycale* sp. from the morphological features. Thirty actinomycetes were isolated from the Arctic sediment samples used in the study.

Of the 62 marine actinomycetes isolated, 27 isolates were developed on Actinomycetes Isolation Agar, 19 on Zobell marine agar, 14 on CSPYME agar and 2 on SGI and, details provided in Table 2.1.

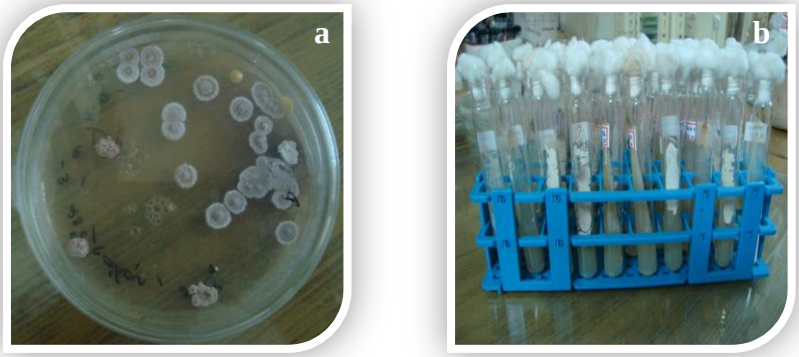


Fig. 2.3: Typical actinomyctes colonies on isolation agar plate (a) and purified culture on nutrient agar slants (b)

Fig. 2.4: Marine sponge *Mycale* sp. collected from Rameswaram coast, South East coast of India

Table 2.1: Details of marine actinomycetes isolated

Sl. No.	Culture code	Source / Station	Isolation media
1.	DM1	Marine sediment (Cruise No – 310)	Zobell Marine Agar
2.	DM2	Marine sediment (Cruise No – 310)	Zobell Marine Agar
3.	DM3	Marine sediment (Cruise No – 310)	AIA

Cont…Table 2.1

Sl. No.	Culture code	Source / Station	Isolation media
4.	DM4	Marine sediment (Cruise No – 310)	AIA
5.	DM5	Marine sediment (Cruise No – 310)	SGI
6.	DM6	Marine sediment (Cruise No – 310)	CSPYME
7.	DM7	Marine sediment (Cruise No – 310)	AIA
8.	DM8	Marine sediment (Cruise No – 310)	AIA
9.	DM9	Marine sediment (Cruise No – 310)	CSPYME
10.	DM10	Marine sediment (Cruise No – 310)	CSPYME
11.	DM11	Marine sediment (Cruise No – 310)	CSPYME
12.	DM12	Marine sediment (Cruise No – 310)	CSPYME
13.	DM13	Marine sediment (Cruise No – 310)	CSPYME
14.	DM14	Marine sediment (Cruise No – 310)	CSPYME
15.	DM15	Marine sediment (Cruise No – 310)	CSPYME
16.	DM16	Marine sediment (Cruise No – 310)	SGI
17.	DM17	Marine sediment (Cruise No – 310)	Zobell Marine Agar
18.	DM18	Marine sediment (Cruise No – 310)	Zobell Marine Agar
19.	DM19	Marine sponge from (Rameswaram coast)	AIA
20.	DM20	Marine sponge from (Rameswaram coast)	AIA

Cont…Table 2.1

Sl. No.	Culture code	Source / Station	Isolation media
21.	DM21	Marine sponge from (Rameswaram coast)	AIA
22.	DM22	Marine sediment (Cruise No – 310)	AIA
23.	DM23	Marine sediment (Cruise No – 310)	AIA
24.	DM24	Marine sediment (Cruise No – 310)	AIA
25.	DM25	Marine sediment (Cruise No – 310)	AIA
26.	DM26	Marine sediment (Cruise No – 310)	AIA
27.	DM27	Marine sediment (Cruise No – 310)	AIA
28.	DM28	Marine sediment (Cruise No – 310)	AIA
29.	DM29	Marine sediment (Cruise No – 310)	AIA
30.	DM30	Marine sediment (Cruise No – 310)	AIA
31.	DM31	Marine sediment (Cruise No – 310)	AIA
32.	DM32	Marine sediment (Cruise No – 310)	AIA
33.	DM33	Arctic sediment	CSPYME
34.	DM34	Arctic sediment	Zobell Marine Agar
35.	DM35	Arctic sediment	Zobell Marine Agar
36.	DM36	Arctic sediment	Zobell Marine Agar
37.	DM37	Arctic sediment	CSPYME
38.	DM38	Arctic sediment	Zobell Marine Agar

Cont…Table 2.1

Sl. No.	Culture code	Source / Station	Isolation media
39.	DM39	Arctic sediment	Zobell Marine Agar
40.	DM40	Arctic sediment	Zobell Marine Agar
41.	DM41	Arctic sediment	Zobell Marine Agar
42.	DM42	Arctic sediment	AIA
43.	DM43	Arctic sediment	AIA
44.	DM44	Arctic sediment	Zobell Marine Agar
45.	DM45	Arctic sediment	Zobell Marine Agar
46.	DM46	Arctic sediment	AIA
47.	DM47	Arctic sediment	Zobell Marine Agar
48.	DM48	Arctic sediment	CSPYME
49.	DM49	Arctic sediment	Zobell Marine Agar
50.	DM50	Arctic sediment	AIA
51.	DM51	Arctic sediment	AIA
52.	DM52	Arctic sediment	Zobell Marine Agar
53.	DM53	Arctic sediment	AIA
54.	DM54	Arctic sediment	CSPYME
55.	DM55	Arctic sediment	Zobell Marine Agar
56.	DM56	Arctic sediment	Zobell Marine Agar

Cont…Table 2.1

Sl. No.	Culture code	Source / Station	Isolation media
57.	DM57	Arctic sediment	AIA
58.	DM58	Arctic sediment	CSPYME
59.	DM59	Arctic sediment	AIA
60.	DM60	Arctic sediment	CSPYME
61.	DM61	Arctic sediment	Zobell Marine Agar
62.	DM62	Arctic sediment	AIA

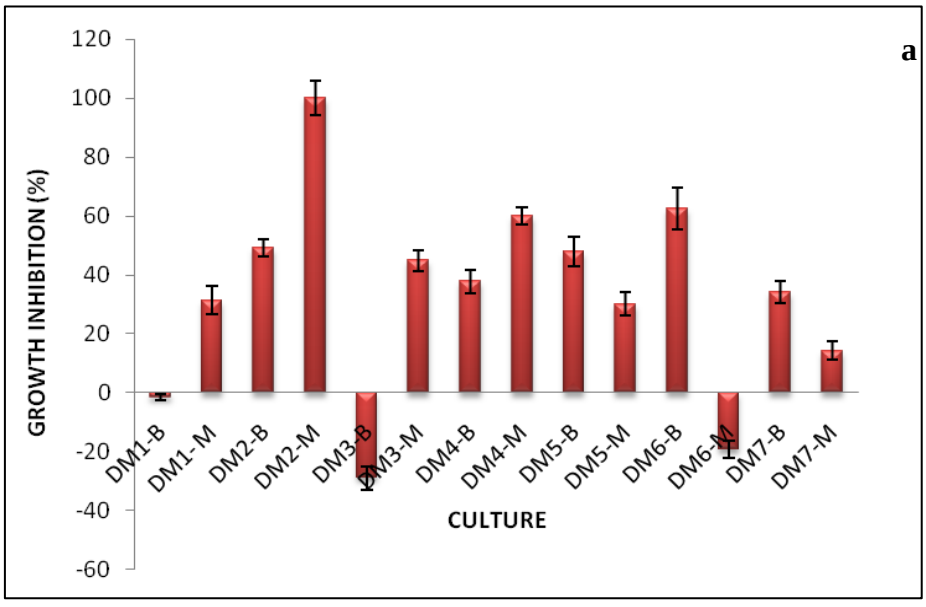
2.3.2 Screening for potential anticancer activity using NCI-H460 cells *in vitro*

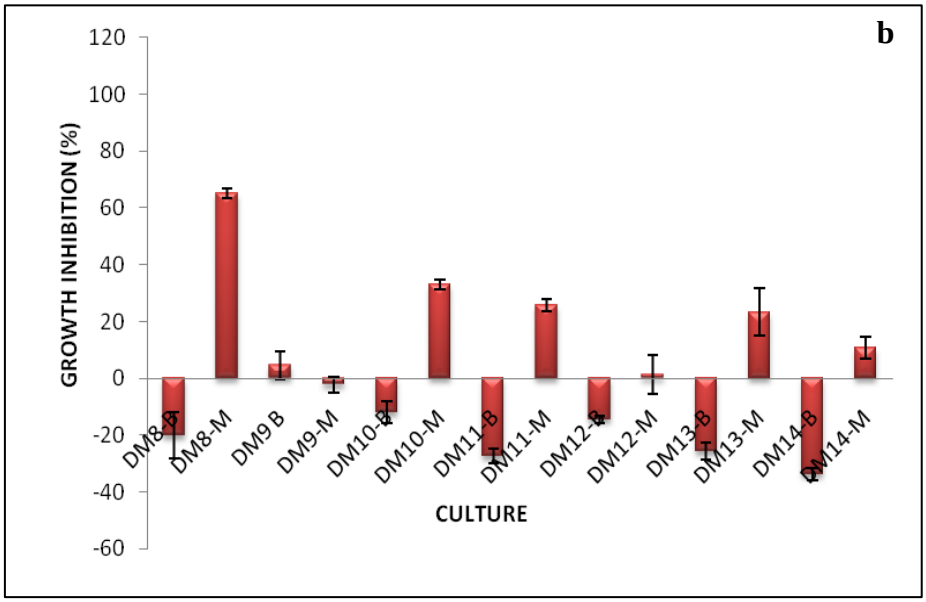
Primary screening of crude extracts of isolated marine actinomycetes were performed on NCI-H460 cell line using SRB assay at a single concentration of 50µg/mL (Fig. 2.5 a-i). The concentration was selected based on previous studies recommending screening the sample at single high concentration as per US National Cancer Institute (NCI) instead of screening the samples in multi-dose strategy (Shoemaker 2006; Anibou et al. 2008). A total of 19 isolates showed activity in the preliminary anticancer activity screening. Among positive isolates, 11 isolates showed complete growth inhibition in both broth and mycelial crude extract, which include DM21, DM30, DM37, DM42, DM43, DM48, DM49, DM50, DM51, DM57, and DM61. Isolate DM2, DM16, DM23, DM27, DM53 and DM54 showed complete growth inhibition in only mycelial crude extract. DM31 showed complete growth inhibition in broth extract whereas no

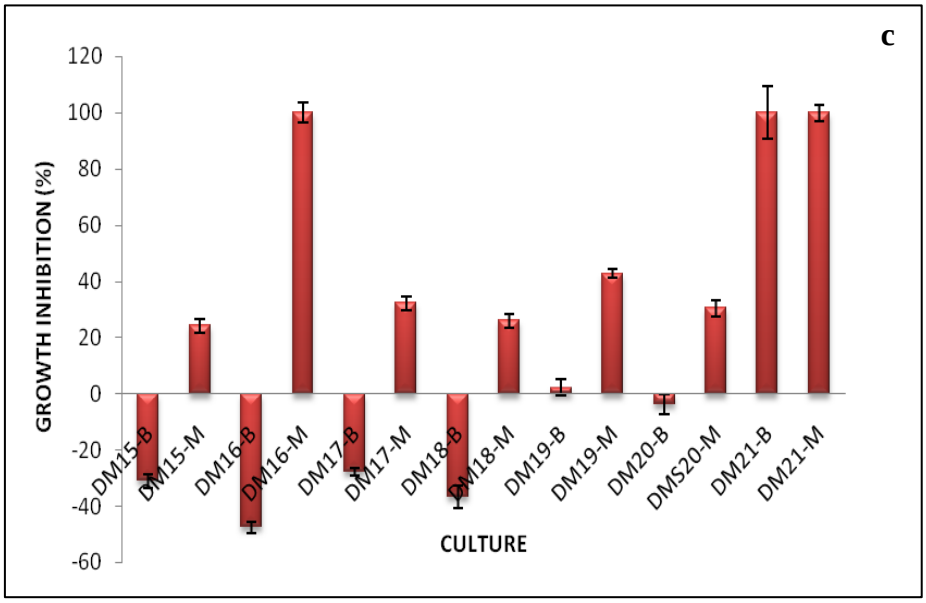
activity was evident in the mycelia extract. Isolate DM32 showed 75% growth inhibition in broth crude extract. Only those isolates which showed complete growth inhibition in broth as well as mycelial extract were selected and used for further study (Table 2.2).

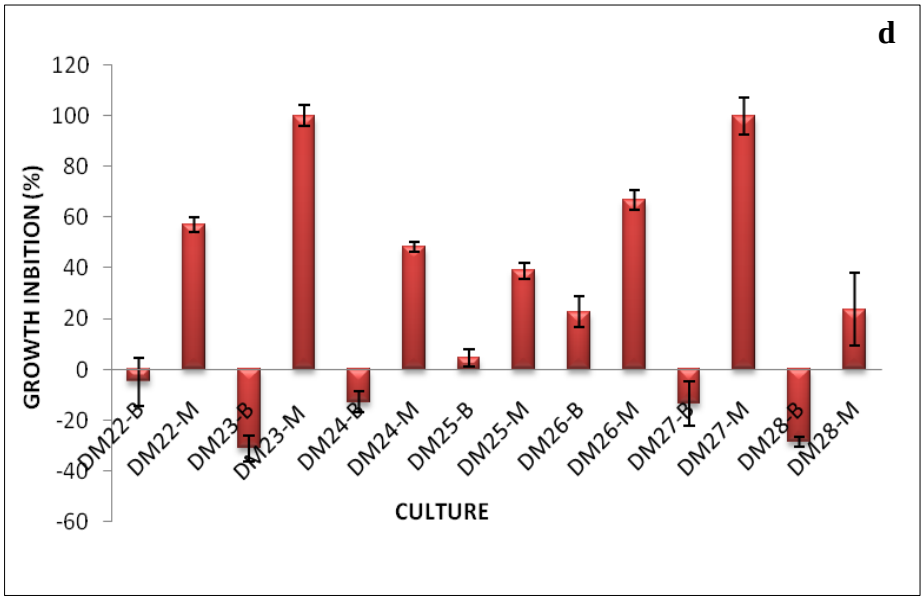
Table 2.2: Isolates showing potential anticancer activity selected for further study

Isolates with promising anticancer activity	Source
DM21	*Mycale* sponge
DM30	Marine sediment South-West Coast of Arabian Sea (Cruise No–310)
DM37, DM42, DM43, DM48, DM49, DM50, DM51, DM57, DM61	Marine sediment (Arctic Kongsfjorden)




b
GROWTH INHIBITION (%)
120
100
80
60
40
20
0
-20
-40
-60
DM8-B
DM8-M
DM9 B
DM9-M
DM10-B
DM10-M
DM11-B
DM11-M
DM12-B
DM12-M
DM13-B
DM13-M
DM14-B
DM14-M
CULTURE


c
GROWTH INHIBITION (%)
120
100
80
60
40
20
0
-20
-40
-60
DM15-B
DM15-M
DM16-B
DM16-M
DM17-B
DM17-M
DM18-B
DM18-M
DM19-B
DM19-M
DM20-B
DMS20-M
DM21-B
DM21-M
CULTURE


d
GROWTH INBITION (%)
120
100
80
60
40
20
0
-20
-40
-60
DM22-B
DM22-M
DM23-B
DM23-M
DM24-B
DM24-M
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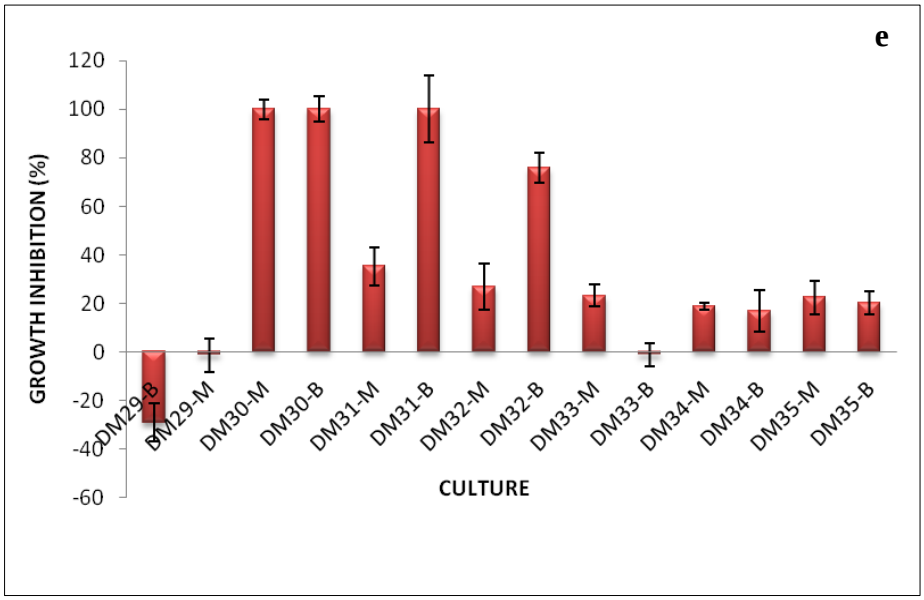
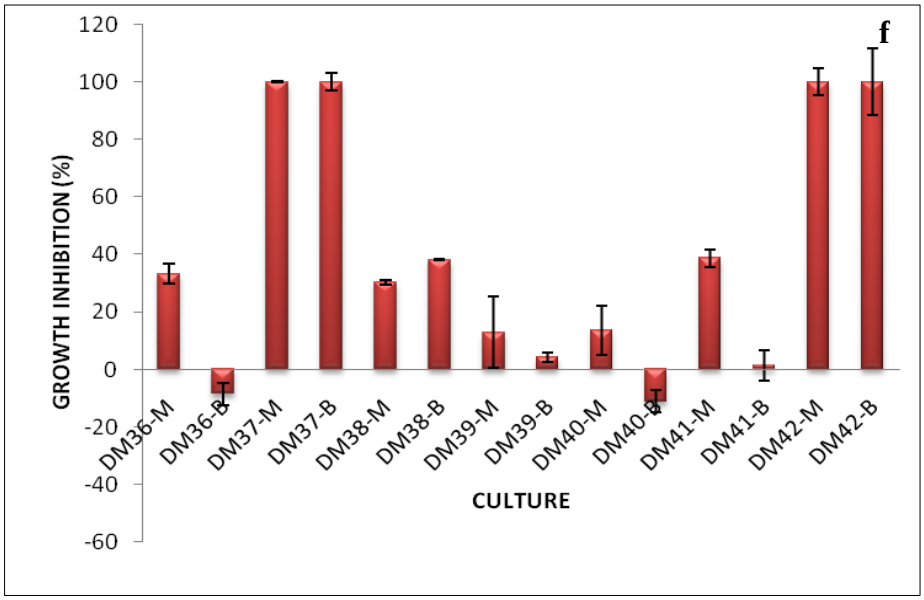
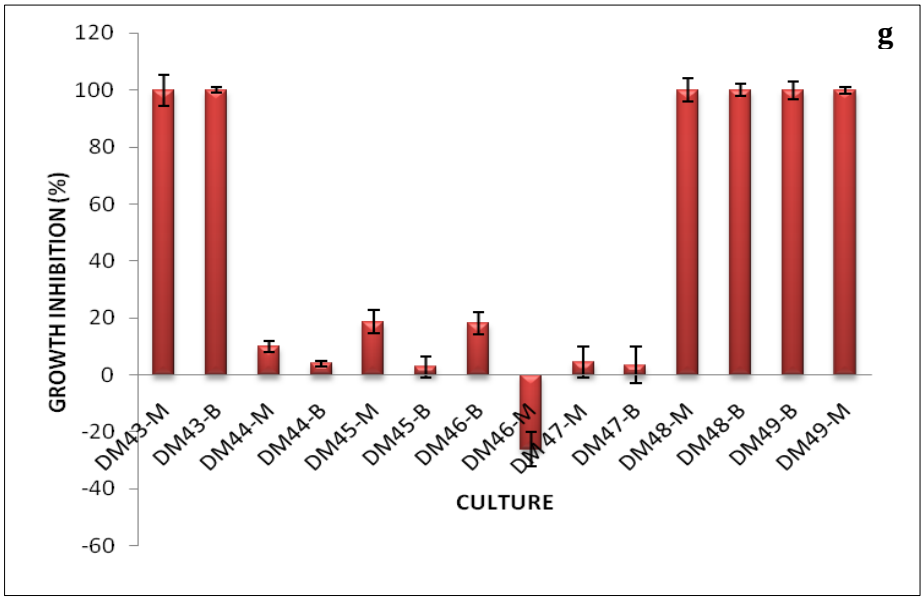
DM25-M
DM26-B
DM26-M
DM27-B
DM27-M
DM28-B
DM28-M
CULTURE


e
GROWTH INHIBITION (%)
120
100
80
60
40
20
0
-20
-40
-60
DM29-B
DM29-M
DM30-M
DM30-B
DM31-M
DM31-B
DM32-M
DM32-B
DM33-M
DM33-B
DM34-M
DM34-B
DM35-M
DM35-B
CULTURE


f
GROWTH INHIBITION (%)
120
100
80
60
40
20
0
-20
-40
-60
DM36-M
DM36-B
DM37-M
DM37-B
DM38-M
DM38-B
DM39-M
DM39-B
DM40-M
DM40-B
DM41-M
DM41-B
DM42-M
DM42-B
CULTURE


g
GROWTH INHIBITION (%)
120
100
80
60
40
20
0
-20
-40
-60
DM43-M
DM43-B
DM44-M
DM44-B
DM45-M
DM45-B
DM46-B
DM46-M
DM47-M
DM47-B
DM48-M
DM48-B
DM49-B
DM49-M
CULTURE

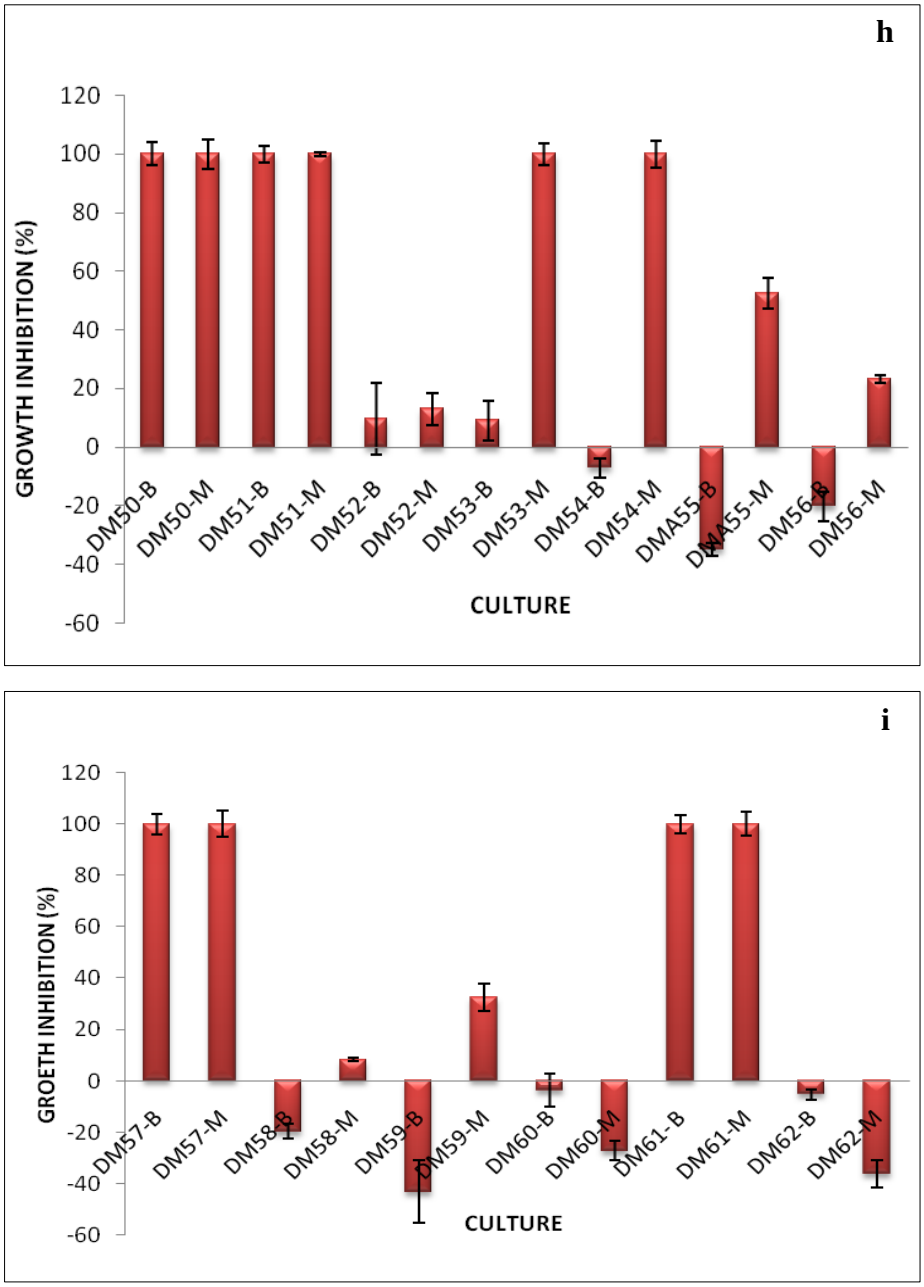


Fig. 2.5 a-i: Percentage growth inhibition by actinomycete crude extracts on NCI-H460 cell lines at 50µg/mL concentration[1] (data shown are mean of three independent observations and its standard deviation)

[1] B indicates broth extract and M indicates mycelia extract

2.4 Discussion

Marine biodiversity is only partly explored, despite being an excellent source of many pharmacologically important products (Kiuru et al. 2014). In marine environment, marine bacteria seem to be promising candidates for drug discovery and among those actinomycetes remain as one of the rich source of natural products (Lam 2006; Goodfellow and Fiedler 2010). Actinomycetes contribute over half of the known bioactive secondary metabolites, especially in the classes of antibiotics, antitumor agents, enzymes and immunosuppressive agents (Bérdy 2005). Many marine actinomycetes isolates were well known to produce novel anticancer agents (Olano et al. 2009). With improved sampling and culture techniques, isolation of new groups of actinomycetes is being made from sediments collected from even the deepest parts of the oceans (Kwon et al. 2006; Pathom-aree et al. 2006). Such efforts are yielding unique chemically rich genera such as *Salinispora* (Feling et al. 2003) and *Marinispora* (Kwon et al. 2006).

Isolation of actinomycetes from environmental samples encounters certain difficulties including contamination with other bacteria and fungi. However, addition of limited quantities of antibiotics and antifungal agents helped to avoid these contaminations to a certain extent without adversely affecting the isolation of actinomycetes. Moreover, the use of calcium carbonate enrichment as pretreatment found to be effective for eliminating contaminating microflora and the selective isolation of actinomycetes in the study. Use of calcium carbonate enrichment causes an alkaline change in pH, which is favorable to actinomycetes. Most of the actinomycetes prefer neutral or slightly alkaline condition for their growth (Ramesh and

Mathivanan 2009). Studies report that composition of medium used for the isolation also greatly influences the actinomycetes colony development (Biji 2003). Current result indicates that Actinomycete Isolation Agar (AIA) is the most effective and suitable medium for the isolation of marine actinomycetes as evidence as around 27 actinomycetes could be isolated on this medium. AIA can be considered as a medium with minimal nutrients only. AIA contains sodium caseinate as a source of nitrogen. Asparagine in addition to being an amino acid is also a source of nitrogen. Sodium propionate is used as a substrate in anaerobic fermentation. Dipotassium phosphate acts as the buffering system. The sulphates present in the medium serve as sources of sulphur and metallic ions. Glycerol serves as an additional source of carbon. Therefore, AIA along with antifungal agents and sufficient pretreatment forms a promising strategy for the isolation of actinomycetes. Ouhdouch et al. (2001) also reports the use AIA as the suitable medium for the isolation of actinomycetes.

Terrestrial soil is considered as the richest source of actinomycetes, whereas studies indicated that marine sediment is also an excellent source of actinomycetes with diverse potential (Sujatha et al. 2005; Fenical and Jensen 2006). These potential marine actinomycetes, contribute only very small fraction of microflora of marine habitats with absolute numbers of actinomycetes much lesser than the terrestrial habitats (Goodfellow and Williams 1983). Even though actinomycetes from marine sediment have been well documented, it is still unclear whether they are the indigenous marine microflora or washout from terrestrial environment.

The bioactive potentials of marine derived actinomycetes are well documented. A marine actinomycete *Pseudonocardia* sp., isolated from

deep-sea sediment of the South China Sea produced the compounds Pseudonocardians A–C and showed potent activity against three tumor cell lines of SF-268, MCF-7 and NCI-H460 (Li et al. 2011). Zhang et al. (2012) identified a deep-sea-derived *Streptomyces sp*. SCSIO 03032 producing compounds spiroindimicins A–D with moderate cytotoxicities against several cancer cell lines. *Salinospora* is another marine actinomycete which is widely distributed in ocean sediments. They produce Salinosporamide A, a compound which is highly cytotoxic against various cancer cell lines (Feling et al. 2003) Though there are numerous reports of antitumor compounds producing marine bacteria isolated from marine sediments, only few reports are available from Indian waters (Selvin et al. 2004; Gandhimathi et al. 2008).

In the present study, a total of 62 morphologically distinct marine actinomycetes were isolated and screened for antitumor activity. For preliminary screening ethyl acetate crude extract of the broth/mycelia were used at a single high concentration of 50 µg/mL using SRB assay. This concentration was selected based on the NCI60 anticancer screening programme of NCI (US National Cancer Institute). As established by the American National Cancer institute (NCI), a crude extract with IC_{50} value <30µg/mL is considered as a potential anticancer extract (Anibou et al. 2008). NCI Screening strategy suggests initial screening of compounds/ extracts at a single high concentration in three selected cell lines panel: MCF7 (breast), NCI-H460 (lung) and SF-268 (glioma). This screening strategy will preserve active agents for multi dose 60 cell line screening (Monga and Sausville 2002). Because of problems occurring during the use of tetrazolium based assays (MTT/XTT) during large scale screening NCI suggested another suitable, non tetrazolium assay SRB for the use in the *in*

vitro primary drug screen. SRB assay yields best combination of stain intensity, signal-to-noise ratio, and linearity with cell number for large scale drug screening (Boyd and Paull 1995). SRB is a bright pink anionic dye that, in dilute acetic acid, binds electrostatically to the basic amino acids of TCA-fixed cells (Skehan et al. 1990; Vichai and Kirtikara 2006).

Among the actinomycetes screened for activity, a total of 19 isolates showed cytotoxicity. Among positive isolates, 11 isolates showed complete growth inhibition in both broth as well as mycelia crude extract, which include DM21, DM30, DM37, DM42, DM43, DM48, DM49, DM50, DM51, DM57 and DM61. Apart from these isolates DM2, DM16, DM23, DM27, DM31, DM32, DM53 and DM54 showed activity either in mycelial extract or broth extract.

In Indian scenario, marine actinomycetes are studied more for their diversity, antimicrobial, anti fungal and enzyme production potential than their ability to produce antitumor or cytotoxic compound production potential (Thumar et al. 2010; Satheeja and Jebakumar 2011). Ramesh and Mathivanan (2009) and Das et al. (2008) isolated and studied actinomycetes population from continental slope sediment of the Bay of Bengal. Kuruvalli and Nadumane (2015) recently reported *in vitro* cytotoxic and antibacterial potential of three marine Actinomycetes isolated from coastal ecosystems of Tanur, Kerala.

From marine environment, actinomycetes are mainly isolated from marine sediment and water. However they were also found in association with marine invertebrates, especially from marine sponges (Hentschel et al. 2003). The recent reports suggest that majority of secondary metabolites from marine invertebrates are actually produced by symbiotic

microorganisms in invertebrate tissues, and actinomycetes are one of the major microbial communities in symbiosis with sponges (Montalvo et al. 2012). Among marine sponges genus *Mycale* is found to produce wide varieties of novel compounds with interesting biological activities (Coello et al. 2009). *Mycale* sponges are producers of cytotoxic compounds such as mycalolides (Matsunaga et al. 1999), pateamine (Northcote et al. 1991) and peloruside (Singh et al. 2010). Studies of Abdelmohsen et al. (2014a) reported that the genus *Streptomyces* is the main actinomycete found in marine sponges with novel chemistry. Apart from that the number of new species reports is also increasing from sponges. For example, marine actinomycetes genus *Salinispora* discovered initially from marine sediment later found in sponges such as *Pseudoceratina clavata* (Kim et al. 2005). Although there are several studies on the isolation and diversity of actinomycetes from marine sponges (Zhang et al. 2006; Jiang et al. 2008), their secondary metabolites production and bioactivity were not extensively studied. Khan et al. (2011) isolated 5 phylogenetically new strains of *Streptomyces* from the marine sponge *Haliclona sp.* and one of them showed promising cytotoxicity against cancer cell lines. Glucosylmannosyl-glycerolipid was isolated from *Microbacterium sp.* strain HP2 associated with the sponge *Halichondria panacea* collected from the Adriatic coast, Rovinj, Croatia. The compounds showed antitumor activity by inhibiting the growth of the tumor cell lines HM02 and Hep G2 (Lang et al. 2004). Two new kijanimicin derivatives were isolated from marine sponge *Hymeniacidon* sp. associated *Streptomyces carnosus* strain AZS17. These compounds showed potent cytotoxic activity against the human liver cancer cell line (Wei et al. 2011). In the current study, 3 sponge associated actinomycetes were isolated from sponge *Mycale* sp. collected from the

West coast of India. Among these, one isolate DM21 showed good anti-tumor activity in the preliminary screening and was selected as a potential isolate for further study.

Studies indicate that novel natural products are discovered when new screening strategies are introduced or when high quality biological materials from unexplored sources are used for isolating microorganisms. This method is an essential strategy for drug discovery programme to come up with new novel drug candidates. In this context an attempt was made to explore diverse marine environments including Arctic Ocean, for isolation of novel microorganisms for biopropsecting. Arctic environment is a comparatively less explored area in terms of bioprospecting. Only a few investigational studies have been carried out in arctic marine environment (Augustine et al. 2012; Yuan et al. 2014). These studies revealed that diverse groups of actinobacteria are present in arctic sediment samples. In the present study, 30 marine actinomycetes isolates were obtained from the sediment samples collected from Arctic sea- Kongsfjorden fjord. Among them 8 marine actinomycetes viz., DM37, DM42, DM43, DM48, DM49, DM50, DM51, DM57 and DM61 showed potent cytotoxic activity in the primary screening. However, these actinomycetes are relatively slow growers and took more time to grow on isolation agar plates. There are only very few studies which have reported the anti cancer compound production potential of arctic actinomycetes. Gao et al. (2012) reported that a marine actinomycete *Nocardia dassonvillei* isolated from Arctic marine sediment has the ability to produce novel anticancer and antifungal phenazine derivative. New benzoxazine secondary metabolites arcticoside and C-1027 chromophore-V were reported by Moon et al. (2014) from culture of an Arctic marine actinomycete *Streptomyces* strain. The compound

Chromophore-V exhibited potent cytotoxic activity against breast carcinoma MDA-MB231 cells and colorectal carcinoma cell line HCT-116. Another arctic isolate *Nocardiopsis* sp. 03N67 showed potent angiogenesis inhibitor activity against human umbilical vein endothelial cells (HUVECs) by producing a compound Cyclo-(l-Pro-l-Met) (Shin et al. 2010a).

In this study, 11 marine actinomycetes isolates showed promising cytotoxicity on NCI-H460 lung cancer cells and were segregated and selected for further detailed morphological, physiological and chemotaxonomical studies and subsequent isolation of lead compounds. All the 62 isolates including the potent 11 isolates were deposited in the microbiology repository of National Centre for Aquatic Animal Health and this could be used in future for screening for bioactivities other than anticancer properties.

Chapter 3

IDENTIFICATION AND CHARACTERIZATION OF ACTINOMYCETES WITH POTENTIAL ANTICANCER ACTIVITY

Contents

3.1 Introduction

3.1.1 Marine actinomycetes

Marine environment has got tremendous potential for the discovery of new natural product lead compounds from marine microorganisms. Out of the large number of bacterial species present in marine environment only a small fraction of microorganism is isolated, cultured, characterized and studied. Among marine microorganisms, actinomycetes are found to be promising and interesting group because of their ability to produce antibiotics.

Actinomycetes are high G-C percentage Gram positive filamentous bacteria with branching mycelium, and are a major phylum of the domain

Bacteria. The mycelium can be of two types, either substrate mycelium or aerial mycelium. Many actinomycetes genera produce spores which are found in aerial hyphae. Among actinomycetes, *Streptomyces* are the dominant genera. They account for 75% bioactive compounds produced by actinobacteria. All other non-*Streptomyces* group, known as rare-actinomycetes, comprises only about 100 genera, accounts for the remaining 25% bioactive compounds (Gopinath et al. 2013; Sujada et al. 2014). When compared with terrestrial actinomycetes, marine actinomycetes are poorly studied.

3.1.2 Identification and characterisation of actinomycetes

Over the past many years, various methods have been applied with the aim of identifying, characterizing, and discriminating actinomycetes strains. The primary objective of such investigations has been to develop the most rapid and effective method for proving the novelty of newly isolated strains. Actinomycetes characterization methods have evolved through several phases over the years, from morphological observations, to classifications based on numerical taxonomic analyses of physiological and biochemical characters and, most recently, to the use of molecular phylogenetic analyses of gene sequences.

3.1.2.1 Classical approach

Classical approaches make use of physiological, morphological, and biochemical characters for the classification of the actinomycetes. The classical method described in the identification key by Shirling and Gottlieb (1966) is very much useful for the identification of *Streptomyces*. They include observation of Aerial mass colour, Melanoid Pigments, Reverse

Side Pigments, Soluble Pigments, Spore Chain Morphology and its surface, assimilation of carbon sources.

3.1.2.2 Chemotaxonomic approach

Chemotaxonomic approach includes the study of chemical variation in the organisms and the use of these chemical characters for the identification and classification of organisms (Frisvad et al. 2008). This is one of the important strategies for the identification of the genera of actinomycetes. The chemical composition of the cell wall provides useful methods of differentiating various types of actinomycetes. In the case of actinobacteria, the presence of Diaminopimelic Acid (DAP) isomers is one of the important cell-wall properties used for classification purpose. Actinobacteria generally contain one of the DAP isomers, the meso-form or the LL-form, mostly located in the peptidoglycan layer. The cell wall sugar composition is also valuable information for the identification and classification of actinomycetes. It plays an important role in the identification of sporulating actinomycetes which have meso-DAP in their cell walls.

FAME analysis is another important parameter for chemotaxonomic analysis based on the fatty acid profile. It has been reported that more than 300 fatty acids and related compounds were found in bacteria. FAME method is adequate for differentiation of members of genus *Streptomyces* from other actinomycetes because of their characteristic profile of fatty acids (Anderson and Wellington 2001).

3.1.2.3 Molecular approach

Molecular characterization is one of the most powerful and reliable approaches in taxonomy. Comparison of nucleic acid of two organisms helps to establish the true connection between two isolates because they are

either the direct gene products or genes themselves. Molecular systematic studies, including both classification and identification, help to study the evolutionary relationship of microorganisms. Importance of phylogenetic analysis studies based on 16S rRNA sequences is increasing in the systematics of bacteria and actinomycetes (Yokota 1997) and helps in the identification of organisms. However, 16S rRNA analysis generally allows the identification of the microorganism up to the genus level only.

It was in 1960s, DNA-DNA hybridization (DDH) values have been used to determine the relatedness between bacterial strains and are still the key criterion in the delineation of bacterial species. The extent of DNA hybridization between two bacterial strains depends on the degree of similarity between their genomic sequences. Klappenbach et al. (2007) reported that a cut-off point of 70% DDH for species delineation corresponded to 95% average nucleotide identity (ANI) and 69% conserved DNA.

3.2 Materials and Methods

3.2.1 Actinomycetes used for the study

Eleven marine actinomycetes, which showed promising activity in the initial antitumor screening (Chapter 2) were selected for further molecular, morphological, phenotypic and chemotaxonomic characterization. The selected isolates include DM21, DM30, DM37, DM42, DM43, DM48, DM49, DM50, DM51, DM57 and DM61.

3.2.2 Genomic DNA extraction from marine actinomycetes

A very simple and rapid method for extracting genomic DNA reported by Cheng and Jiang (2006) was used for extraction. To extract DNA, 2 mL

of 48h old actinomycetes culture was centrifuged at 8000 rpm for 5 min and supernatant was removed. The cell pellet was then washed twice with 400µl STE Buffer (100mM NaCl, 10mM Tris/HCl, 1mM EDTA, pH 8.0) twice. The cell pellet was then re-suspended in 200 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). 50 mg of 425–600 µm size-fractionated glass beads (Sigma) were added to the cell suspension. 100 µl Tris-saturated phenol (pH 8.0) was added to these tubes, followed by a vortex-mixing step of 60-200s to lyse the cells. The samples were subsequently centrifuged at 13,000 rpm for 5 min at 4ºC to separate the aqueous phase from the organic phase. 160µl upper aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube. 40µl TE buffer water was added to make up to 200µl and then 100µl chloroform was added to the tube. The contents were mixed by inverting the microcentrifuge tube several times and centrifuged for 5 min at 8000 rpm at 4 ºC**.** Aqueous layer containing the DNA was purified by chloroform extraction until the white interface was no longer present; this procedure was repeated two to three times. 160 µl upper aqueous phase was transferred to a clean 1.5 ml tube. 40 µl milli Q water and 5 µl RNase (10 mg/ml) were added and incubated at 37ºC for 10 min to digest RNA. Then 100 µl chloroform was added to the tube, mixed well and centrifuged for 5 min at 13,000rpm at 4ºC. 150 µl upper aqueous phase was transferred to a clean 1.5 ml tube. The aqueous phase contained purified DNA and was stored in -20ºC.

DNA concentration and purity of extracted DNA was assessed spectrophotometrically by comparing absorbance at 260 and 280nm followed by 1% agarose gel electrophoresis. Concentration of DNA was calculated using the following formula.

Concentration of DNA= OD_{260}×50×dilution factor where, OD is the Optical Density

3.2.3 16S rRNA gene amplification and phylogenetic tree construction

PCR amplification of 16S rRNA was performed using universal primers 16S1 (GAGTTTGATCCTGGCTCA) and 16S2 (ACGGCTACCTT GTTACGACTT). The PCR was performed in a 25µl reaction mixture containing 1 µl template DNA, 12.5 µl Emerald PCR mix (Takara Bio Inc., Japan) 9.5µl milli Q, 1 µl each primer. The thermal profile applied was an initial denaturation of 95°C for 3 minutes, 30 cycles of denaturation (98°C for 10 s), annealing (58°C for 40 s), extension (72°C for 1 minute 30s), and a final extension (68°C for 10 minutes). The amplified PCR products were electrophoresed on 1% agarose gel, containing ethidium bromide (10 µg/ml) and were visualized on a Gel Documentation System (Bio-Rad). The PCR product was further purified by Exosap (Affymetrix, USA) and sequenced with an ABI prism model 3700 Big Dye Sequencer (Applied Biosystems, USA) at SciGenom Labs Pvt. Ltd, Kochi, India. The nucleotide sequences obtained were assembled using Gene Tool software and nucleotide sequence data were analyzed using BLAST and were deposited in NCBI GenBank (http://www.ncbi.nlm.nih.gov/BankIt). The nucleotide sequences were aligned using ClustalW on MEGA 6.0 (Tamura et al. 2013). The 16S rRNA sequences of closely related actinomycetes were retrieved from GenBank, and their similarity to the present isolate was assessed at the nucleotide level. A phylogenetic tree was constructed using the neighbor-joining method with boot strap values based on 1000 replicates.

3.2.4 Scanning Electron Microscopic (SEM) imaging of marine actinomycetes

Morphological features of intact spores of actinomycetes were observed under scanning electron microscopy. For SEM imaging, it is important to prepare the actinomycete samples with minimum disturbances for getting intact spore structure and spore chain arrangements. For this coverslip culture technique was used. Briefly, glass cover slips were sterilized by autoclaving and then inserted into solidified culture medium at an angle of about 45º until about half the cover slip was gone into the medium. Actinomycete spores were inoculated along the line where the upper surface of the cover slip met the medium, using a loop. The plates were then incubated for 14 days at 28ºC for sporulation. After incubation period cover slips were carefully withdrawn from the medium. The cover slip with undisturbed culture was directly sputter coated and viewed under scanning electron microscopy (VEGA3 TESCAN).

3.2.5 Carbon source utilization test

Washed inocula of actinomycetes were used to inoculate the carbon source utilization media. Washed inocula were prepared as per the protocol described by Shirling and Gottlieb (1966). Twenty two different types of carbon sources were used for the study and those were Xylose, Galactose, Melibiose, Tyrosine, Inositol, Raffinose, Ribose, Leucin, Adonitol, Salicin, Rhamnose, Cellobiose, Proline, Glycine, Mannose, Lactose, Maltose, Fructose, Lysine, Glycerol and Dextrose.

The actinomycetes spores were inoculated into 5mL of sterile distilled water and sufficient spore materials were transferred to make a very turbid suspension in the distilled water. Transferred this suspension to 50 ml of

tryptone-yeast extract broth (3.2.5.1 C) in a 250 ml Erlenmeyer flask. The flasks were agitated at 200 rpm with sterile glass beads at 28 ºC for 48 h. After incubation about 5-10mL of the broth was centrifuged and supernatant was discarded. The pellet was washed with sterile distilled water and then re-suspended in distilled water and used as inoculum. Approximately 0.05 ml of washed inoculum was inoculated into Basal mineral salts medium (3.2.5.1 B) containing 1% carbon source. Plates were kept for 10-16 days of incubation.

3.2.5.1 Composition of media used for carbon utilization test

A. Pridham and Gottlieb trace salts (1 ml of this solution per liter of final medium) (Shirling and Gottlieb 1966)

$CuSO_4.5H_2O$	0.64 g
$FeSO_4.7H_2O$	0.11 g
$MnCl_2.4H_2O$	0.79 g
$ZnSO_4.7H_2O$	0.15 g
Distilled water	100 ml

Store at 3-5° C until use and bring to room temperature before usage.

B. Basal mineral salts medium (used analytical reagent grade chemicals) (Shirling and Gottlieb 1966)

$(NH4)_2\ O_4$	2. 6 4 g
KH_2P0_4. anhydrous	2.38 g
$K_2HPO_4 \cdot 3H_2O$	5.65 g
$MgS0_4.7H_20$	1.00 g
Pridham and Gottlieb trace salts (A)	1 mL
Distilled water	1 L
pH	7.0

5ml aliquots of the basal medium with 1% carbon source were sterilized at 115ºC for 10 min.

C. Tryptone-yeast extract broth for preparation of washed inoculum

Tryptone	5.0 g
Yeast Extract	3.0 g
Distilled water	1.0 L
pH	7.0

3.2.6 Cultural characteristics on different media

Cultural characteristics of actinomycetes, including aerial mass colour, substrate mycelium pigmentation and diffusible pigments production were studied on ISP 2, ISP 3, ISP 4 and ISP 5, Czapek's agar, Nutrient Agar (NA) and Potato Dextrose Agar (PDA). Actinomycetes were inoculated on these media and incubated at 28 ºC for 14 days and the colony colour was compared with the colour chips from the ISCC–NBS colour charts (Kelly 1964).

3.2.7 Analysis of cell wall fatty acids by gas chromatography

Cellular fatty acid methyl ester analysis was performed using the protocol of the Sherlock Microbial Identification System (MIDI) by Gas Chromatography (model 6890N network GC system; Agilent). Biomass used for the analysis was obtained by culturing the actinomycetes isolates in Trypticase Soy Broth Agar (TSBA), for 7 days at 28 ºC. The results were compared with the TSBA 6 database of fatty acids in Sherlock version 6. Briefly cells were harvested and placed on a culture tube and 1 mL of Reagent 1 (45g sodium hydroxide + 150mL methanol + 150 mL distilled water) was added. Tubes were briefly vortexed and placed in a boiling water bath for 30 minutes with frequent vortexing of 5-10s every 5 minutes. Tubes

were taken out and after cooling, 2mL of Reagent 2 (325mL 6.0N hydrochloric acid + 275 mL methyl alcohol) was added. Tubes were briefly vortexed and heated for 10 ± 1 min at 80° ± 1°C. Then 3 mL Reagent 3 (200 mL hexane + 200mL methyl tert-butyl ether) was added to cooled tubes and gentle tumbling on a clinical rotator for about 10 minutes. The aqueous (lower) phase was discarded. Finally 3ml of Reagent 4 (10.8g sodium hydroxide in 900ml distilled water) was added to the organic phase in the tubes and used for the analysis.

3.2.8 Analysis of G+C content

A fluorimetric method for the estimation of G+C mol% content by thermal denaturation reported by Gonzalez (2002) using Real-Time PCR platform (Applied Biosystems ABI7500 with StepOne Plus V2.0 software) was used for the study. Briefly, DNA extraction was performed using PureLink Genomic DNA mini Kit (Invitrogen, USA) as per manufacturer's protocol and stored in 0.1X SSC buffer (0.015M NaCl and 0.0015M trisodium citrate, pH 7.0). The DNA concentration was quantified by NanoDrop spectrophotometer ND 1000 (Thermo scientific, USA). Thermal denaturation was performed using approximately 0.1 X standard saline citrate (SSC) with approximately 5µg genomic DNA and SYBR Green I at a final dilution of 1: 100000. *Pseudonocardia kongjunensis* (DSM No.44525) type strain obtained from Leibniz-Institute DSMZ, Germany with known G+C mol % was used as control DNA for the experiment. Thermal conditions consisted in a ramp from 25 ºC to 100 ºC at a rate of 1 ºC min^{-1}. Fluorescence measurements were performed at each step during this ramp. From the T_m value observed, G+C mol% was calculated as per the below equation. %G+C =1.98 Tm – 106.91

3.2.9 Evaluation of DNA-DNA hybridization

DNA-DNA hybridization was carried out only for *Pseudonocardia sp.* MCCB268 which showed low sequence similarity with other reported isolates after BLAST analysis. DNA-DNA hybridization study performed as per the method reported by Loveland-curtze et al. (2011). *Pseudonocardia kongjuensis* (DSM No.44525) type strain obtained from Leibniz-Institute DSMZ, Germany which showed highest sequence similarity (98%) with *Pseudonocardia sp.* MCCB268 was used as reference strain. Both reference DNA and sample DNA were extracted in 2X SSC buffer (Saline sodium citrate) by PureLink Genomic DNA mini Kit (Invitrogen, USA) as per manufacturer's protocol. Intact DNA was quantified using NanoDrop spectrophotometer. Uniformly small-sized segments, optimally about 400–1500 bp is essential for the experiment and it was standardized by giving different pulses at lowest setting using sonifier (Sonics Vibracell, VCX 500, Newtown,CT). Different sonication conditions were attempted for standardization as given below;

1. DNA sonication in 2X SSC for 12 pulses (1 sec) at an output of 50% Sonication.
2. DNA sonication in 2X SSC for 12 pulses (1 sec) at an output of 50% Sonication + 5 min ultra sonication water bath.
3. 5 times DNA sonication in 2X SSC for 12 pulses (1 sec) at an output of 50% Sonication + 5 min ultra sonication water bath.
4. 10 times DNA sonication in 2X SSC for 12 pulses (1 sec) at an output of 50% Sonication + 5 min ultra sonication water bath.
5. 7 times DNA sonication in 2X SSC for 12 pulses (2 sec) at an output of 50% Sonication.

Experiments were done with sheared DNA of approximately 0.1 mg/mL, resulting in a final DNA concentration of 1 mg/well. DNA samples in 2X SSC buffer were added in 10 µL aliquots (single) for individual samples (*Pseudonocardia kongjuensis* and *Pseudonocardia* sp. MCCB 268) or two 5 µL aliquots for DNA pairs (Mix) to a 96 well PCR plate (Applied Biosystems, USA).

10 µL of SYBR Green I nucleic acid stain (SG) diluted in 2X SSC (1: 10000) was added to each well for a total final volume of 20 µL. Samples were thoroughly mixed by repeated pipetting. All works were carried out under low light condition and centrifuged briefly prior to melting and reassociation experiment of the DNA samples on the Real Time PCR machine (Applied Biosystems systems ABI 7500). Before starting the experiment, optimal renaturation temperature [T_{or} =50.516(% G+C) +47.0] was calculated according to De et al. (1970).

The hybridization cycle was started with an initial temperature set at 25 ºC (5 s), and further the temperature was increased to 99 ºC (100% ramp), then temperature was maintained at 99 ºC for 10 min to ensure the melting of DNA. After DNA melted, the temperature was decreased quickly (100% ramp) to optimal renaturation temperature and maintained at that temperature for 40 min for reassociation to take place. After that, the temperature was again reduced (100% ramp) to 25 ºC and held for 30 s. Fluorescence readings were taken on every 7–10 seconds throughout the experiment. Hybridization was performed with five replications for each sample. Fluorescence was recorded throughout the renaturation step and plotted against time in minutes. Linear regression trend lines were fitted in the plot. The straightest portions of the curves, with the highest correlation

coefficients, were used for the calculations. The slopes of the linear regression lines, representing the renaturation rates (increase in fluorescence per minute) V', were used to determine the degree of binding (%) using the equation of De et al. (1970).

$$D= [4\ V'_M - (V'_A + V'_B) / 2* (\sqrt{V'_A\ V'_B})]* 100$$

The subscripts ''M'', ''A'', and ''B'' refer to the mixture of a pair of DNAs and the 2 individual samples, respectively.

3.2.10 Effect of physico-chemical parameters on the growth of actinomycetes

3.2.10.1 Preparation of inoculum

Actinomycetes inoculum was prepared by harvesting spores of actinomycetes into sterile seawater of salinity 15ppt. From this suspension 100 µl was used as inoculum for the following optimization processes.

3.2.10.2 Effect of pH

To find out the pH optima, Nutrient broth (Peptone-0.5g, Beef extract-0.5 g, seawater (30ppt)-100mL) with pH of 4, 5, 6, 7, 8, 9, 10, 11 and 12 were prepared. The pH of the medium was adjusted either by using 0.1N NaOH or 0.1N HCl solution.

The broth was autoclaved at 121ºC for 15 min. 100 µl of actinomycete spores were inoculated into the 20 mL broth and were incubated at 28 ± 2 °C for 14 days. The growth of the actinomycete culture was estimated as cell dry weight after incubation. For this the culture was centrifuged at 8000 rpm at 4^{o}C and the cell pellets were washing with sterile PBS dried at 60^{o}C overnight and recoded the mass.

3.2.10.3 Effect of temperatures

Nutrient agar (Peptone-0.5g, Beef extract-0.5 g, Agar-2.0g, seawater (30ppt)-100mL, pH-7.5) plates were prepared and sterilized. 100 µl of inoculums of actinomycetes spores were inoculated into the plates and were incubated at 4, 10, 20, 28, 37, 45 and 55°C for 14 days. After incubation, the plates were observed for growth of actinomycetes and recorded.

3.2.10.4 Effect of NaCl on growth of actinomycetes

Nutrient broth based culture medium of nine different salinities including 0, 10, 20, 30, 40 and 50 ppt were prepared and sterilized. 100 µl of actinomycetes spores were inoculated into 20 mL broth and the tubes were incubated at 28 ± 2 °C for 14 days. The growth of the actinomycete culture as cell dry weight was recorded as per Section 3.2.10.2

3.2.11 Catalase test

A clear glass slide was taken and a loopfull of culture was placed on it. Few drops of hydrogen peroxide (H_2O_2) were added to the cultures. The evolution of air bubbles from the culture indicated a positive reaction.

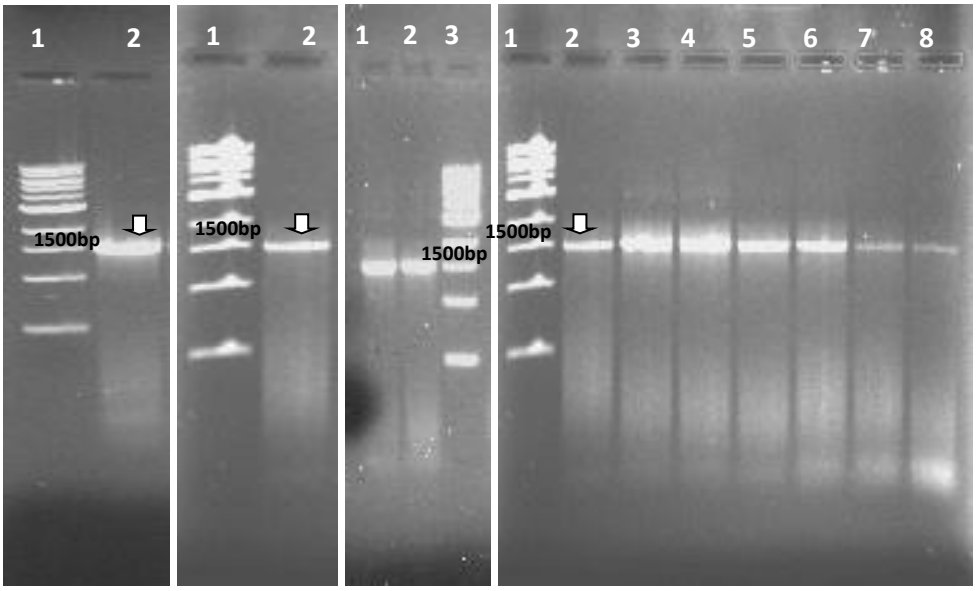
3.2.12 Oxidase test

Small peices of filter paper were soaked in 1% aqueous tetra methyl para phenylene diamine dihydrochloride and the papers were dreid. A small portion of the actinomycete culture was placed on the test paper containing the test solution with a clean platinum loop and the colour change was observed and blue colouration indicated positive result for oxidase.

3.3 Results

3.3.1 Molecular identification of the isolates and phylogenetic analysis

Molecular identification of the isolates were carried out by the PCR amplification of 16S rRNA gene. Amplified PCR products were visualized in agarose gel (Fig. 3.1) and sequenced. Obtained nucleotide sequences were analyzed by BLAST and phylogenetic tree was constructed. Sequences were submitted in GenBank. The molecular identification details are given in Table 3.1. All isolates were deposited in Microbial Culture Collection repository of National Centre for Aquatic Animal Health, Cochin University of Science and Technology, India and assigned an unique culture identification number as given in Table 3.1



Lane 2: DM21	Lane 2: DM30	Lane1-DM37 Lane 2- DM42	Lane 2-8:DM43, DM48, DM49, DM50, DM51, DM57, DM61

Fig. 3.1: Gel electrophoresis image of amplified 16S rRNA gene of isolates

3.3.2 Identification of DM21

The isolate DM21 was submitted to Microbial Culture Collection repository of National Centre for Aquatic Animal Health, as MCCB 267. BLAST search result shows that the isolate DM21 comes under the genus *Streptomyces* and has got highest sequence similarity (99%) with one of the recently reported species *Streptomyces zhaozhouensis* NEAU-LZS-5 (He et al. 2014) and *Streptomyces sedi* strain YIM 65188 (Li et al. 2009). *Streptomyces avicenniae* MCCC 1A01535 showed 96% sequence similarity with the MCCB 267 (Xiao et al. 2009). All other representatives of the genus *Streptomyces* showed lower sequence similarity to MCCB 267. Since MCCB 267 formed a distinct clade with *Streptomyces zhaozhouensis* NEAU-LZS-5 in phylogenetic analysis using neighbour joining method (Fig. 3.2), it was designated as *Streptomyces* sp. MCCB 267.

Table 3.1: Molecular identification of selected actinomycetes isolates

Sl. No.	Actinomycetes	Isolate number	Source	GenBank accession number
1	*Streptomyces* sp. MCCB 267	DM21	Sponge from Rameswaram, South East coast of India	KR057432
2	*Streptomyces* sp. MCCB246	DM30	Indian ocean Sediment	KP322127
3	*Pseudonocardia* sp. MCCB 268	DM37	Arctic sediment	KR057433
4	*Streptomyces* sp. MCCB 248	DM42, DM43, DM48, DM49, DM50, DM51, DM57, DM61	Arctic sediment	KP313874

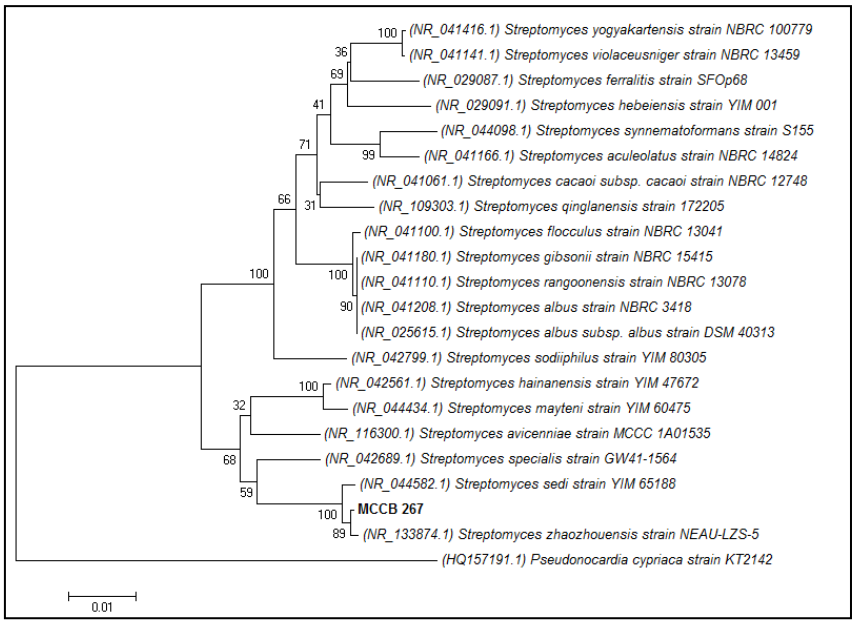


Fig. 3.2: Neighbour-Joining phylogenetic dendrogram based on 16S rRNA gene sequences, showing relationships between the isolated *Streptomyces* sp. MCCB 267 and related taxa

3.3.3 Characterization of *Streptomyces* sp. MCCB 267

3.3.3.1 Morphological and culture characterization of *Streptomyces* sp. MCCB 267

Nutrient agar (NA) was used for routine subculture of isolates. Morphological apperaence of *Streptomyces* sp. MCCB 267 in NA and appearance of actimomycetes hyphae and spores in SEM imaging is given in Fig. 3.3. Scanning electron microscopy revealed the presence of simple smooth spiral spore chains in MCCB 267 (Fig. 3.3b), similar to members of the genus *Streptomyces* which possess spores arranged in chains (Kim et al. 2003).

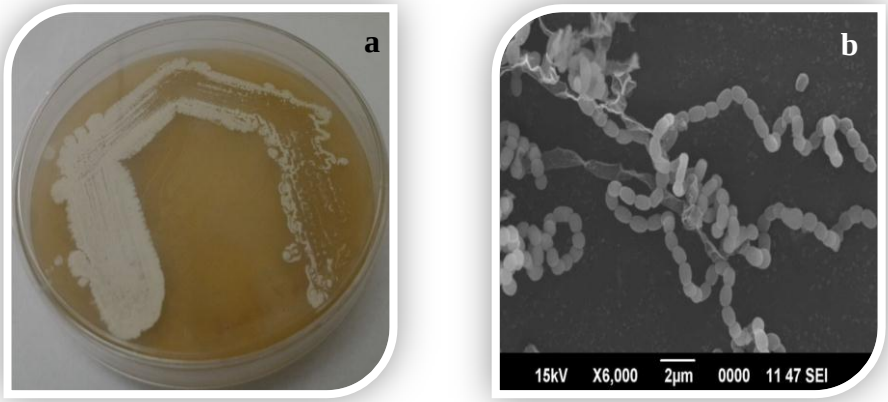


Fig. 3.3 : a) Colony morphology and b) scanning electron micrograph of aerial mycelia of *Streptomyces* sp. MCCB 267 after incubation for 14 days on Nutrient agar at 28 ºC

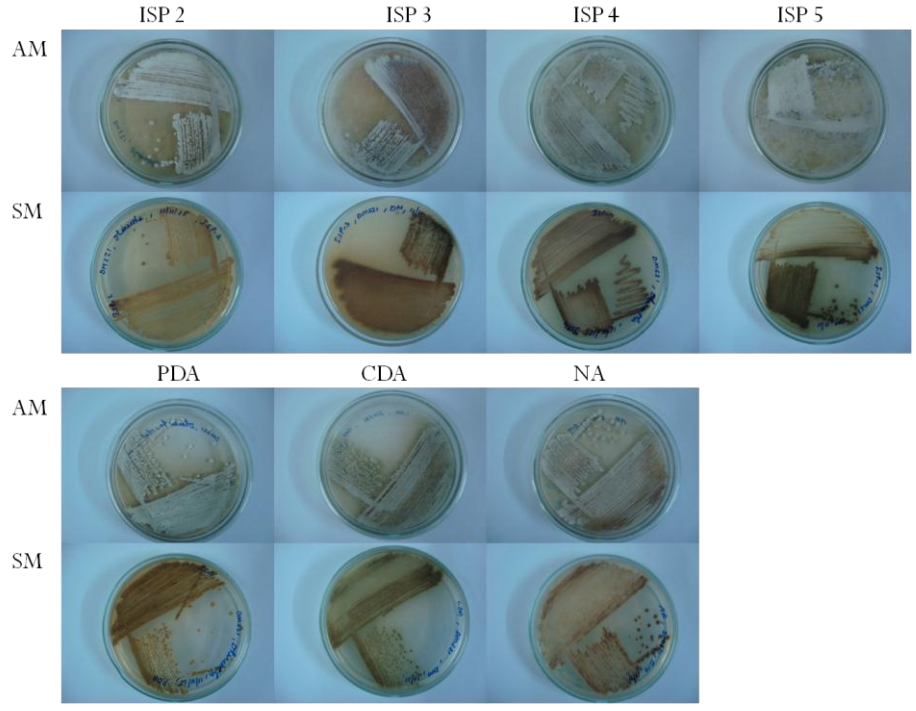


Fig. 3.4: Cultural characteristics *Streptomyces* sp. MCCB267 various media after 2 weeks at 28º C

Table 3.2: Cultural characteristics *Streptomyces* sp. MCCB 267 various media after 2 weeks at 28 ºC

Agar medium	Colour of mycelium				Soluble pigment	
	Aerial mycelium		Substrate mycelium			
	***Streptomyces* sp. MCCB 267**	***Streptomyces zhaozhouensis*** (He et al. 2014)	***Streptomyces* sp. MCCB 267**	***Streptomyces zhaozhouensis*** (He et al. 2014)	***Streptomyces* sp. MCCB 267**	***Streptomyces zhaozhouensis*** (He et al. 2014)
ISP 2	White	White	Moderate yellow	White	No	No
ISP 3	Pale yellow green	White	Light olive brown	Vivid yellowish green	No	No
ISP 4	Moderate yellow green	None	Moderate olive	White	No	No
ISP 5	White	White	Strong yellow green	Light Yellow Green	No	No
PDA	Moderate yellowish green	NA	Dark greenish yellow	NA	No	NA
CDA	Light yellow green	None	Moderate yellow green	Yellowish white	No	No
NA	Greenish white	Pale yellow	Brownish pink	Pale yellowish	No	No

The isolate *Streptomyces* sp. MCCB 267 was also studied for its cultural characteristics on seven different media including ISP 2, ISP 3, ISP 4 and ISP 5, Czapek's agar (CDA), nutrient agar (NA) and potato dextrose agar (PDA) at 28ºC for 14 days and colour of aerial mycelia and substrate (reverse side) mycelia (Fig. 3.4). The results were compared with type strain *Streptomyces zhaozhouensis* NEAU-LZS-5 (He et al. 2014) and tabulated in Table 3.2.

Streptomyces sp. MCCB 267 showed good growth on all the seven-tested media, whereas *S. zhaozhouensis* NEAU-LZS-5 grew well on ISP 2, ISP 3, ISP 5, ISP 7 and nutrient agar and showed poor growth on ISP 4, ISP 6 and Czapek's agar. *S. zhaozhouensis* NEAU-LZS-5 grew with white substrate mycelia and doesn't formed any aerial mycelia on ISP 4 media, whereas MCCB267 formed moderate yellow green aerial mycelia in ISP 4 and moderate olive colored substrate mycelia. Similarly, on CDA also *S. zhaozhouensis* NEAU-LZS-5 grew without aerial mycelia whereas MCCB 267 grew with light yellow green aerial mycelia. MCCB267 produced no diffusible pigment production in any of these media.

3.3.3.2 Carbon source utilization ability of *Streptomyces* sp. MCCB267

The isolates were checked for their ability to utilize 21 different carbon sources as their sole carbon source. Carbon source utilization ability of isolates was observed after 10-16 days at 28ºC. Results have indicated that *Streptomyces* sp. MCCB267 has the ability to utilize Xylose, Galactose, Melibiose, Raffinose, Ribose, Salicin, Rhamnose, Cellobiose, Proline, Maltose, Fructose, Glycerol and Dextrose as sole carbon source. Carbon source utilization profile was compared with *S. zhaozhouensis* NEAU-LZS-5 and summarized in Table 3.3.

Table 3.3: Ability of *Streptomyces* sp. MCCB 267 to use different carbon sources

Carbon source	*Streptomyces* sp. MCCB 267	*Streptomyces zhaozhouensis* NEAU-LZS-5 (He et al. 2014)
Xylose	+	+
Galactose	+	+
Melibiose	+	NA
Tyrosine	_	_
Inositol	_	_
Raffinose	+	_
Ribose	+	_
Leucin	_	NA
Adonitol	_	NA
Salicin	+	NA
Rhamnose	+	_
Cellobiose	+	NA
Proline	+	NA
Glycine	_	NA
Mannose	_	_
Lactose	_	+
Maltose	+	+
Fructose	+	_
Lysine	_	NA
Glycerol	+	
Dextrose	+	+

3.3.3.3 Fatty acid profile of actinomycetes isolates

Fatty acid profile of the *Streptomyces* sp. MCCB 267, was compared with S. *zhaozhouensis* NEAU-LZS-5 and given in Table 3.4. The major fatty acids in the cell wall of *Streptomyces* sp. MCCB 267 were found to be $C_{16:0}$ iso (40.69%), $C_{16:1}$ iso G (12.63%), $C_{17:0}$ anteiso (9.08%), $C_{15:0}$ anteiso (6.31%) and $C_{15:0}$ iso (5.05%).

Table 3.4: Fatty acid profile of the *Streptomyces* sp. MCCB 267

Fatty acids	Fatty acid profile of *Streptomyces* sp. MCCB 267 (%)	Fatty acid profile of *S. zhaozhouensis* NEAU-LZS-5 (%) (He et al. 2014)
12:0 iso	-	-
12:0	-	-
13:0 iso	-	-
13:0 anteiso	-	-
14:0 iso	0.85	-
14:0	-	-
15:0 iso	5.05	7.8
15:0 anteiso	6.31	-
15:1 iso G	0.00	-
15:1 anteiso A	-	-
15:1 w6c	1.55	
15:0	0.00	9.98
16:1 iso H	-	-
16:1 iso G	12.63	-
16:0 iso	40.69	-
16:1 w5c	0.45	-
16:1 w7c	-	7.49
16:0	2.30	35.48
17:1 anteiso w9c	4.23	-
17 : 1 w7c	-	12.97
17:0 iso	2.28	-
17:0 anteiso	9.08	-
17:1 w8c	4.31	-
17:0 cyclo	2.46	-
17:0	2.02	7.24
17:0 10-methyl	0.51	-
18:1 w9c	0.68	-
18:0	2.67	7.64

3.3.3.4 Analysis G+C mol% content of *Streptomyces* sp. MCCB 267

Estimation of G+C mol% content was performed by thermal denaturation using Real-Time PCR. Tm values after melt curve analysis and GC values of all selected isolates were summarized in Table 3.5.

After melt curve analysis *Streptomyces* sp. MCCB 267 was found to have G+C content of 68%.

Table 3.5 : Tm values after melt curve analysis and G+C mol% actinomycetes isolates

Sl. No.	Isolate name	Tm value (ºC)	G+C (mol %)
1	*Streptomyces* sp. MCCB 267	88.2	68%
2	*Streptomyces* sp. MCCB246	86.73	65%
3	*Pseudonocardia* sp. MCCB 268	89.59	70.5%
4	*Streptomyces* sp. MCCB 248	88.19	67 %

3.3.4 Identification of DM 30

The culture DM30 was submitted in NCAAH culture collection as MCCB 246. The16S rRNA gene sequence of strain MCCB 246 and further BLAST search result showed the isolate belongs to the genus *Streptomyces* and got highest sequence similarity (99%) with the type strain *Streptomyces wuuyuanensis* FX61 (Zhang et al. 2013). However, phylogenetic analysis using neighbour joining method showed that the isolate formed a distinct clade with *Streptomyces wuuyuanensis* FX61 (Fig. 3.5). These similarities indicate that the isolate MCCB 246 belongs to the genus *Streptomyce*s and hence designated as *Streptomyces* sp. MCCB246 (Gen bank accession number KP322127).

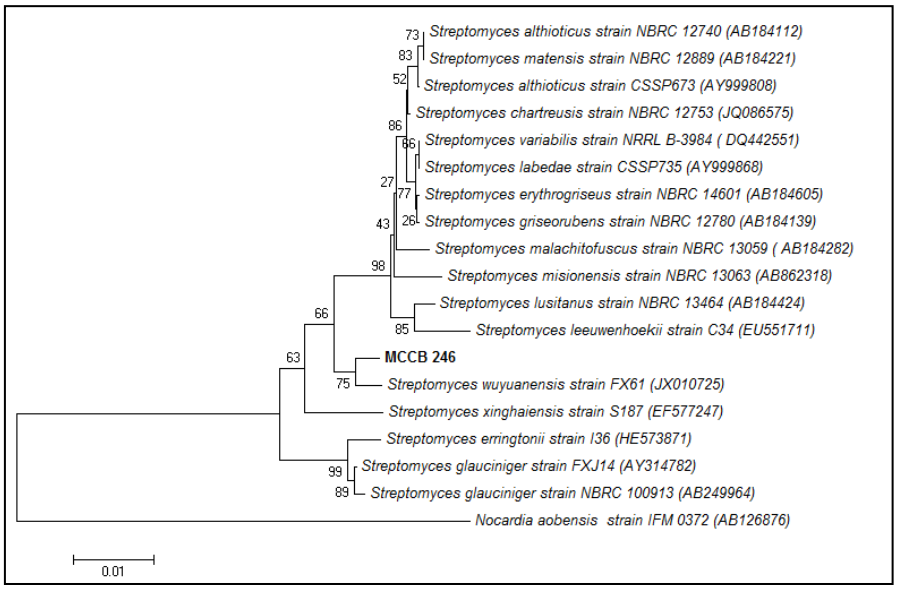


Fig. 3.5: Neighbour-Joining phylogenetic dendrogram based on 16S rRNA gene sequences, showing relationships between the isolated *Streptomyces* sp. MCCB246 and related taxa

3.3.5 Characterization of *Streptomyces* sp. MCCB 246

3.3.5.1 Morphological and culture characterization of *Streptomyces* sp. MCCB 246

Morphological apperaence of *Streptomyces* sp. MCCB 246 in NA and appearance of actimomycetes hyphae and spores in SEM imaging were taken (Fig. 3.6). In SEM analysis *Streptomyces* sp. MCCB246 was found to produce biverticillus spiral smooth spore chains. The isolate *Streptomyces* sp. MCCB246 was studied for its cultural characteristics including colour of aerial mycelia and substrate (reverse side) mycelia on seven different media including ISP 2, ISP 3, ISP 4 and ISP 5,Czapek's agar (CDA), nutrient agar (NA) and potato dextrose agar (PDA) at 28ºC for 14 days (Fig. 3.7).

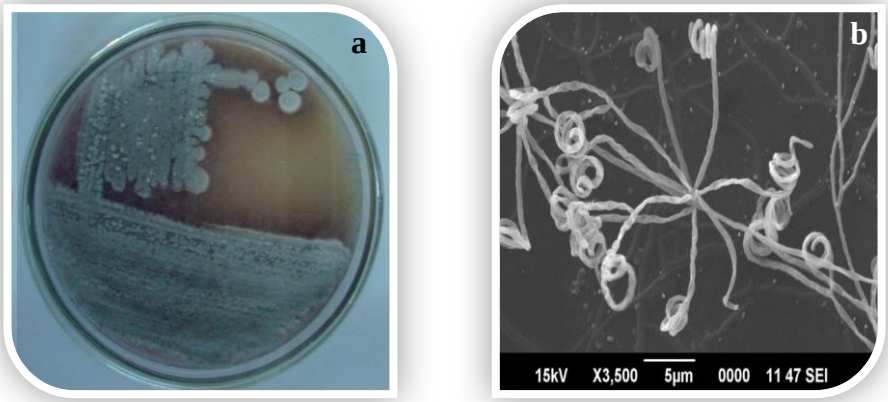


Fig. 3.6 : a) Colony morphology and b) scanning electron micrograph of aerial mycelia of *Streptomyces* sp. MCCB 246 after incubation for 14 days growth on Nutrient agar at 28 ºC

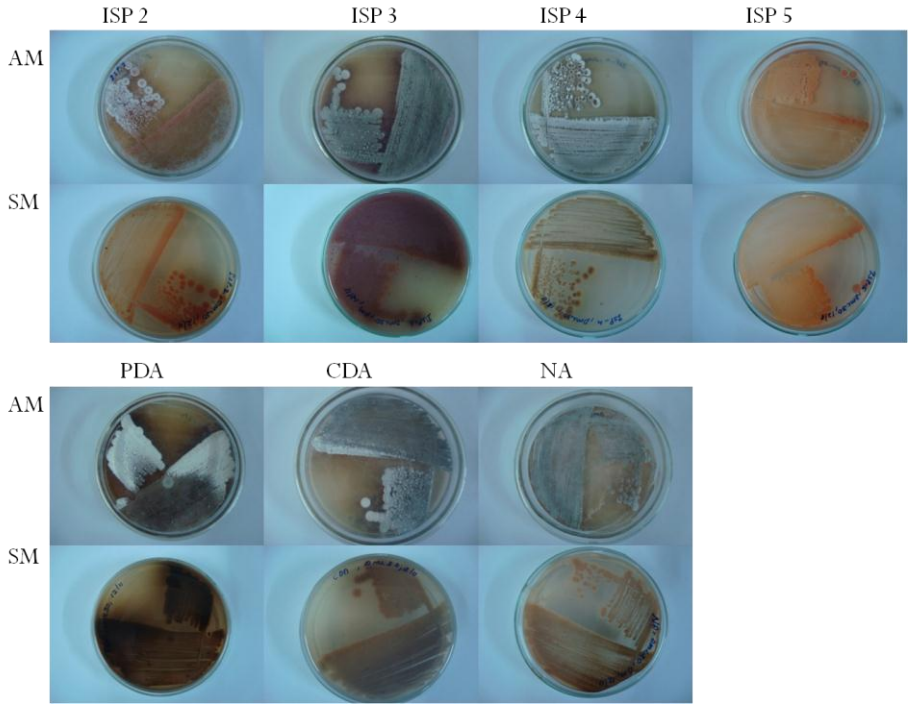


Fig. 3.7: Cultural characteristics of *Streptomyces* sp. MCCB246 on various media after 14 days of growth at 28º C

Table 3.6: Cultural characteristics *Streptomyces* sp. MCCB246 on various media after 14 days at 28º C

Agar medium	Colour of mycelium				Soluble pigment	
	Aerial mycelium		Substrate mycelium			
	***Streptomyces* sp. MCCB 246**	***Streptomyces wuyuanensis*** (Zhang et al. 2013)	***Streptomyces* sp. MCCB 246**	***Streptomyces wuyuanensis*** (Zhang et al. 2013)	***Streptomyces* sp. MCCB 246**	***Streptomyces wuyuanensis*** (Zhang et al. 2013)
ISP 2	Yellowish white	Grey	Strong orange yellow	Yellow	Yes	No
ISP 3	Light greenish grey	Grey	Light greenish red	Yellowish-white	Yes	No
ISP 4	Light grey	Oyster white	Deep yellow	Yellow	No	No
ISP 5	Light orange yellow	Oyster white	Light orange	Yellow	Yes	No
PDA	White	Grey	Deep brown	Yellow	Yes	No
CDA	Greyish green	Grey	Light brown	White	Yes	No
NA	Light greenish grey	Absent	Greenish red	Yellow	Yes	No

The results were compared with nearest type strain *Streptomyces wuyuanensis* (Zhang et al. 2013) and tabulated in Table 3.6. The isolate *Streptomyces* sp. MCCB246 showed good growth on ISP 3, ISP 4, PDA, CDA and NA media. The isolate showed growth in ISP 2, ISP 5 with very less sporulation. In all the seven media used, the isolate showed distinct aerial and substrate mycelial colouration and showed soluble pigment production except in ISP 4 medium. In contrast , the nearest type strain *Streptomyces wuuyuanensis* FX61 showed either white or yellow substrate mycelium and white or oyster white in the tested media except NA where it grown without aerial mycelium.

3.3.5.2 Carbon source utilization of *Streptomyces* sp. MCCB246

Carbon source utilization ability of *Streptomyces* sp. MCCB246 was observed after 10-16 days growth at 28ºC. Carbon source utilization profile was compared with *Streptomyces wuyuanensis* and summarized in Table 3.7.

The result has indicated that *Streptomyces* sp. MCCB246 had the ability to utilise Xylose, Melibiose, Tyrosine, Inositol, Raffinose, Ribose, Cellobiose, Proline, Glycine, Mannose, Lactose, Maltose, Fructose, Lysine and Dextrose but not Galactose, Leucin, Adonitol, Salicin and Glycerol.

3.3.5.3 Fatty acid profile of *Streptomyces* sp. MCCB246

Fatty acid profile of the *Streptomyces* sp. MCCB 246, is given in Table 3.8. The major fatty acids present in the isolates were $C_{16:0}$ iso (27.095%), $C_{15:0}$ anteiso (14.4%), $C_{15:0}$ iso (12.41%), $C_{17:0}$ anteiso (7.58%), $C_{16:0}$ (7.77%), and $C_{14:0}$ iso (5.84%).

Table 3.7: Ability of *Streptomyces* sp. MCCB246 to use different carbon sources

Carbon source	*Streptomyces* sp. MCCB 246	*Streptomyces wuyuanensis* (Zhang et al. 2013)
Xylose	+	+
Galactose	_	+
Melibiose	+	NA
Tyrosine	+	NA
Inositol	+	+
Raffinose	+	+
Ribose	+	NA
Leucin	_	NA
Adonitol	_	NA
Salicin	_	NA
Rhamnose	_	NA
Cellobiose	+	NA
Proline	+	NA
Glycine	+	NA
Mannose	+	NA
Lactose	+	NA
Maltose	+	NA
Fructose	+	NA
Lysine	+	NA
Glycerol	_	NA
Dextrose	+	NA

Table 3.8: Fatty acid profile of the *Streptomyces* sp. MCCB 246

Fatty acids	Fatty acid profile of *Streptomyces* sp. MCCB246 (%)	Fatty acid profile of *Streptomyces wuyuanensis* (%) (Zhang et al. 2013)
12:0 iso	0.50	-
12:0	1.01	-
13:0 iso	0.39	-
13:0 anteiso	0.32	-
14:0 iso	5.84	5.81
14:0	1.40	-
15:0 iso	12.41	8.79
15:0 anteiso	14.40	10.55
15:1 iso G	-	-
15:1 anteiso A	-	-
15:1 w6c	-	-
15:0	-	-
16:1 iso H	2.51	8.3
16:1 iso G	-	-
16:0 iso	27.09	31.03
16:1 w5c	-	-
16:0	7.77	6.33
17:1 anteiso w9c	2.29	-
17:0 iso	4.82	-
17:0 anteiso	7.58	5.2
17:1 w8c	-	-
17:0 cyclo	2.9	-
17:0	-	-
17:0 10-methyl	-	-
18:1 w9c	-	-
18:0	-	

3.3.5.4 Analysis G+C mol% content of *Streptomyces* sp. MCCB 246

Estimation of G+C mol % content was performed by thermal denaturation using Real-Time PCR. Tm values after melt curve analysis of *Streptomyces* sp. MCCB 246 is given in Table 3.5. After melt curve analysis *Streptomyces sp.* MCCB 246 was found to have Tm value of 86.73^{o}C and G+C content of 65%.

3.3.6 Identification of DM42, DM43, DM48, DM49, DM50, DM51 and DM57

Upon blast analysis isolates viz. DM42, DM43, DM48, DM49, DM50, DM51 and DM57 were revealed as one and same. So these isolates were submitted in NCAAH culture collection as MCCB 248. BLAST analysis identified MCCB 248 belonging to the genus *Streptomyces*, having highest sequence similarity (99%) to the type strain *Streptomyces artemisiae* YIM 63135. Moreover, phylogenetic analysis using the neighbour joining method placed it in a distinct clade along with *Streptomyces artemisiae* YIM 63135 (Fig. 3.8). Accordingly, the isolate MCCB 248 was designated as *Streptomyces* sp. MCCB 248 (GenBank accession number KP313874).

3.3.7 Characterization of *Streptomyces* sp. MCCB 248

3.3.7.1 Morphological and culture characterization of *Streptomyces* sp. MCCB 248

Morphological apperence of *Streptomyces* sp. MCCB248 in NA and appearance of actimomycetes hyphae and spores in SEM imaging are given (Fig. 3.9). The isolate *Streptomyces* sp. MCCB 248 was studied for its cultural characteristics including colour of aerial mycelia and substrate mycelia on seven different media at 28ºC for 14 days (Fig. 3.10). The

results were compared with nearest type strain *Streptomyces artemisiae* (Zhao et al. 2010) and tabulated in Table 3.9. Results of cultural characterestics of *Streptomyces* sp. MCCB 248 and *Streptomyces artemisiae* indicate that they are very much distinct in cultural charecterestics in all the seven tested media. Notably, *Streptomyces* sp. MCCB248 doesn't produce any soluble pigments in the tested media, whereas Streptomyces artemisiae produced soluble pigments in ISP 2 and ISP 3.

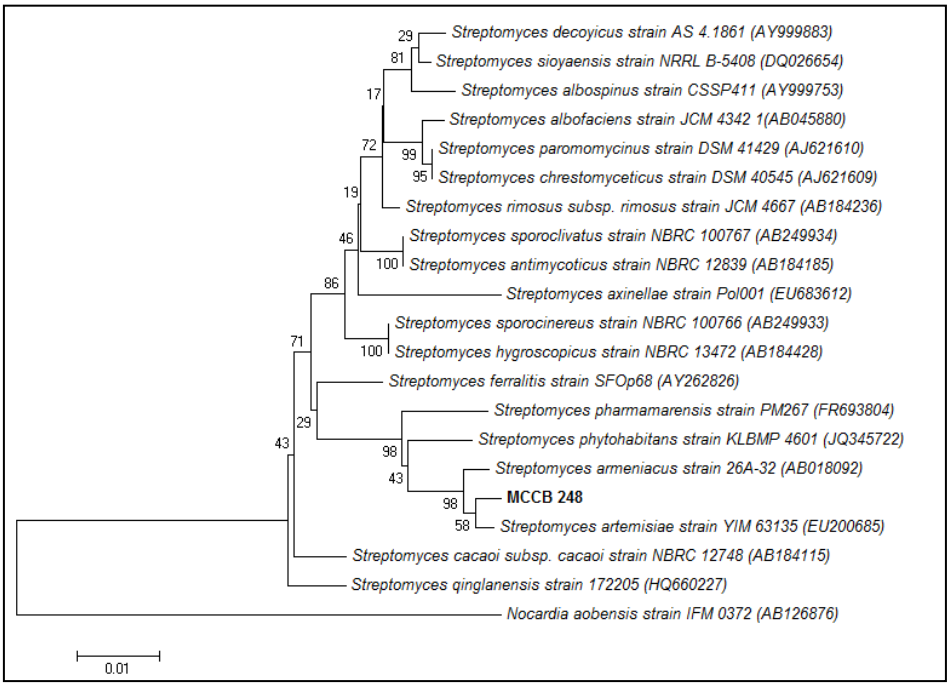


Fig. 3.8: Neighbour-Joining phylogenetic dendrogram based on 16S rRNA gene sequences, showing relationships between the isolated *Streptomyces* sp. MCCB 248 and related taxa

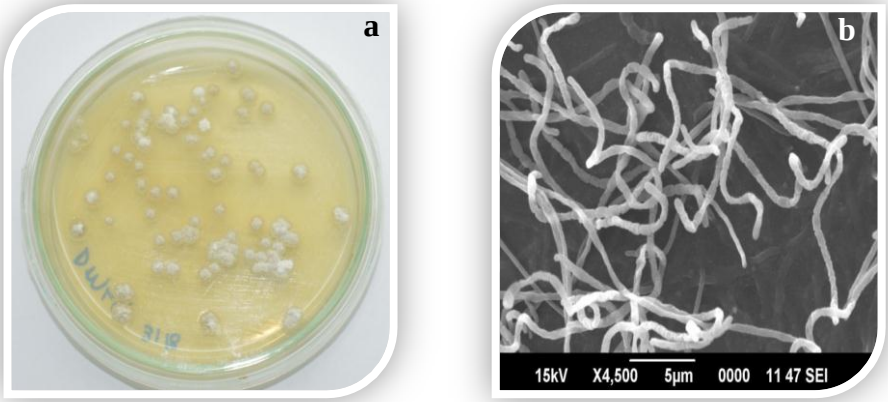


Fig. 3.9 : a) Colony morphology and b) scanning electron micrograph of aerial mycelia of *Streptomyces* sp. MCCB 248 after incubation for 14 days on Nutrient agar at 28 ºC

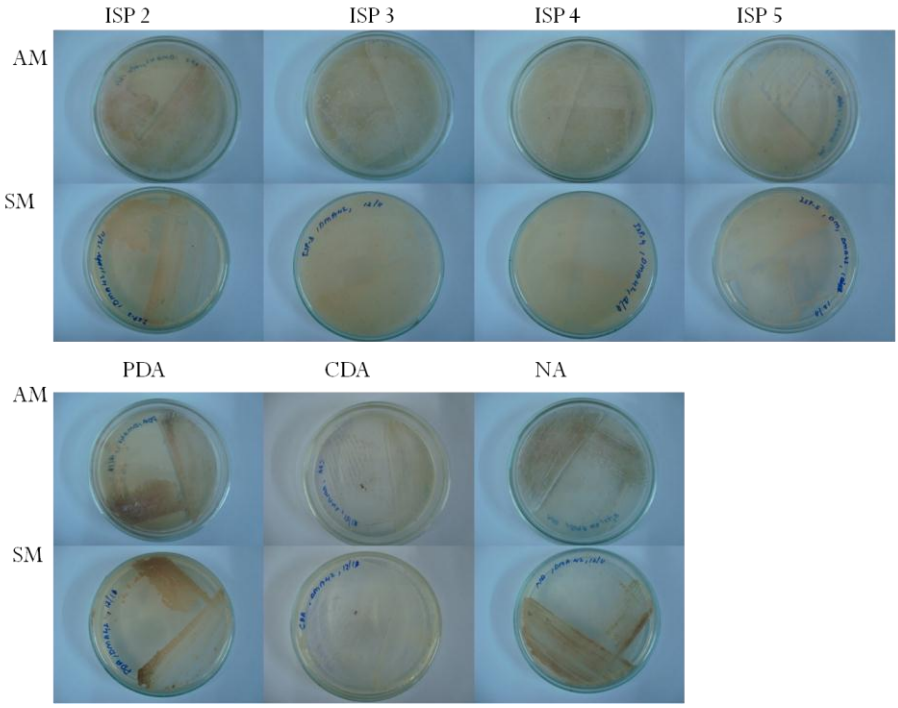


Fig. 3.10: Cultural characteristics *Streptomyces* sp. MCCB 248 on various media after 2 weeks at 28º C

Table 3.9: Cultural characteristics *Streptomyces* sp. MCCB248 various media after 2 weeks at 28º C

Agar medium	Colour of mycelium				Soluble pigment	
	Aerial mycelium		Substrate mycelium			
	***Streptomyces* sp. MCCB 248**	***Streptomyces artemisiae*** (Zhao et al. 2010)	***Streptomyces* sp. MCCB 248**	***Streptomyces artemisiae*** (Zhao et al. 2010)	***Streptomyces* sp. MCCB 248**	***Streptomyces artemisiae*** (Zhao et al. 2010)
ISP 2	Yellowish white	White	Pale yellow	Yellow–white	No	Yes
ISP 3	Yellowish white	Pink	Pale yellow	Pink–brown	No	Yes
ISP 4	White	White	Pale yellow	Yellow–white	No	No
ISP 5	Yellowish white	White	Pale yellow	Yellow–white	No	No
PDA	Pale yellow	Yellow–white	Moderate yellow	Pink	No	No
CDA	White	Pink	Light yellow	White	No	No
NA	Yellowish grey	White	Light greyish yellowish brown	Yellow–white	No	No

3.3.7.2 Carbon source utilization ability of *Streptomyces* sp. MCCB248

Carbon source utilization ability of *Streptomyces* sp. MCCB248 was observed after 10-16 days at 28ºC and compared with *Streptomyces artemisiae* (Zhao et al. 2010) and shown in (Table 3.10). Results have indicated that *Streptomyces* sp. MCCB248 has the ability to utilise Xylose, Melibiose, Tyrosine, Inositol, Raffinose, Ribose, Cellobiose, Proline, Glycine, Mannose, Lactose, Maltose, Fructose, Lysine and Dextrose but not Galactose, Leucin, Adonitol, Salicin and Glycerol.

Table 3.10: Ability of *Streptomyces* sp. MCCB248 to use different carbon sources

Carbon source	***Streptomyces* sp. MCCB 248**	***Streptomyces artemisiae*** (Zhao et al. 2010)
Xylose	+	NA
Galactose	+	+
Melibiose	_	NA
Tyrosine	+	NA
Inositol	+	_
Raffinose	_	NA
Ribose	+	NA
Leucin	+	NA
Adonitol	+	NA
Salicin	_	NA
Rhamnose	_	+
Cellobiose	+	NA
Proline	+	NA
Glycine	_	NA
Mannose	+	+
Fructose	+	NA
Lysine	_	NA
Glycerol	+	+
Dextrose	+	+

3.3.7.3 Fatty acid profile of *Streptomyces* sp. MCCB248

Fatty acid profile of *Streptomyces* sp. MCCB 248 is given in Table 3.11. The major fatty acids present in the isolates were $C_{16:0}$ iso (16.77%), $C_{17:0}$ anteiso (15.48%), $C_{15:0}$ anteiso (14.05%), $C_{16:0}$ (13.18%), $C_{18:0}$ (6.53%), $C_{16:1}$ iso G (6.03%), $C_{17:1}$ anteiso w9c (5.89%).

Table 3.11: Fatty acid profile of the *Streptomyces* sp. MCCB 248

Fatty acids	Fatty acid profile of *Streptomyces* sp. MCCB 248 (%)	Fatty acid profile of *Streptomyces artemisiae* (%) (Zhao et al. 2010)
12:0 iso	-	-
12:0	-	-
13:0 iso	-	-
13:0 anteiso	0.49	-
14:0 iso	1.87	-
14:0	2.34	-
15:0 iso	3.64	-
15:0 anteiso	14.05	17
15:1 iso G	0.38	-
15:1 anteiso A	0.87	-
15:1 w6c	-	-
15:0	-	-
16:1 iso H	-	-
16:1 iso G	6.03	-
16:0 iso	16.77	30
16:1 w5c	-	-
16:0	13.18	-
17:1 anteiso w9c	5.89	-
17:0 iso	2.37	-
17:0 anteiso	15.48	27.3
17:1 w8c	-	-
17:0 cyclo	0.88	-
17:0	-	-
17:0 10-methyl	-	-
18:1 w9c	1.96	-
18:0	6.53	-

3.3.7.4 Analysis G+C mol% content of *Streptomyces* sp. MCCB 248

Estimation of G+C mol% content was performed by thermal denaturation using Real-Time PCR. Tm values after melt curve analysis of *Streptomyces* sp. MCCB 248 is given in Table 3.5. After melt curve analysis, *Streptomyces* sp. MCCB 248 was found to have tm value of 88.19 and G+C content of 67 %.

3.3.8 Identification of DM37

The culture DM37 was submitted in NCAAH culture collection as MCCB 268. The 16S rRNA gene sequence of the isolate and further BLAST search result showed that the isolate belongs to the genus *Pseudonocardia* and has shown only 98% sequence similarity with type strain *Pseudonocardia konjuensis* (Min et al. 2001). All other isolates showed low sequence similarity. Phylogenetic analysis using neighbour joining method showed that the isolate formed a separate branch with *Pseudonocardia konjuensis* in the tree (Fig. 3.11). Hence, the isolate was designated as *Pseudonocardia* sp. MCCB 268 (Gen bank accession number KR057433).

3.3.9 Characterization of *Pseudonocardia* sp. MCCB 268

In molecular taxonomy, *Pseudonocardia* sp. MCCB 268 showed less sequence similarity with other reported strains. So this isolate was selected for further detailed characterization including DNA-DNA hybridization with its nearest neighbor *Pseudonocardia kongjuensis* which showed highest sequence similarity. *Pseudonocardia kongjuensis* (DSM No.44525) type strain obtained from Leibniz-Institute DSMZ, Germany was used as reference strain.

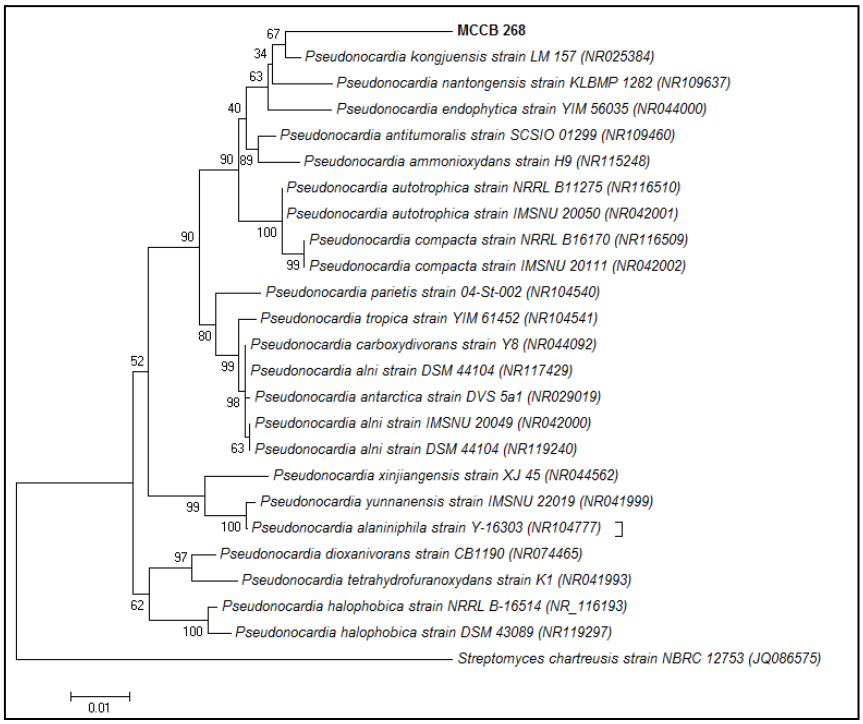


Fig. 3.11: Neighbour-Joining phylogenetic dendrogram based on 16S rRNA gene sequences, showing relationships between the isolated *Pseudonocardia* sp. MCCB 268 and related taxa

3.3.9.1 Morphological and culture characterization of *Pseudonocardia* sp. MCCB 268

Morphological apperaences of *Pseudonocardia* sp. MCCB 268 and *Pseudonocardia kongjuensis* (DSM No.44525) in NA were compared and given in Fig. 3.12. Appearance of actimomycetes hyphae and spores of *Pseudonocardia* sp. MCCB 268 in SEM imaging is given (Fig. 3.13). The isolate showed smooth rod shaped spores, which is a typical morphology of genus *Pseudonocardia.* The isolate was studied for its cultural characteristics including colour of aerial mycelia and substrate (reverse side) mycelia on seven different media at 28ºC for 14 days (Fig. 3.14). The results were compared with nearest type strain *Pseudonocardia kongjuensis*

and tabulated in Table 3.12. *Pseudonocardia* sp. MCCB 268 grows well on all media except ISP 3 and PDA. Both *Pseudonocardia* sp. MCCB 268 and *Pseudonocardia kongjuensis* did not form soluble pigment on any of the media tested.

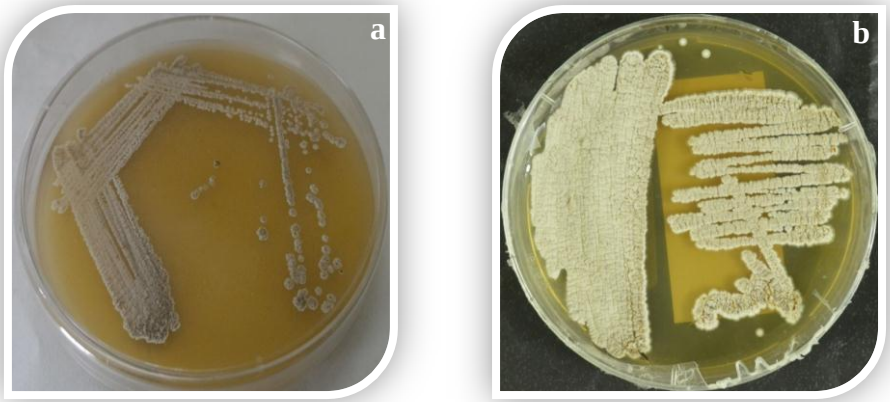


Fig. 3.12: Colony morphology of a) *Pseudonocardia sp.* MCCB 268 and b) *Pseudonocardia kongjuensis* after incubation for 14 days on Trypticase Soy Broth Agar (TSBA) at 28 ºC

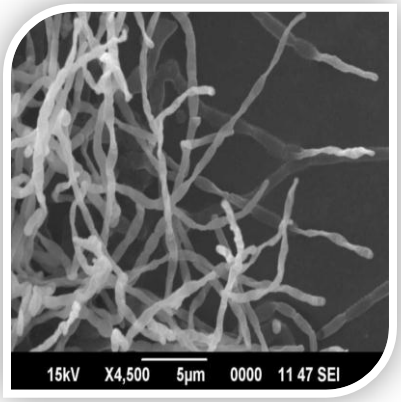


Fig. 3.13: Scanning electron micrograph of aerial mycelia and spore chain of *Pseudonocardia* sp. MCCB 268 after incubation for 14 days on Nutrient Agar at 28 ºC

Table 3.12 : Cultural characteristics *Pseudonocardia* sp. MCCB 268 various media after 2 weeks at 28º C

Agar medium	Colour of mycelium				Solub le pigment	
	Aerial mycelium		Substrate mycelium			
	***Pseudonocardia sp.* MCCB 268**	***Pseudonocardia kongjuensis*** (Min et al. 2001)	***Pseudonocardia sp.* MCCB 268**	***Pseudonocardia kongjuensis*** (Min et al. 2001)	***Pseudonocardia sp.* MCCB 268**	***Pseudonocardia kongjuensis*** (Min et al. 2001)
ISP 2	Pale greenish yellow	White	Light yellow	Yellowish white	No	No
ISP 3	White	White	Pale yellow	Pale orange yellow	No	No
ISP 4	White	White	Light yellow	White	No	No
ISP 5	White	Yellowish white	Pale yellow	Yellowish white	No	No
PDA	Light yellow	Yellowish white	Vivid yellow	Dark orange yellow	No	No
CDA	Pale yellow	White	Light yellow	Light orange yellow	No	No
NA	White	Yellowish white	Pale yellow	Light orange yellow	No	No

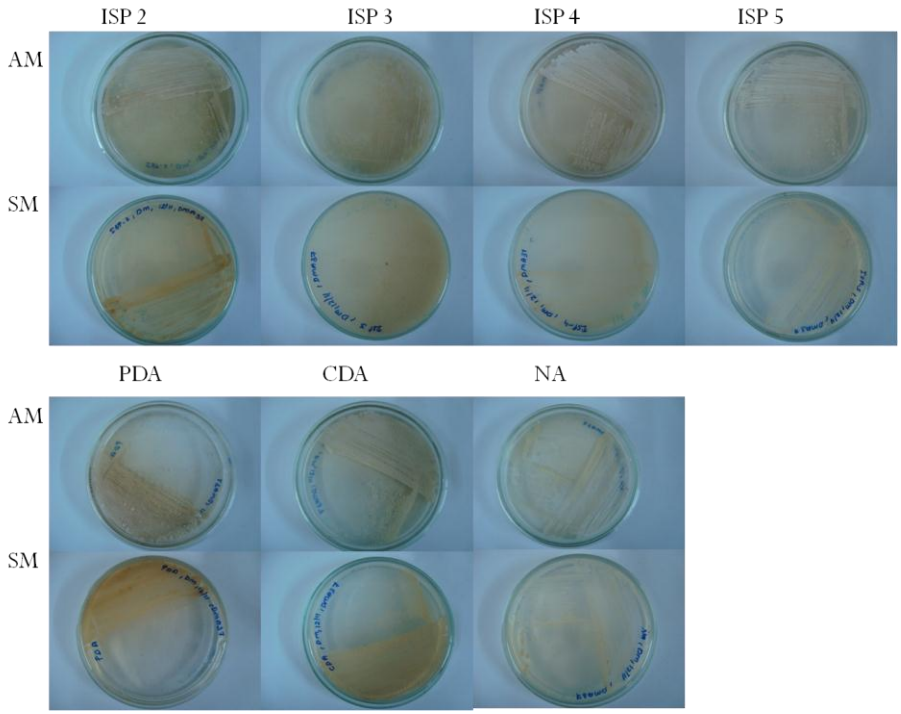


Fig. 3.14: Cultural characteristics *Pseudonocardia* sp. MCCB 268 various media after 2 weeks at 28º C

3.3.9.2 Carbon source utilization ability of *Pseudonocardia* sp. MCCB 268

Carbon source utilization ability of *Pseudonocardia* sp. MCCB 268 was observed after 10-16 days at 28ºC and shown in Table 3.13. *Pseudonocardia* sp. MCCB 268 uses Xylose, Tyrosine, Leucin, Adonitol, Cellobiose, Proline, Mannose, Fructose, Glycerol and Dextrose as sole carbon source but not Galactose, Melibiose, Inositol, Raffinose, Ribose, Salicin, Glycine, Lactose, Maltose and Lysine. *Pseudonocardia kongjuensis* was found to have the ability to utilize inositol and maltose whereas *Pseudonocardia* sp. MCCB 268 was found incapable of utilizing it.

Table 3.13: Ability of *Pseudonocardia* sp. MCCB 268 to use different carbon sources

Carbon source	*Pseudonocardia sp. MCCB 268*	*Pseudonocardia kongjuensis* (Min et al. 2001)
Xylose	+	+
Galactose	_	NA
Melibiose	_	NA
Tyrosine	+	NA
Inositol	_	+
Raffinose	_	NA
Ribose	_	NA
Leucin	+	NA
Adonitol	+	+
Salicin	_	_
Rhamnose	+	NA
Cellobiose	+	+
Proline	+	NA
Glycine	_	NA
Mannose	+	+
Lactose	_	NA
Maltose	_	+
Fructose	+	NA
Lysine	_	NA
Glycerol	+	NA
Dextrose	+	+

Table 3.14: Fatty acid profile of *Pseudonocardia* sp. MCCB 268 along with its closest phylogenetic neighbour *Pseudonocardia kongjuensis*

Fatty acids	Fatty acid profile of *Pseudonocardia* sp. MCCB 268 (%)	Fatty acid profile of *Pseudonocardia kongjuensis* (DSM No.44525) (%)
12:0 iso	-	-
12:0	-	-
13:0 iso	-	-
13:0 anteiso	-	-
14:0 iso	-	-
14:0	0.51	2.66
15:0 iso	2.85	4.37
15:0 anteiso	-	-
15:1 iso G	-	-
15:1 anteiso A	-	-
15:1 w6c	-	1.55
15:0	-	-
16:1 iso H	22.66	9.17
16:1 iso G	-	-
16:0 iso	45.64	21.7
16:1 w5c	-	-
16:0	-	15.34
17:1 anteiso w9c	-	-
17:0 iso	1.38	3.63
17:0 anteiso	2.45	2.93
17:1 w8c	1.89	5.05
17:0 cyclo	-	-
17:0	-	-
17:0 10-methyl	2.56	6.79
18:1 w9c	0.32	-
18:0	0.72	11.10

3.3.9.3 Fatty acid profile of *Pseudonocardia* sp. MCCB 268

Fatty acid profile of *Pseudonocardia* sp. MCCB 268 along with its closest phylogenetic neighbour *Pseudonocardia kongjuensis* is given in Table 3.14. The major fatty acid components of MCCB 268 were $C_{16:0}$ iso (45.64%) and $C_{16:1}$ iso H (22.66%). In case *Pseudonocardia kongjuensis* , major fatty acid components were $C_{16:0}$ iso (21.7%), $C_{16:0}$ (15.34%), $C_{16:1}$ iso H (9.1%), $C_{17:1}$ w8c (5.05%), $C_{17:0}$ 10-methyl (6.79%) and $C_{18:0}$(11.10%). Fatty acids $C_{16:0}$ and $C_{15:1}$ w6c were present only in *Pseudonocardia konjuensis* but not in *Pseudonocardia* sp. MCCB 268 .

3.3.9.4 Analysis G+C mol% content of *Pseudonocardia* sp. MCCB 268

Tm values after melt curve analysis and G+C mol % values of *Pseudonocardia* sp. MCCB 268 are given in Table 3.5. After melt curve analysis, *Pseudonocardia sp.* MCCB 268 was found to have Tm value of 89.59 °C and G+C content of 70.5%.

3.3.9.5 DNA-DNA hybridization studies of *Pseudonocardia* sp. MCCB 268 and *Pseudonocardia kongjuensis*

For hybridization study, uniformly fragmented DNA segments (optimally about 400-500bp) were necessary. Different sonication conditions were tested to get the DNA fragments of desired size. DNA sonication in 2X SSC for 7 pulses (2 s) at an out an output of 50% gave DNA fragments of 400-1500bp (Fig. 3.15). After DNA-DNA hybridization, fluorescence measurements recorded throughout the renaturation step and plotted against time in minutes (Fig. 3.16-3.18). The straightest portions of the curves, with the highest correlation coefficients, were used for the calculations. The slopes of the linear regression lines, representing the

renaturation rates (increase in fluorescence per minute) V', were used to determine the degree of binding (%) using the equation of (De et al. 1970). DNA–DNA hybridization analysis showed that the isolate *Pseudonocardia sp*. MCCB 268 exhibited low DNA–DNA relatedness of 41.5% with its closest phylogenetic neighbour *Pseudonocardia kongjuensis.*

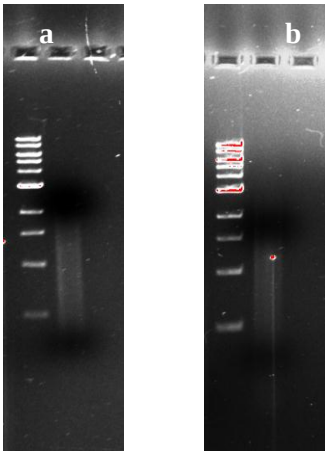


Fig. 3.15 : DNA fragments of 400-1500bp size of a) *Pseudonocardia* sp. MCCB 268 and b) *Pseudonocardia kongjuensis*

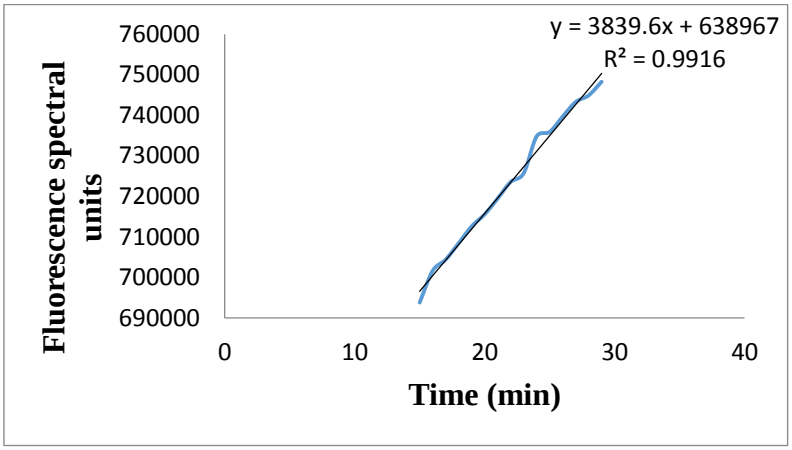


Fig. 3.16 : Fluorescence measurements Vs Time in minutes graph of *Pseudonocardia kongjuensis*

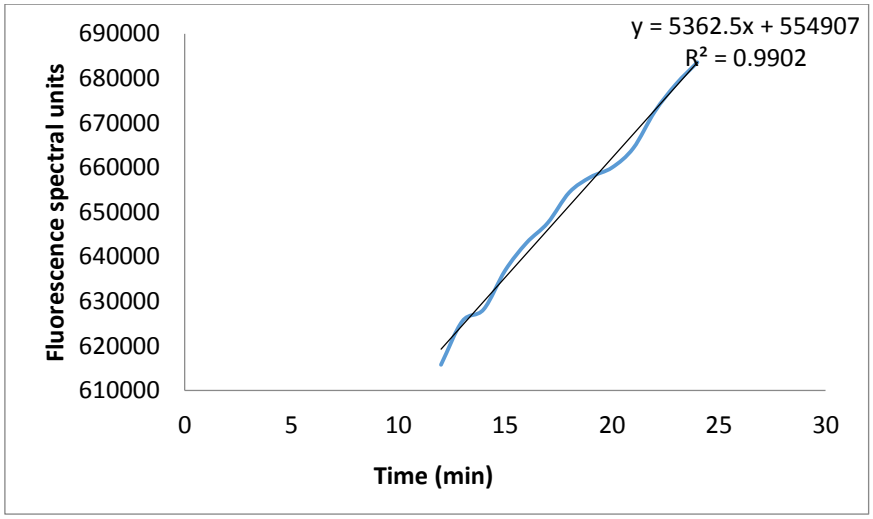


Fig. 3.17 : Fluorescence measurements Vs Time in minutes graph of *Pseudonocardia* sp. MCCB 268

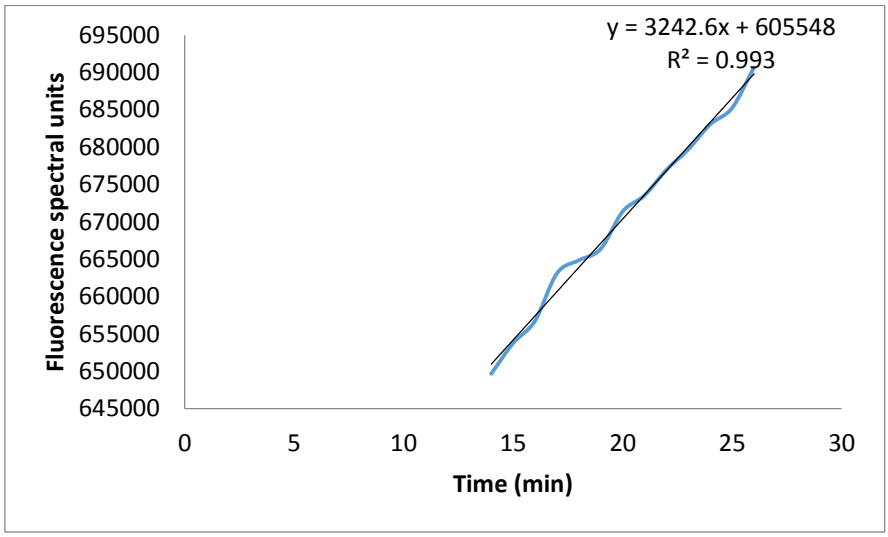


Fig. 3.18 : Fluorescence measurements Vs Time in minutes graph of *Pseudonocardia kongjuensis* and *Pseudonocardia* sp. MCCB 268 mixture

3.3.9.6 Effect of pH, temperature and salinity on the growth of *Pseudonocardia* sp. MCCB 268

The growth of *Pseudonocardia* MCCB268 was evaluated at 9 different pH conditions ranging from pH 4 to 12. The isolate showed growth at pH 6-8 with optimal growth at pH 7 (Fig. 3.19). Out of 7 different temperatures (4, 10, 20, 28, 37, 45 and 55ºC) tested the isolate showed optimal growth at 28ºC, however it did not grow at 4ºC and 55ºC (Table 3.15). The isolate showed growth at wide range of salinity (0-40ppt) (Fig. 3.20) with optimal growth at 30ppt.

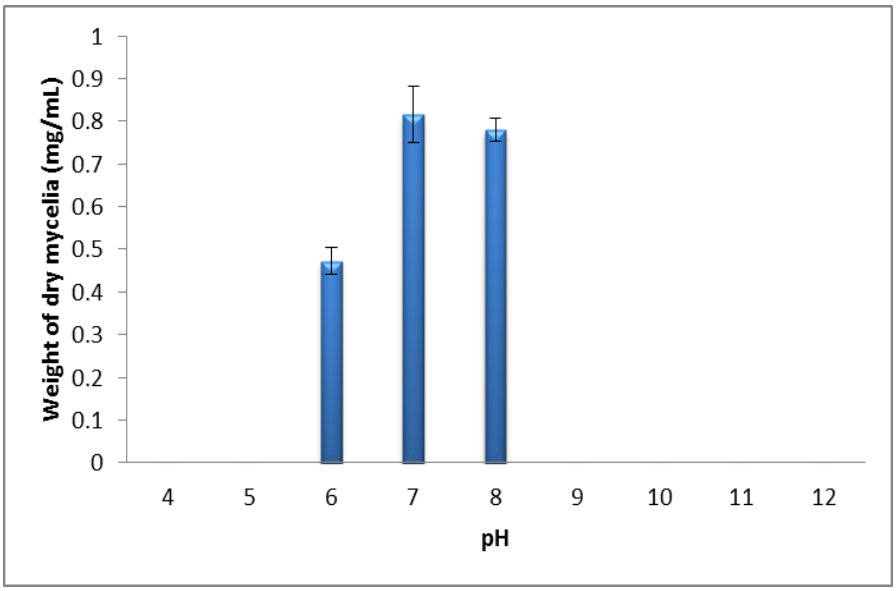


Fig. 3.19: Effect of pH on growth of *Pseudonocardia* sp. MCCB 268

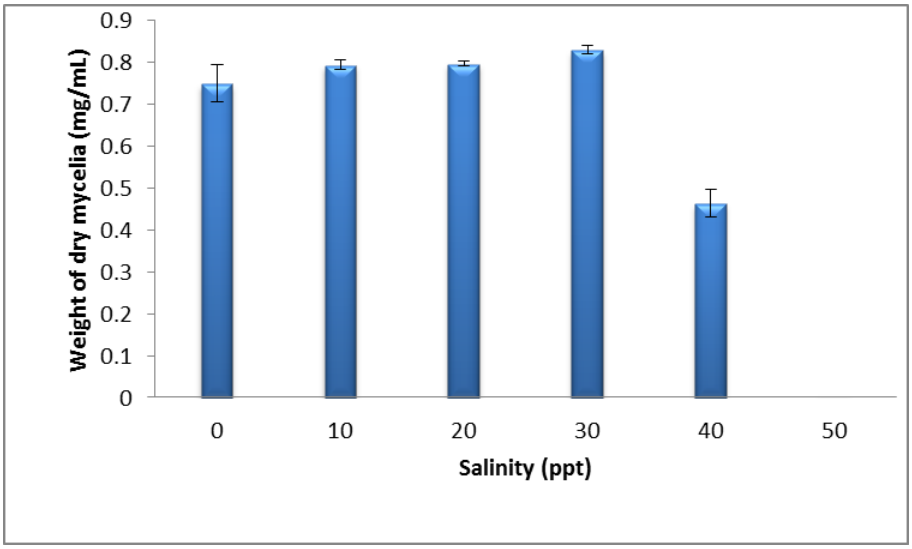


Fig. 3.20: Effect of Salinity on growth of *Pseudonocardia* sp. MCCB 268

Table 3.15: Effect of temperature on growth of *Pseudonocardia* MCCB 268

Sl. No.	Temperature (°C)	Observation
1	4	No growth
2	10	Poor growth
3	20	Good growth
4	28	Very good growth
5	37	Good growth
6	45	Good growth
7	55	No growth

3.3.10 Oxidase and catalase activity of isolates

Pseudonocardia sp. MCCB 268 showed positive reactions for both oxidase and catalase.

3.4 Discussion

Actinobacteria is one of the dominant bacterial phyla which comprise one of the largest bacterial genera, *Streptomyces*. Waksman and Henrici (1943) initially introduced genus *Streptomyces* as aerobic, spore forming actinomycetes. They described *Streptomyces* as Gram-positive bacteria with high DNA G+C content with production of extensively branched substrate and aerial mycelia, and the presence of LL-diaminopimelic acid and absence of characteristic sugars in the cell wall (Anderson and Wellington 2001). They are the most abundant actinobacteria and well known for their ability to produce bioactive metabolites including antibiotics, enzyme inhibitors, antitumour agents and antifungal compounds (Lucas et al. 2013). In the current study, of the four potent isolates of actinomycetes were isolated identified and characterized with anticancer activity, three of them belong to *Streptomyces* genus (*Streptomyces* sp. MCCB 267, *Streptomyces* sp. MCCB246, and *Streptomyces* sp. MCCB 248) and one belongs to a rare actinomycete genus *Pseudonocardia* (*Pseudonocardia* sp. *MCCB 268*).

Streptomyces sp. MCCB 267 was isolated from a marine sponge *Mycale sp* collected from Rameswaram, South East coast of India Ocean. After BLAST and phylogenetic analysis, the isolate was found to have more sequence similarities with *Streptomyces zhaozhouensis* NEAU-LZS-5, an endophytic actinomycete isolated from the leaf of *Candelabra aloe* (He et al. 2014). Detailed morphological studies using slide culturing and observation under scanning electron microscopy revealed the presence of

simple, smooth, spiral spore chains of the *Streptomyces* sp. MCCB 267. However, the type strain *Streptomyces zhaozhouensis* was reported for its smooth single elliptical spores on aerial mycelium (He et al. 2014). The strain MCCB267 showed growth on Xylose, Galactose, Melibiose, Raffinose, Ribose, Salicin, Rhamnose, Cellobiose, Proline, Maltose, Fructose, Glycerol and Dextrose as sole carbon source. *Streptomyces sp.* MCCB 267 was unable to utilize Tyrosine, Inositol, Leucin, Adonitol, Glycine, Mannose, Lactose, and Lysine as sole carbon source. In this study, the major fatty acids found in the cell wall of *Streptomyces* sp. MCCB 267 were $C_{16:0}$ iso, $C_{16:1}$ iso G, $C_{17:0}$ anteiso, $C_{15:0}$ anteiso and $C_{15:0}$ iso, whereas the major fatty acids reported from *Streptomyces zhaozhouensis* were $C_{16:0}$, $C_{17:1}$ w7C, $C_{15:0}$, iso-$C_{15:0}$, $C_{18:0}$, $C_{16:1}$ w7c and anteiso-$C_{17:0}$ (He et al. 2014) . The G+C content of the isolate was found to be 67.7 mol %, which was similar to *Streptomyces zhaozhouensis.* Even though *Streptomyces sp*. MCCB 267 showed close resemblance to its nearest neighbour *Streptomyces zhaozhuensis*, it showed distinct physiological and biochemical characteristic features especially the spore structure, carbon utilization and cellular lipid profile suggesting that it might be a previously un-described subspecies of *Streptomyces zhaozhuensis* and hereby designated it as *Streptomyces zhaozhuensis* subsp. *mycale*. subsp. nov. Studies have indicated that marine sponges are rich sources of different marine actinomycetes. In recent years, the role of associated microorganism in the production of natural products, originally reported from marine invertebrates, is increasingly recognized. Of 20 compounds derived from marine natural products that are currently in clinical trials, 15 were isolated from marine invertebrates, specifically from sponges, tunicates, or mollusks, and only the remaining 5 were isolated from microorganism. However,

further studies including genome mining and subsequent direct isolation of the compound from a producing microorganism revealed that, 16 of these molecules are actually derive from microbial sources and only 4 are derive from macroorganisms. For example, dolastatin 10 originally isolated from the marine gastropod *Dolabella auricularia*, was later shown to be produced by a cyanobacteria *Symploca* and *Lyngbya* upon which they feed (Luesch et al. 2001).

A study of Abdelmohsen et al. (2010) reported that 90 actinomycetes isolates belong to 18 different genera from 11 different species of marine sponges collected from offshore Ras Mohamed (Egypt) and from Rovinj (Croatia). Among them 14 isolates were putatively novel species. Gandhimathi et al. (2008) reported that sponge *Callyspongia diffusa* contains 38.46% actinomycetes among the total culturable microbial symbionts associated with the sponge. These actinomycetes exhibited good antimicrobial activity against the growth of human pathogens.

Streptomyces sp. MCCB246 is a gram positive marine actinomycte isolated from marine sediment from South-West coast of Indian Ocean. After molecular identification the isolate was named as *Streptomyces* sp. MCCB246 and showed more similarity with *Streptomyces wuyuanensis* FX61 (Zhang et al. 2013). *Streptomyces wuyuanensis* is not yet reported for any bioactive compound prodution till date. Analysis of carbon source utilisation pattern of type strain *Streptomyces wuuyuanensis* revealed that it has the ability to utilise galactose, whereas *Streptomyces* sp. MCCB246 is unable to utilize galactose . The major fatty acids present in the isolate MCCB246 were $C_{16:0}$ iso, $C_{15:0}$ anteiso, $C_{15:0}$ iso, $C_{17:0}$ anteiso, $C_{16:0}$ and $C_{14:0}$ iso. The phylogenetically related type strain *Streptomyces*

wuuyuanensis FX61 also showed similar fatty acid as major fatty acids. The isolate MCCB246 was found to contained DNA G+C content of 65% and the type strain *Streptomyces wuuyuanensis* FX61 showed a G+C content of 72 mol%. Even though, *Streptomyces* sp. MCCB246 showed 99% sequence similarity with *Streptomyces wuuyuanensis* FX61 its detailed morphological, physiological and some chemotaxomic characteristics are different from the type strain *Streptomyces wuuyuanensis* FX61 and suggests that it might be a previously un-described subspecies of *Streptomyces wuyuanensis.*

After BLAST analysis and phylogenetic analysis of the isolate MCCB 248 isolated from the Arctic fjord Kongsfjorden showed highest sequence similarity (99%) to the type strain *Streptomyces artemisiae* YIM 63135. The latter strain was isolated from surface-sterilized tissue of *Artemisia annua* L., collected in Yunnan Province, southwest China,and described by (Li et al. 2010). The isolate *Streptomyces* sp. MCCB 248 is an aerobic gram positive actinomycete which forms extensively branched aerial and substrate mycelium that produces smooth surface spores with rod shape similar to type strain. *Streptomyces* sp. MCCB 248 doesn't produce any diffusable pigments in any of the medium, whereas nearest strain *Streptomyces artemisiae* produces soluble pigments in ISP 2 and ISP 3. Only difference found in carbon utilization patteren *Streptomyces* sp. MCCB 248 and *Streptomyces artemisiae* is that *Streptomyces* sp. MCCB 248 is capable of utilizing inositol but not rhamnose, where as type strain *Streptomyces artemisiae* has the abiliy to utilize rhamnose but not inositol. The major fatty acids profile of *Streptomyces* sp. MCCB 248 is very similar to that of type strain of *Streptomyces artemisiae.* The G+C content of isolate is found to be 67 mol%, whereas *Streptomyces artemisiae* has G+C content of 72.6

mol% (Zhao et al. 2010). Detailed morphological, physiological and chemotaxomic characterisation studies indicate that *Streptomyces* sp. MCCB 248 might be a previously un-described subspecies of *Streptomyces artemisiae.*

Pseudonocardia sp. MCCB 268 was isolated from the Arctic fjord Kongsfjorden marine sediment. The genus *Pseudonocardia* is mycolateless nocardioform actinomycetes, originally proposed by Henssen in 1957. Until now, the genus *Pseudonocardia* contains only 55 species (http://www.bacterio.net/pseudonocardia.html). After the BLAST analysis of 16S rRNA gene and further phylogenetic analysis, isolate MCCB 268 showed 98% sequence similarity and it formed a separate branch with the type strain *Pseudonocardia kongjuensis* in the phylogenetic tree (Min et al. 2001). Because of low sequence similarity with other related strains, *Pseudonocardia* sp. MCCB 268 was selected for further detailed identification studies by comparing with its nearest neighbour *Pseudonocardia kongjuensis. Pseudonocardia* sp. MCCB 268 is a gram-positive actinomycete which produce smooth surfaced rod shaped spores, typical morphological feature of *Pseudonocardia* genus. *Pseudonocardia* sp. MCCB 268 utilizes Xylose, Tyrosine, Leucin, Adonitol, Cellobiose, Proline, Mannose, Fructose, Glycerol and Dextrose as sole carbon source but not Galactose, Melibiose, Inositol, Raffinose, Ribose, Salicin, Glycine, Lactose, Maltose or Lysine, whereas the type strain *Pseudonocardia konjuensis* utilizes Galactose, Inositol and Maltose (Min et al. 2001). The isolates also differ in terms of their major fatty acid profile. Apart from the common fatty acids the type strain *Pseudonocardia konjuensis* contained $C_{15:1}$ w6c, and $C_{16:0}$ (15.34%). The isolate MCCB 268 was having a G+C content of 70.5 mol%, whereas *Pseudonocardia konjuensis* showed a G+C

content of 71 mol%. DNA-DNA hybridization studies of *Pseudonocardia* sp. MCCB 268 and *Pseudonocardia kongjuensis* showed only low DNA–DNA relatedness of 41.5 %. This value is well below the cut off value of 70%, which is reported for the delineation of genomic species (Klappenbach et al. 2007). Reports indicate that even though many species of the genus share 98.5–99.6% 16S rRNA gene sequence similarity, they show DNA-DNA hybridization values well below the 70% cut-off point. This observation clearly indicates that *Pseudonocardia* sp. MCCB 268 represents a novel species.

There are only very few reports of bioactive compounds producing species of *Pseudonocardia* species from marine environment. Maskey et al. (2003) reported a *Pseudonocardia* sp. B6273, which produces new phenazine derivative, phenazostatin D. Another deep sea actinomycete species *Pseudonocardia antitumoralis* (Tian et al. 2013), produces three new diazaanthraquinone derivatives Pseudonocardians A–C, along with previously reported compound deoxynyboquinone. These compounds showed potent cytotoxic activities against three tumor cell lines of SF-268, MCF-7 and NCI-H460(Li et al. 2011). Five curvularin macrolides, which suppress the proliferation of cancer cell lines, were isolated from marine actinomycete *Pseudonocardia* sp. HS7 (Ye et al. 2016).

Arctic environment is considered as one of the least explored ecosystems for bioactive compounds. It acts as a source of lots of novel microorganisms. *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* sp. nov., polar marine bacteria were isolated from sea ice and water from the Arctic and the Antarctic by Gosink et al. (1998). Knoblauch et al. (1999) reported five

novel psychrophilic Gram-negative, sulfate-reducing bacteria isolated from marine sediments off the coast of Svalbard Arctic.

In the present work four actinomycetes from marine environment with potential anticancer activity were identified, segregated and characterized in detailed. Among these, two actinomycetes were from Arctic marine sediment and they include a novel isolate belongs to *Pseudonocardiaceae* family *Pseudonocardia* sp. MCCB 268 and *Streptomyces* sp. MCCB 248. An endosymbiotic actinomycetes *Streptomyces* sp. MCCB 267 from a marine sponge *Mycale* sp. and *Streptomyces* sp. MCCB 246 isolated from the sediment samples collected from the south-west coast of India were other promising isolates and they showed disticnt morphological and biochemical charactertics with its close type strain and could be considered as a potential isolates for detailed study. It can be concluded with the observation that marine environment continues to be a rich source of potential novel actinomycetes, which could be explored for different bioactive compounds with biomedical applications.

Chapter 4

CONFIRMATION OF ANTICANCER ACTIVITY, CHEMICAL DEREPLICATION AND SCREENING FOR BIOSYNTHETIC GENES ENCODING SECONDARY METABOLITES IN ACTINOMYCETES

Contents

4.1 Introduction

This chapter deals with three important aspects, with respect to anticancer potential of the actinomycetes isolates such as (i) confirmation of anticancer activity of selected actinomycetes (ii) Chemical de-replication of crude actinomycetes extract, and (iii) screening for biosynthetic gene(s) involved in secondary metabolite production in selected actinomycetes.

4.1.1 Evaluation of anticancer activity

Isolation and characterisation of anticancer lead compound from a natural source are labour intensive and time consuming effort. Prioritizing and selecting the right starting materials are very curial for any successful bioprospecting work. Hence the best practical approach for *selection* of

isolates for bioprospecting work is confirming the biological activity and possible elimination of false positive or artefacts. Usual procedure for confirmation of activity includes the evaluation of anticancer activity of crude extract using MTT, XTT or SRB assay along with a standard anticancer drug as a positive control on cancer cell line. As per US National Cancer Institute (NCI) recommendations SRB assay is the recommended assay for evaluating preliminary anticancer activity of both natural products and natural product extracts (Boyd 1997). This is followed by calculating one or several cytotoxicity parameters (e.g., IC_{50}, IC_{90} and LC_{50}) for crude extract/compound after estimating cell viability with a cytotoxicity test. In order to evaluate the mode of cytotoxicity various cell based assays such as Hoechst 33342 staining, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick and Annexin V – Propidium iodide double staining etc could be carried out.

4.1.2 Marine natural product de-replication

The rapid identification of already known bioactive metabolites or compounds by comparison of experimental spectral data to databases is referred to as de-replication. It helps to eliminate already known active compounds in the initial screening process of active crude extracts or fractions. During the last decade, significant technological advances made in analytical instrumentation, besides the development of suitable databases, have enabled the development of rapid de-replication processes. The techniques such as ultrahigh performance liquid chromatography (UHPLC) and benchtop high-resolution mass spectrometry (HRMS) detectors help to get the detailed information on the chemical composition of crude extracts. The routine analysis of samples generates lots of data, which is too large for

manual analysis. Although, several natural product (NP) databases such as Dictionary of Natural Products, MarinLit and AntiBase help in de-replication (identification of known compounds), these resources have certain limitations including high recurring cost of data updation and their inherent inability to process the mass spectral (MS) data. On the other hand, MS databases such as Metlin (https://metlin.scripps.edu/), mzCloud (https://www.mzcloud.org/), MassBank *(www.massbank.jp/?lang=en)*, and ReSpect (http://spectra.psc.riken.jp/), can host and process MS/MS spectra, these databases limits analysis of data to few individual spectra or a less amount of liquid chromatography (LC)–MS files (Wang et al. 2016). Though Metlin and mzCloud are open source databases available online, their reference libraries are not available free of cost.

4.1.2.1 De-replication using Global Natural Products Social (GNPS) molecular networking

The field of natural products research is undergoing renaissance through the utilization and development of new tools and techniques such as genome mining, compound activity mapping, and high-content biological screening along with integrated bioinformatics, as well as molecular networking, principal component analysis, and other cheminformatics approaches (Yang et al. 2013).

Among these different new approaches, metabolite de-replication based on the mass spectral data through molecular networking (MN) is the most effective way to organize MS/MS fragmentation spectra (Allard et al. 2016). Molecular network (MN) is the visual display of chemical space present in mass spectroscopic experiment with the underlying concept that

structurally related molecules will fragment in similar ways to give analogous patterns (Naman et al. 2017).

This Global Natural Products Social Molecular Networking (GNPS; http://gnps.ucsd.edu) can be used for developing the molecular network. GNPS is an open-access knowledge base for community-wide organization and sharing of raw, processed or identified tandem mass (MS/MS) spectrometry data (Wang et al. 2016). Through this researchers can include their newly described and structurally annotated data sets into a publicly available database, thereby enabling future compound de-replication against authentic, structurally annotated MS/MS spectra. It will form spectral network with related molecules or even when the spectra themselves are not matched to any known compounds. GNPS is the only public database that enables molecular networking at present. The GNPS analysis infrastructure further enables rapid online de-replication, variable de-replication (approximate matches to spectra of related molecules), and identification of spectra in molecular networks. In the first stage of de-replication, querying of newly acquired MS/MS spectra against all the accumulated reference spectra in spectral libraries of GNPS will help high-throughput de-replication of natural product research. In the second stage of de-replication, GNPS uses variable de-replication, which enables detection of significant matches to either putative analogues of known compounds or compounds belonging to the same general class of molecules. Variable de-replication is not available in any of the other de-replication platforms (Yang et al. 2013; Wang et al. 2016).

4.1.3 Biosynthetic genes for secondary metabolite production

Actinomycetes are considered as one of the rich producers of metabolites with biological activity and many of these are described as being produced by the action of many biosynthetic genes in secondary metabolites production (Ayuso et al. 2005). There are a number of examples where PCR based screening for genes associated with secondary metabolism are used to evaluate the biosynthetic potential of actinomycetes including type I and II polyketide synthases (PKS I and II), non-ribosomal peptide synthetase (NRPS) aminodeoxyisochorismate synthase (phzE), dTDP-glucose-4, 6-dehydratase (dTGD), halogenase (Halo) and cytochrome P450 hydroxylase (CYP) genes (Metsä-Ketelä et al. 1999; Du et al. 2004; Ayuso-Sacido and Genilloud 2005; Lee et al. 2006; Hornung et al. 2007; Schneemann et al. 2011). The products of these genes are involved in biosynthetic pathway, the sequence of enzyme-catalyzed process in which substrates is converted into more complex specific products (here secondary metabolites) in living organisms.

Polyketides represent one of the most important classes of natural products and they are biosynthesized from acyl CoA precursors by polyketide synthases (PKSs). Polyketide synthases are enzymes which synthesize the backbone of these compounds by an iterative way. Later, a group of diverse tailoring enzymes is responsible for the introduction of various modifications in polyketides (Rix et al. 2002). PKSs are classified into three groups: Type I, Type II and Type III polyketide synthases.

Type I polyketide synthases are large and highly modular enzymes where, each enzymatic function required for polyketide chain elongation is represented by a distinct enzyme domain. Macrolide type polyketides are

usually synthesized by the type I polyketide synthases (Marahiel et al. 1997). In contrast to this, aromatic polyketides are mostly built by another type of polyketide synthases i.e., Type II polyketide synthases (PKS II). PKS II are aggregates of monofunctional enzymes, which include two ketosynthases (KSα, KSβ) and an acyl carrier protein (ACP). This enzyme complex is used iteratively to extend the polyketide chain (Hertweck et al. 2007). Type III PKSs are homodimeric enzymes that basically function as condensing enzymes. Type I and II PKSs use acyl carrier protein (ACP) to activate the acyl CoA substrates and to channel the growing polyketide intermediates, whereas type III PKSs, act directly on the acyl CoA substrates and are independent of ACP (Moore and Hopke 2001). In spite of structural and mechanistic differences, all polyketide synthases biosynthesize polyketides by step by step decarboxylative condensation of the acyl CoA precursors. The ketoacyl synthase (KS) domain (for type I PKSs) or subunit (for type II and III PKSs) in the PKSs catalyzes the C–C bond-forming step in biosynthesis of polyketides.

Non-ribosomal peptide synthetases (NRPS) are group of enzymes which produce the class of peptide secondary metabolites of non ribosomal origin (independent of messenger RNA) found in microorganisms like bacteria and fungi. Like PKSs, structurally NRPS are multifunctional polypeptides encoded by a variable number of modules with multiple enzymatic activities. NRPS modules contain the domains corresponding to the condensation, adenylation, and thiolation steps involved in the recognition and condensation of the substrate (Ayuso-Sacido and Genilloud 2005). Non ribosomal peptides are found in higher organisms such as nudibranchs, but they are thought to be produced by associated microorganisms. Non ribosomal peptides frequently have cyclic and/or

branched structures, and they can contain non-proteinogenic amino acids including D-amino acids. They may be synthesized by one or more specialized nonribosomal peptide-synthetase (NRPS) enzymes. Genes responsible of them are usually organized in one operon in bacteria. Due to the similarity with polyketide synthases (PKS), many secondary metabolites are, in fact, fusions of NRPs and polyketides.

So far the main targets in the detection of secondary metabolite pathways were genes encoding for polyketide synthases (PKSs) or non-ribosomal peptide synthetases (NRPSs). Apart from these polyketides and non-ribosomal peptides, another interesting compound class is phenazines (Schneemann et al. 2011). Phenazines are heterocyclic, nitrogenous compounds that are substituted at different sites of the core ring system and as a result, display a wide range of structural derivatives and biological activities. Use of appropriate oligonucleotide primers will help to investigate the diversity of phenazine producing bacteria.

The polyene antibiotics are a group of compounds comprising a family of type I polyketide macrolide ring system with 20–40 carbon backbones containing 3–8 conjugated double bonds (Brautaset et al. 2002). In polyene antibiotics, cytochrome P450 hydroxylases (CYPs) gene performs the hydroxylation as post-PKS modification, after the formation of the polyketide backbone (Munro and Lindsay 1996). Genes encoding polyene specific CYP with highly conserved regions are present in all polyene gene clusters. Hence polyene-specific PCR screening approach will help isolation and identification of the cryptic polyene gene cluster (Lee et al. 2006).

In this chapter, anticancer activities of selected actinomycetes were confirmed through specific cell based assays and the biosynthetic potentials for the production of secondary metabolites were evaluated through PCR based screening. In addition to these de-replication was attempted using Global Natural Products Social Molecular Networking to analyze possible metabolites in the selected isolates.

4.2 Materials and Methods

4.2.1 Confirmation of *in vitro* anticancer activity of crude extracts of selected actinomycetes

Specific *in vitro* cell based assays were performed to establish the potential anticancer nature of the crude extracts as cytotoxicity can also be due to necrosis caused by the constituents in the extract, it is imperative to prove that the mode of cell lysis is not due to necrosis.

4.2.1.1 Determination of IC_{50} of crude extracts

Evaluation of *in vitro* anticancer activity of crude extracts were performed using Sulforhodamine B (SRB) colorimetric assay on 96-well culture plates (Skehan et al. 1990) as discussed in chapter 2 (2.2.5). The IC_{50} values of the extracts on NCI-H460 human lung cancer cells as well as on a normal diploid cell lines derived from epithelial kidney cells of African green monkey (BS-C-1) were determined. Both cell lines procured from NCCS, Pune and maintained at NCAAH on RPMI-1640 (Himedia, India) supplemented with 10% fetal bovine serum (FBS) (Himedia, India) were used for the assay. Briefly, two fold serial dilution of the crude extract of isolate was prepared to have concentrations ranging from 1 mg/mL to 15.625 µg/mL in 10% DMSO, and 10 µl of these were added to 190 µl of cells as described in the SRB experiment section (2.2.5). The plates were

incubated at 37 ºC in a CO_2 (5%) incubator for 48 h. After performing the SRB assay, optical density (OD) was read at 510 nm in a microplate reader (Tecan, Switzerland). Percentage of cell-growth inhibition (GI) was calculated according to the following equation (Vichai and Kirtikara 2006): The IC_{50} value was determined based on probit analysis (Brownlee et al. 1952).

4.2.1.2 Hoechst 33342 staining

To evaluate morphological changes induced by the actinomycete secondary metabolites, NCI-H460 cells were stained with Hoechst 33342 and observed for characteristic apoptotic features using a fluorescence microscope (Zhang et al. 2007). Briefly, NCI-H460 cells seeded in a chamber slide with 1.9×10^4 cells per well, were treated with the culture extract at its IC_{50} value. After 24 h of treatment, the culture medium was removed and cells were washed twice with PBS and stained with DNA specific Hoechst 33342 dye (Sigma Chemicals, USA) (2 µg/mL in PBS) for 10 min at 37 ºC. At the end of staining, cells were observed under a fluorescence microscope for apoptotic features. Doxorubicin (Sigma, USA) was used as a positive control and DMSO was used as a negative control.

4.2.1.3 Terminal Deoxynucleotidyl Transferase dUTP Nick End Labelling (TUNEL) assay

To detect cells undergoing apoptosis, *in situ* end labeling of the 3′ OH end of the DNA fragments generated during apoptosis was performed using the In Situ Cell Death Detection Kit (TUNEL) (Roche Diagnostics, Switzerland) according to the manufactures instructions. Briefly, NCI-H460 cells in their log phase were treated with the crude extract at its IC_{50} value and incubated for 24 h. Subsequently, cell culture medium was removed and

cells were washed with PBS, air dried and fixed with 4% paraformaldehyde (in PBS) for 1 h at 25 °C. Fixed cells were washed 3 times with PBS and incubated with permeabilisation solution (1% Triton-X in 1% sodium citrate) for 2 min at 4 °C. As positive control, NCI-H460 cells were fixed, permeabilised and treated with recombinant DNAase I (New England Biolabs) for 10 min to induce DNA strand nicks. Both control and treated cells were washed twice with PBS, and 50 µL of TUNEL reaction mixture was added to each well. The cells were incubated for 1 h at 37 °C in a humidified dark chamber and subsequently washed three times with PBS and observed under a fluorescence microscope.

4.2.1.4 Annexin V – Propidium iodide (PI) double staining

Externalization of membrane phosphatidylserine (PS) is an early event occurring in cells undergoing apoptosis, and can be visualized with FITC labeled Annexin V staining. Annexin V is a Ca2+ dependent phospholipid-binding protein that has high affinity for PS, and binds to cells with exposed PS. To confirm apoptosis induction by crude actinomycetes extract , Annexin-V-FLOUS/Propidium iodide (PI) staining was performed as per manufacturer's instructions (Roche Diagnostics, (Switzerland)). Briefly, cells grown in chamber slides (Millicell EZ slide, Millipore Corporation, US) were treated with the extract at its IC_{50} concentration. After the addition of the extract, cells were observed under a fluorescent microscope for apoptotic features at regular intervals of treatment (0, 6, 12 and 24 h). Cells treated with 10% DMSO were used as a negative control.

4.2.2 Chemical de-replication of crude extracts using Global Natural Products Social (GNPS) Molecular Networking

As an attempt for early de-replication, Global Natural Products Social (GNPS) Molecular Networking was performed as explained hereafter.

4.2.2.1 Sample preparation for LC-PDA-MS/MS

An aliquot (1 mg/ml) of the crude ethyl acetate extract of each of the four isolates (*Streptomyces* sp. MCCB 267, *Streptomyces* sp. MCCB246, *Pseudonocardia sp.* MCCB 268, *Streptomyces* sp. MCCB 248) was prepared for chemical analysis by filtration over GracePure C18-Max 100 mg/1 mL SPE cartridges (Grace Technologies, USA). Compounds were eluted from the column using acetonitrile and methanol to produce a final volume of 1 mL.

4.2.2.2 Acquiring MS Spectra by LC-PDA-MS/MS for molecular networking

The prepared samples were taken for nominal mass resolution LC-PDA-MS/MS analysis on a Thermo Finnigan system with a Surveyor PDA Plus Detector, Autosampler Plus, and LC Pump Plus coupled to an LCQ Advantage Plus mass spectrometer (Thermo Fisher Scientific, USA), and with a Phenomenex Kinetex (150 mm x 10 mm x 5 μm) C18 analytical column(Phenomenex, USA). Gradient solvent system used was as follows: 0.6 mL/min of 30% acetonitrile: 70% water with 0.1% v/v formic acid for 5 min, increased linearly to 99% acetonitrile in 17 min, held at 99% acetonitrile for 5 min, returned to 30% acetonitrile in 1 min. The UV-Vis spectrum from 200-600 nm and positive mode ESI mass spectrum from m/z 190-2000 were recorded, and the mass spectrometer was configured for an automated sample-dependent MS/MS scan.

4.2.2.3 Chemical dereplication efforts by LC-PDA-MS/MS and molecular networking

Data were analyzed both manually and by mass spectrometric molecular networking using the Global Natural Products Social Molecular Networking (GNPS)(Wang et al. 2016). The data were initially converted to mzXML format, and uploaded to ProteoSAFe (http://proteomics.ucsd. edu/ProteoSAFe/) using an ftp client. Molecular networks were created using the online workflow at GNPS (http://gnps.ucsd.edu). This network was generated using standard settings selected except for minimum matched fragment ions = 2, minimum cluster size = 1, and main pairs cos = 0.5. The results were visualized online as well as downloaded the completed work flow and visualized the annotations of network using Cytoscape 3.4. This was performed in duplicate using both the native library searching and analogue detection search modes.

4.2.3 Amplification of biosynthetic genes encoding for secondary metabolites

PCR based screening for polyketide synthases (PKS I & PKS II), nonribosomal peptide synthetases (NRPS), aminodeoxyisochorismate synthase (phzE), and cytochrome P450 hydroxylase (CYP) genes was performed using the degenerate primers reported previously (Izumikawa 2003; Wawrik et al. 2005; Ayuso-Sacido and Genilloud 2005; Lee et al. 2006; Schneemann et al. 2011). The details of denenerative primers were given in Table 4.1. The composition of the reaction mixture for all PCR amplifications was: 0.4 µL template, 5 µL 2X EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Japan), and 0.4 µL each of the primers (10X). PCR conditions were as described below.

PKS I (10 µL): 2 min at 95˚C, followed by 25 cycles of 40 s at 95 °C, 40 s at 62 °C and 45 s at 72 °C, followed by a 5 min extension at 72 °C.

PKS II (10 µL): 5 min at 95 °C, followed by 40 cycles of 1 min at 95 °C, 30 s at 62 °C and 1 min at 72 °C, followed by a 10 min extension at 72 °C.

NRPS (10 µL): 5 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 2 min at 59 °C and 4 min at 72 °C, followed by a 10-min extension at 72 °C (Yuan et al. 2014).

phzE (10µL): 2 min at 94 °C, followed by 36 cycles of 1 min at 94 °C, 1 min at 54.7 °C and 2 min at 72 °C, followed by a 7-min extension at 72 °C(Yuan et al. 2014)

CYP (10µL): 5 min at 96 °C, followed by 45 cycles of 1 min at 96 °C, 30 s at 60 °C and 45 s at 72 °C, followed by a 5 min extension at 72 °C (Yuan et al. 2014)(Yuan et al. 2014).

Similarity searching for the gene sequences was performed using BLASTX against the NCBI GenBank. For phylogenetic analysis, the nucleic acid sequences were translated to protein sequences using ExPASy translate tool (https://www.expasy.org) and aligned with other similar biosynthetic proteins in the NCBI database using the ClustalW program on MEGA 6 (Tamura et al. 2013). A phylogenetic tree of the corresponding biosynthetic genes was constructed by the neighbor-joining method with bootstrap values based on 1000 replications.

Table 4.1: Primers used screening of biosynthetic genes involved in secondary metabolites production

Biosynthetic gene	Primer	Length (bp)
PKS I (KSMA-F, KSMB-R)	5′-TSGCSATGGACCCSCAGCAG-3′ 5′-CCSGTSCCGTGSGCCTCSAC-3′	700
PKS II (540F, 1100R)	5′-GGITGCACSTCIGGIMTSGAC-3′ 5′-CCGATSGCICCSAGIGAGTG-3	554
NRPS (A3F, A7R)	5′-GCSTACSYSATSTACACSTCSGG-3′ 5′-SASGTCVCCSGTSCGGTAS-3′	700
*Phz*E (phzEf, phzEr)	5′-GAAGGCGCCAACTTCGTYATCAA-3′ 5′-GCCYTCGATGAAGTACTCGGTGTG-3′	450
CYP (PEH-1, PEH-2)	5′-TGGATCGGCGACGACCGSVYCGT-3′ 5′-CCGWASAGSAYSCCGTCGTACTT-3′	350

4.3 Results

4.3.1 Confirmation of *in vitro* anticancer of crude extracts

The bioactivity of selected actinomycetes extract was studied in detail to confirm their anticancer nature.

4.3.1.1 IC_{50} determination of the crude extracts of selected Actinomycetes

IC_{50} values of crude extracts of *Streptomyces* sp. MCCB 267, *Streptomyces* sp. MCCB 246, *Pseudonocardia* sp. MCCB 268 and *Streptomyces* sp. MCCB 248 were determined through probit analysis (Fig. 4.1 to 4.4). The IC_{50} values exhibited by the extracts on NCI-H460 and BS-C-1 cells were compiled and given in Table 4.2.

Table 4.2: IC_{50} values of crude extracts on NCI-H460 lung cancer cell line and BS-C-1 normal epithelial kidney cells

Sample	IC $_{50}$ (µg/mL)	
	NCI-H460	BS-C-1
MCCB 267	2.21	4.77
MCCB 246	3.50	3.70
MCCB 268	33.11	43.45
MCCB 248	8.99	8.09

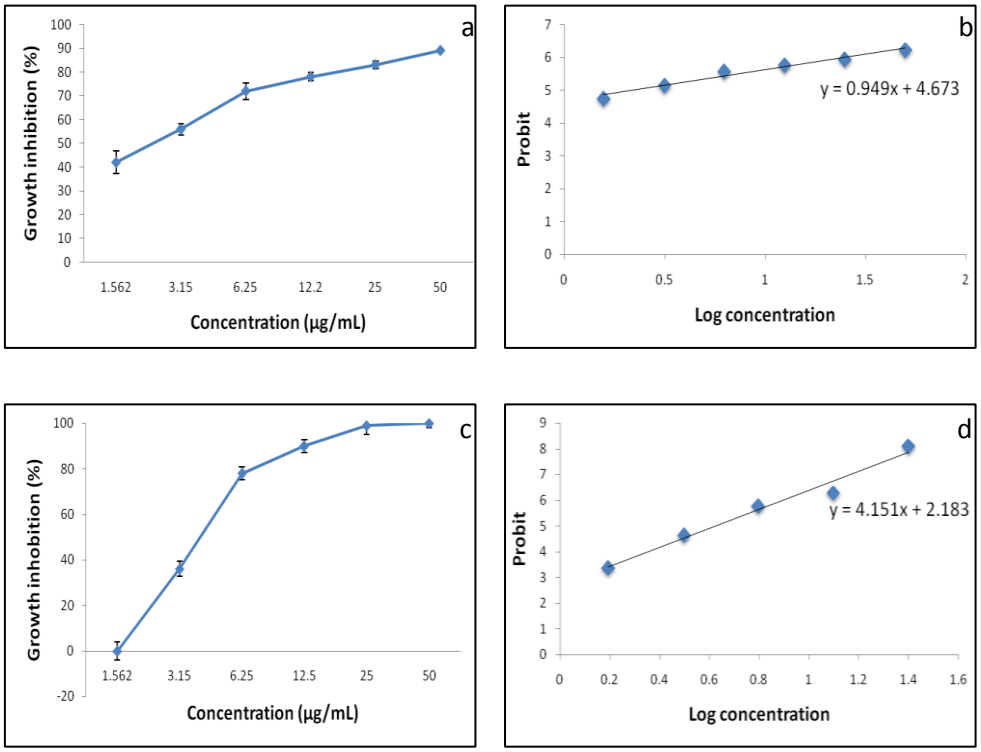


Fig. 4.1: Growth inhibitory effect and Probit analysis of *Streptomyces* sp. MCCB 267 crude extract after 48h incubation on NCI-H460 cells (a, b) and BS-C-1 cells (c, d) (Results were shown as means ± SD of at least triplicate observations)

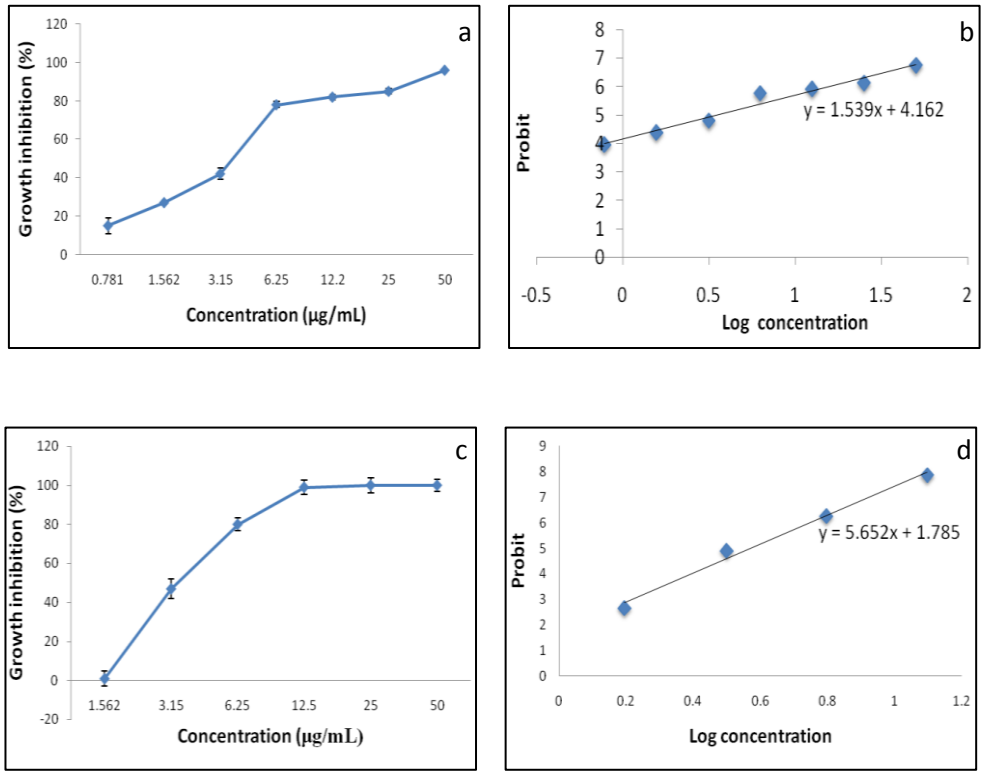


Fig. 4.2: Growth inhibitory effect and Probit analysis of *Streptomyces* sp. MCCB 246 crude extract after 48h incubation on NCI-H460 cells (a, b) and BS-C-1 cells (c, d) (Results were shown as means ± SD of at least triplicate observations)

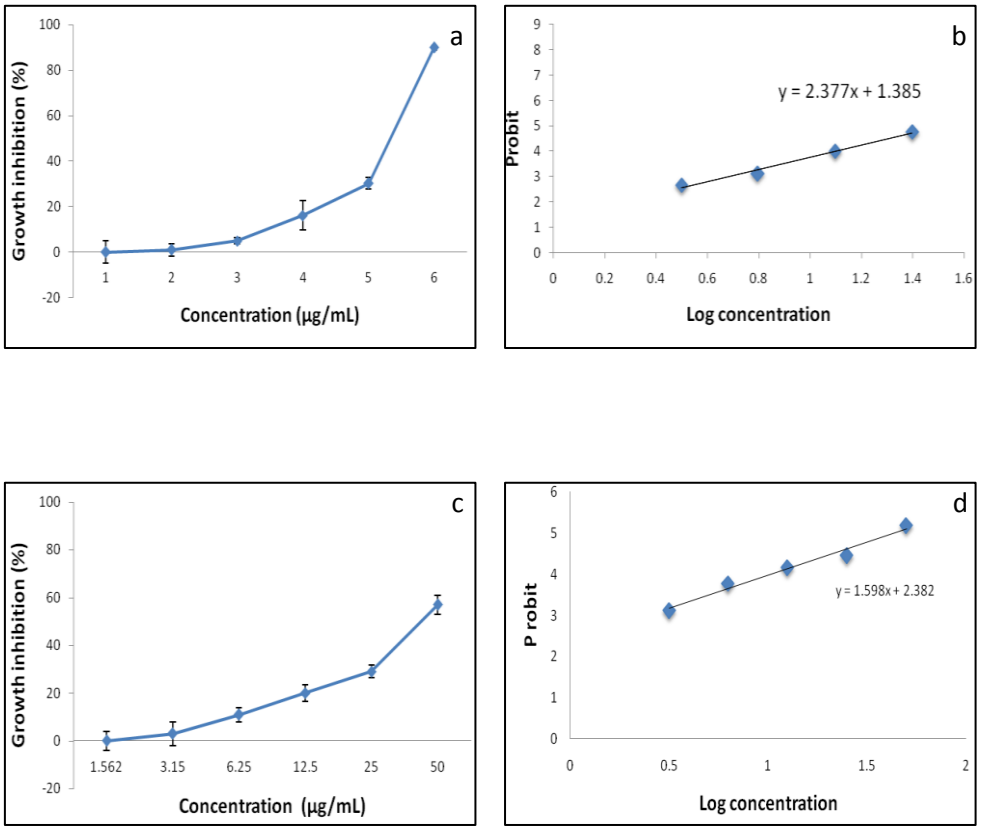


Fig. 4.3: Growth inhibitory effect and Probit analysis of *Pseudonocardia* sp. MCCB 268 crude extract after 48h incubation on NCI-H460 cells (a, b) and BS-C-1 cells (c, d) (Results were shown as means ± SD of at least triplicate observations)

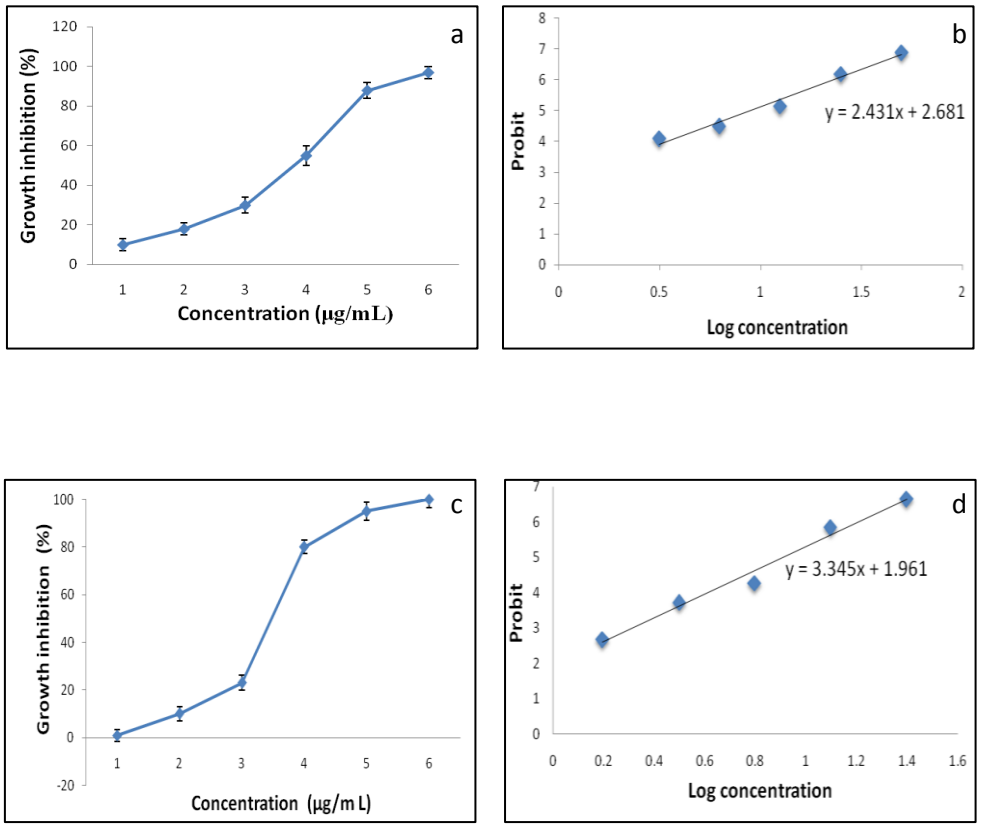
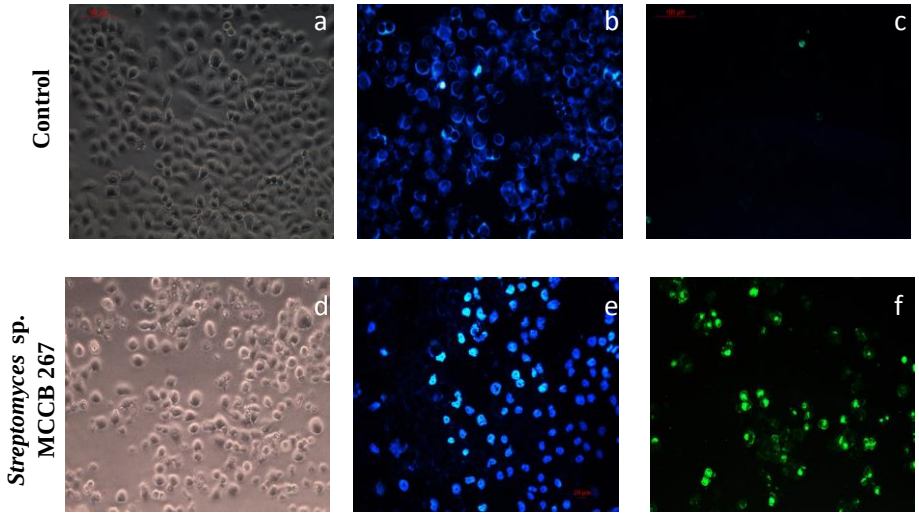


Fig. 4.4: Growth inhibitory effect and Probit analysis of *Streptomyces* sp. MCCB 246 crude extract after 48h incubation on NCI-H460 cells (a, b) and BS-C-1 cells (c, d) (Results were shown as means ± SD of at least triplicate observation)

4.3.1.2 Evaluation of morphological changes and DNA damage induced by extracts

Under phase contrast microscope, it was observed that NCI-H460 cells exposed to crude extracts from potent actinomycetes were less confluent after 24 h of incubation, and attached to the substratum with pronounced shrinkage and morphological changes (Fig.4.6 d, g, j, m). DNA damage induced by the extracts were studied by Hoechst 33342 staining and TUNEL assay (Fig. 4.6). NCI-H460 cells exposed to the active extracts exhibited a characteristic apoptotic morphology, such as shrinkage of cell nuclei, chromatin condensation and nuclear fragmentation (Fig. 4.6 e, h, k, n) as evident in Hoechst 33342 staining, which indicated that one or more components of these extracts induce apoptosis, whereas the control cells showed round and intact cellular nuclei (Fig. 4.6 b). This was further asserted using a TUNEL assay, which demonstrated condensed TUNEL positive chromatin within the cell nuclei (Fig. 4.6 f, i, l, o) when treated with the active extracts indicating DNA fragmentation, a hall mark of apoptosis.



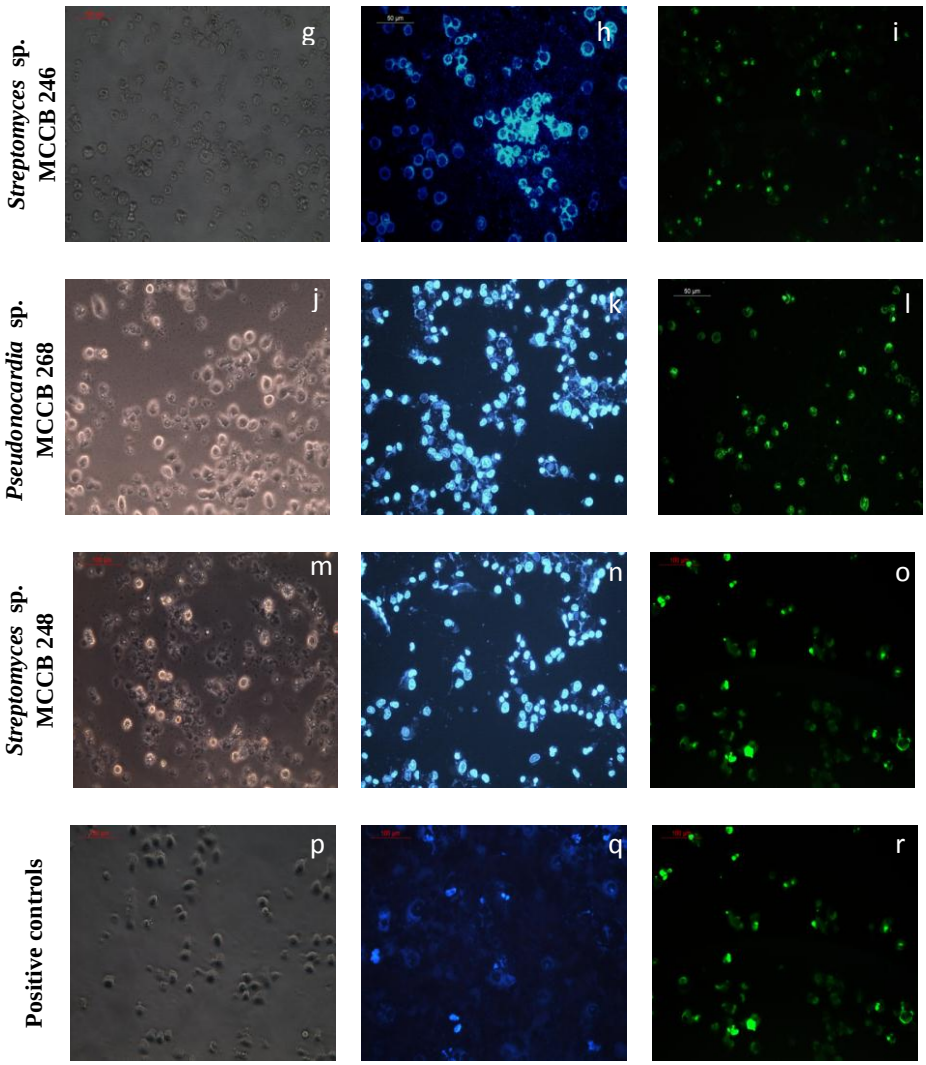


Fig. 4.6: Morphological changes and DNA damage in NCI-H460 cells treated with crude extracts. Phase contrast microscopic image, Hoechst 33342 assay and TUNEL assay control NCI-H460 (a, b, c), *Streptomyces* sp. MCCB 267 (d, e, f), *Streptomyces* sp. MCCB 246 (g, h, i), *Pseudonocardia* sp. MCCB 268, (j, k, l) *Streptomyces* sp. MCCB 248 (m, n, o) and positive controls (p, q, r)

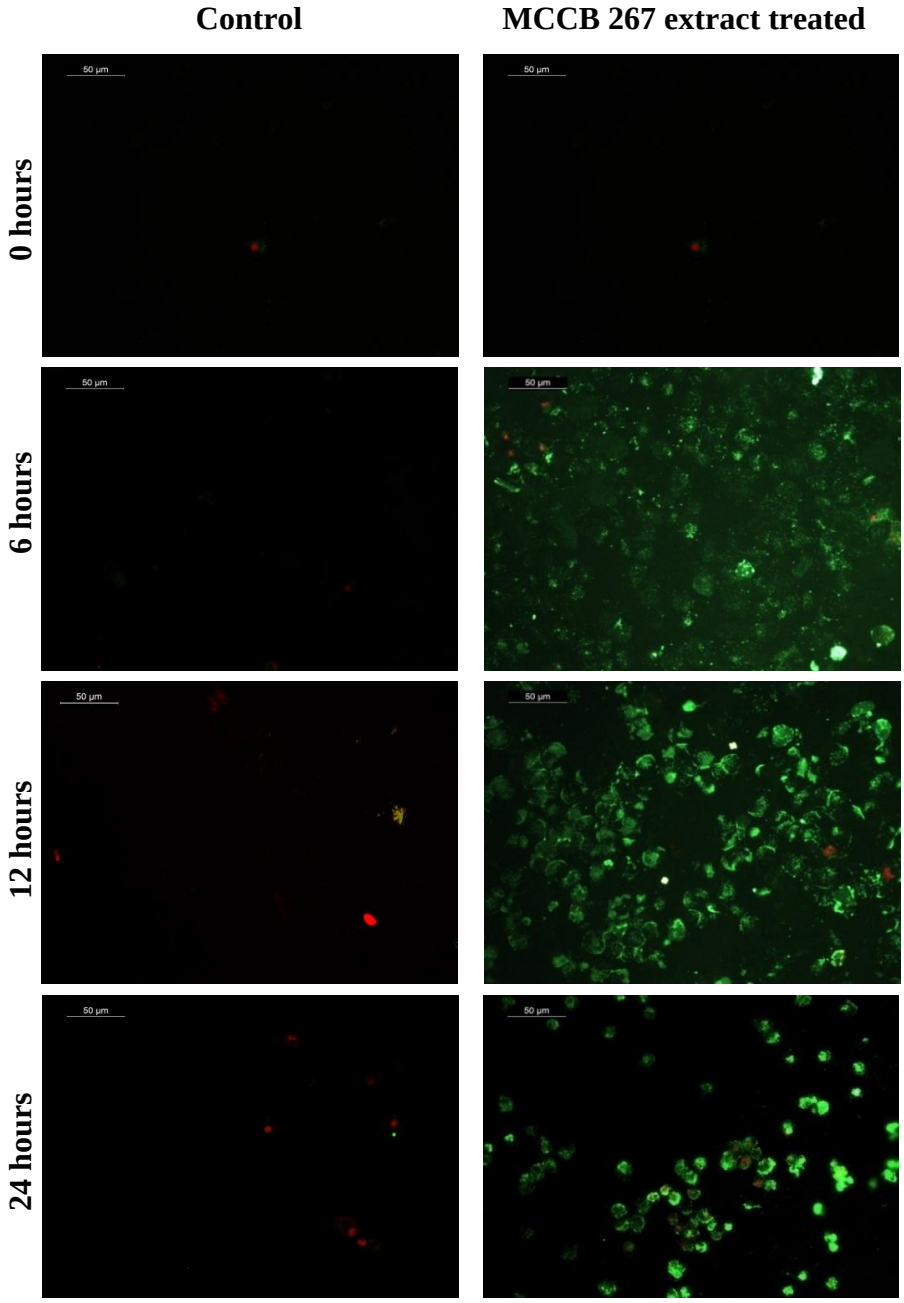


Fig. 4.7: Fluorescent microscopic images (20 X) of Annexin-V/PI double-staining assay. After treating with *Streptomyces* sp. MCCB 267 extract, cells were stained with Annexin V-FITC and propidium iodide and analyzed after 0, 6, 12 and 24 h of treatment

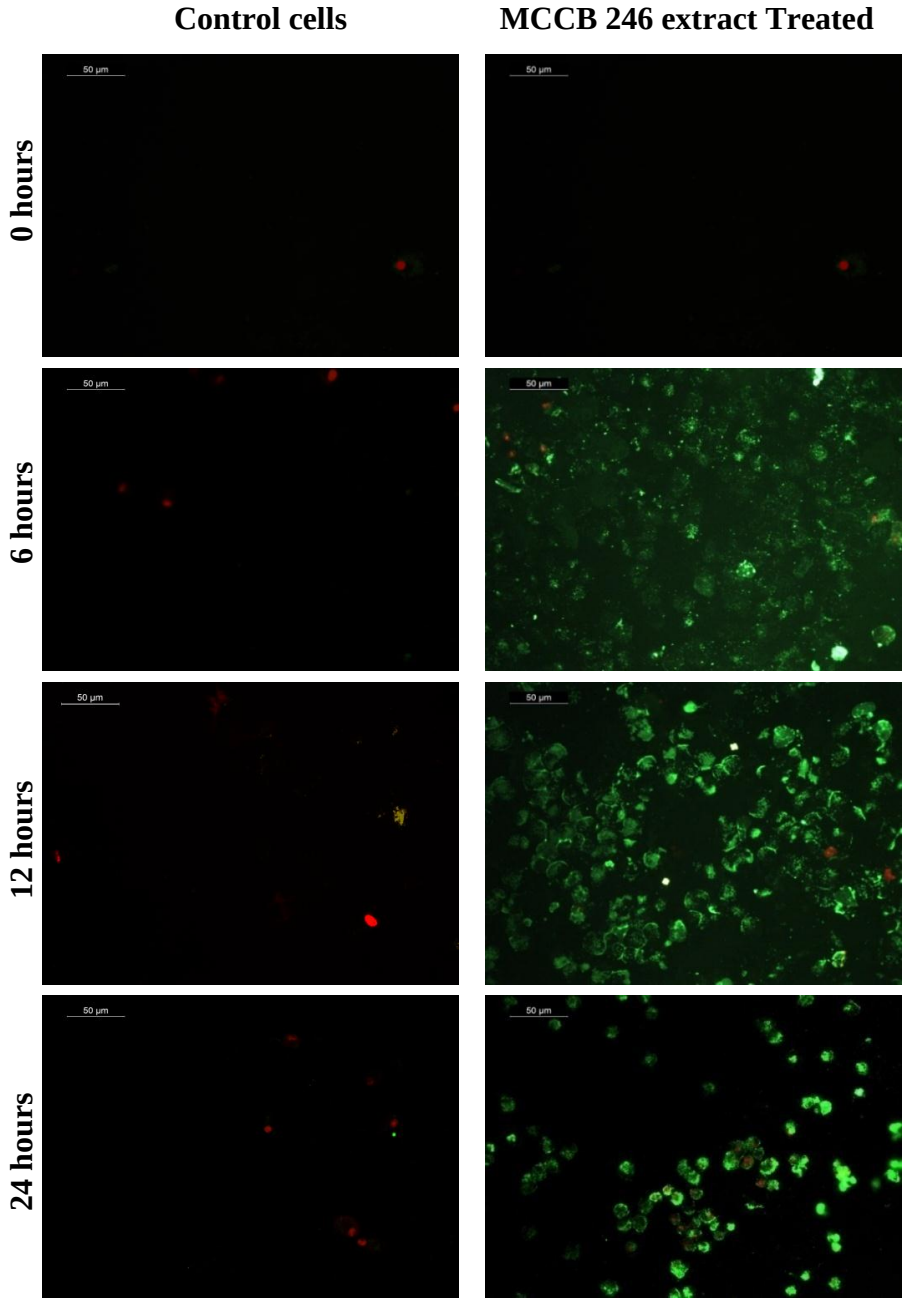


Fig. 4.8: Fluorescent microscope image (20 X) of Annexin-V/PI double-staining assay. After treating with *Streptomyces* sp. MCCB 246 extract, cells were stained with Annexin V-FITC and propidium iodide and analyzed after 0, 6, 12 and 24 h of treatment

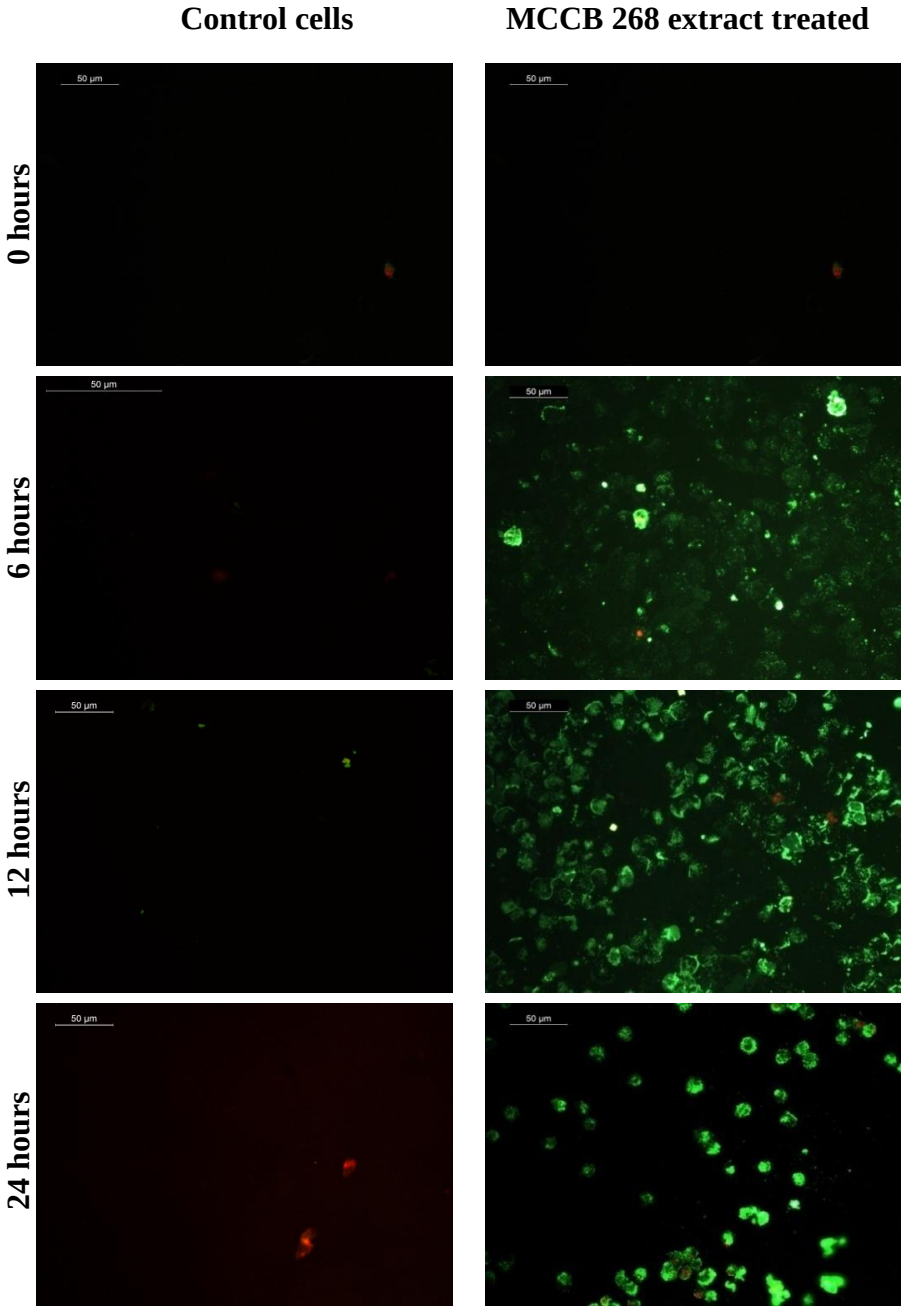


Fig. 4.9: Fluorescent microscope image (20 X) of Annexin-V/PI double-staining assay. After treating with *Pseudonocardia sp*. MCCB 268 extract, cells were stained with Annexin V-FITC and propidium iodide and analyzed after 0, 6, 12 and 24 h of treatment

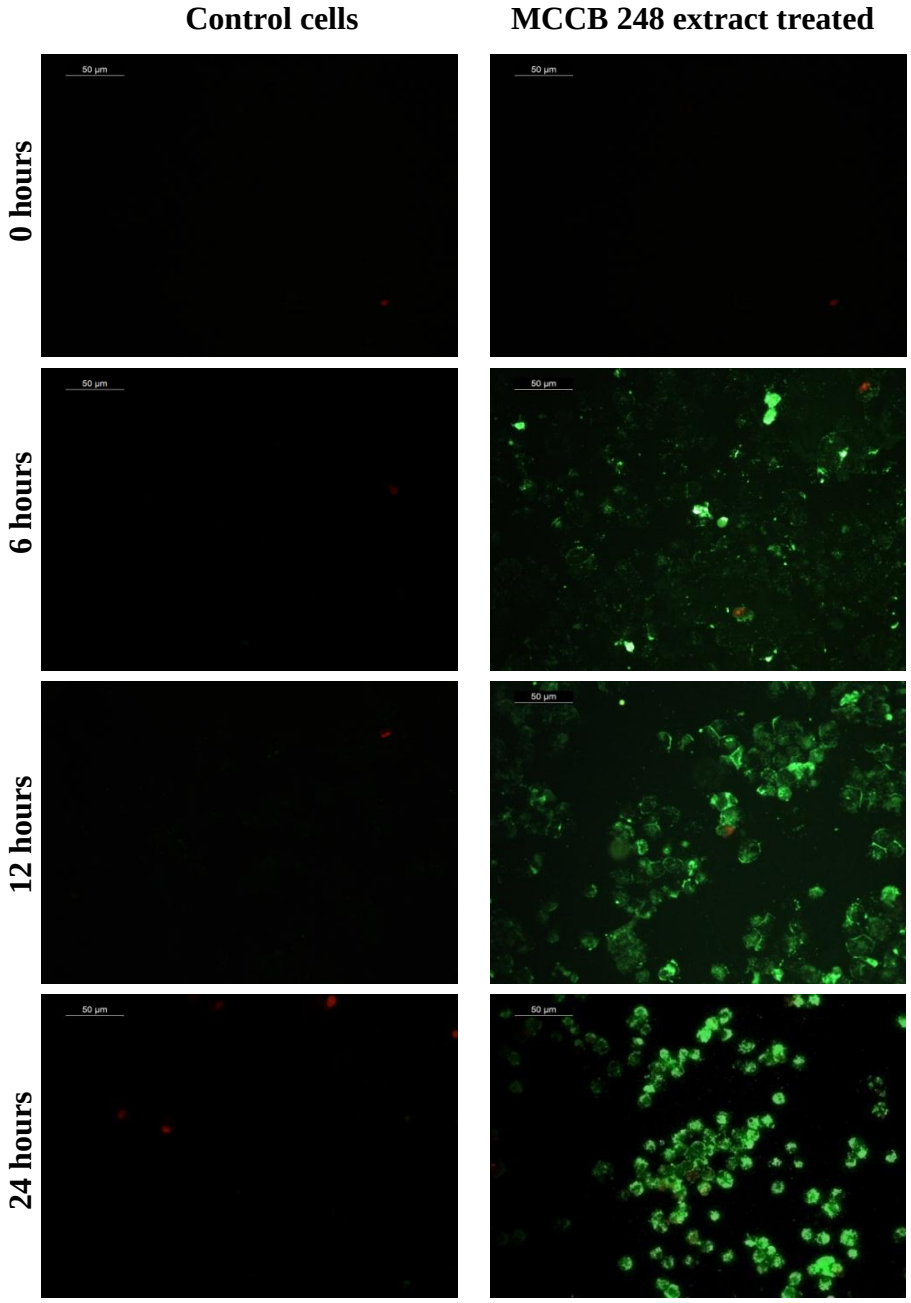


Fig. 4.10: Fluorescent microscope image (20 X) of Annexin-V/PI double-staining assay. After treating with *Streptomyces* sp. MCCB 248 extract, cells were stained with Annexin V-FITC and propidium iodide and analyzed after 0, 6, 12 and 24 h of treatment

4.3.1.3 Evaluation of Apoptosis induction by extracts using Annexin-V/PI double-staining assay

Translocation of phosphatidylserine (PS) from inner to outer plasma membrane is one of the earliest events taking place during apoptosis. Annexin V is a cellular protein which has got high affinity towards PS. Normal cell membrane is impermeable for Propidium iodide (PI) and only cells which are either dead or at the later stages of apoptosis will take up PI.As a result cells in early stages of apoptosis will be only Annexin V positive and cells in later stages of apoptosis will be both Annexin V and PI positive. In Annexin V-PI assays, a time -dependent increase in Annexin V positive cells were observed in cells treated with extracts, implying that there was an increased PS translocation to the outer leaflet of the plasma membrane (Fig. 4.7-10). At the beginning of treatment (0h), a majority of the cells were negative for both Annexin V and propidium iodide (PI) staining. More cells turned to positive for both annexin V and PI in the end of treatment.

4.3.2 Chemical dereplication of crude actinomycetes extracts

As an attempt for early dereplication, Global Natural Products Social (GNPS) Molecular Networking was performed and results are given below.

4.3.2.1 Chemical dereplication efforts of *Streptomyces* sp. MCCB 267, by LC-PDA-MS/MS and molecular networking

The crude ethyl acetate extract of *Streptomyces* sp. MCCB 267 was initially subjected to a C18 SPE clean-up step, and then analyzed by positive ionization LC-MS/MS analysis. Twelve major chemical components were found to be present in the crude extract. These molecules had mass spectra

indicating protonated mass ions of m/z 437.2, 507.26, 621.18, 679.43, 818.72, 1042.87, 1143.17, 1197.19, 1197.19, 1255.31, 1401.49, 1816.1 (Fig. 4.11). These dominant MS/MS spectra did not match with any library entry upon molecular networking, whereas some minor peaks were detected in de-replication indicated the presence of Diketopiperazines (m/z 261.1, 227.082, 211.09), Genistein (271) and Alteramide B (495.27) in the extract.

In molecular networking, one of the dominant mass ions of m/z 507.689 formed a prominent cluster of related compounds peaks connected with nodes including mass ion of m/z 495.235 (m/z similar to Alteramide B), which is a polycyclic tetramate macrolactam (Fig. 4.12 &4.13).

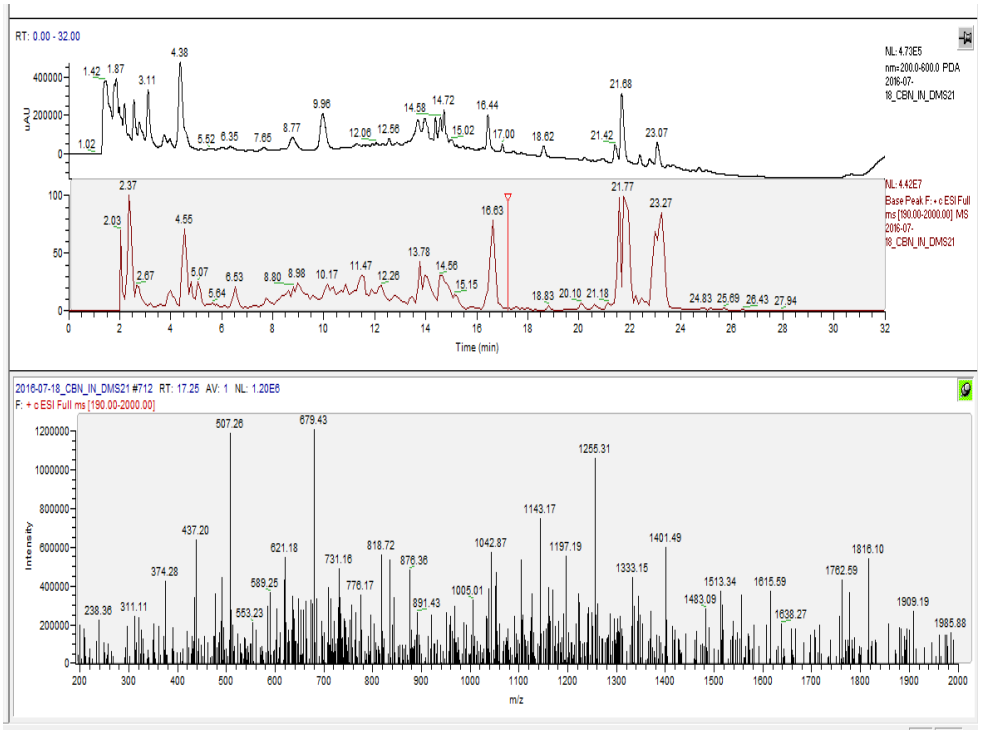


Fig. 4.11: LC-PDA-MS chromatogram of *Streptomyces* sp. MCCB 267 crude extract

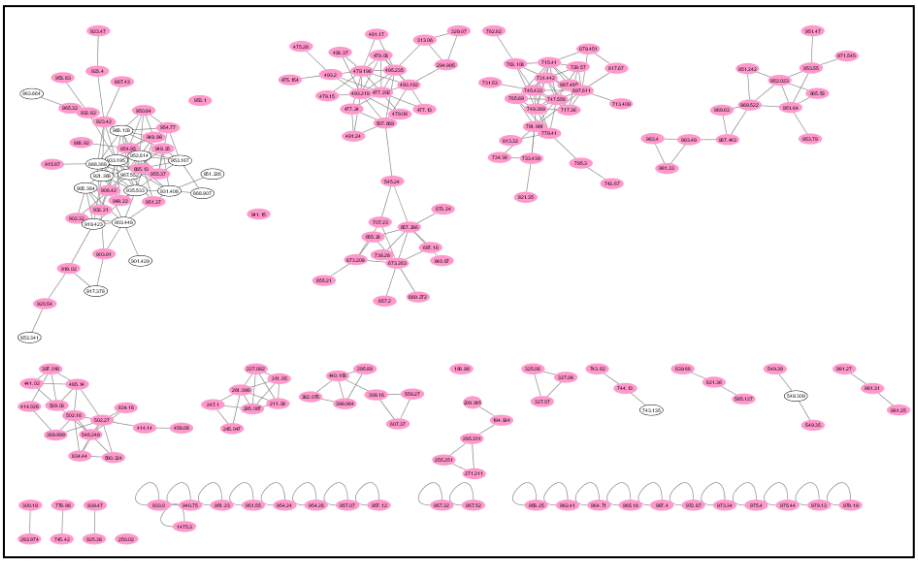


Fig. 4.12: LC-MS/MS based GNPS molecular networking of *Streptomyces* sp. MCCB 267 crude extract. Nodes have been labeled with their MS parent ion, and many self-loop nodes (single peak clusters) were abridged here for brevity. White nodes are peaks detected in the background file generated by injecting acetonitrile blank by the same method before sample.

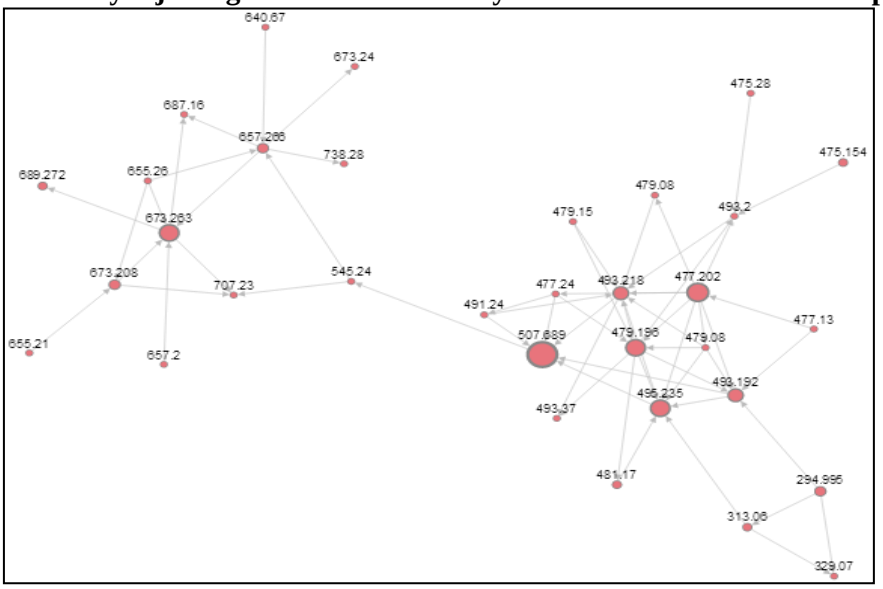


Fig.4.13: Cluster showing major peaks (m/z 507.689 and 479.19) in the GNPS molecular networking of *Streptomyces* sp. MCCB 267 crude extract. Nodes have been labelled with their MS parent ion, scaled to size according to the intensity of their MS peak integration

4.3.2.2 Chemical de-replication efforts of *Streptomyces* sp. MCCB246 by LC-PDA-MS/MS and molecular networking

After C18 SPE clean-up step, the crude ethyl acetate extract of *Streptomyces* sp. MCCB246 was analyzed by positive ionization on LC-MS/MS (Fig. 4.14). Eight major chemical components were found to be present in the crude extract. These molecules had mass spectra indicating protonated mass ions of m/z 511.96, 591.03, 741.92, 940.83, 1101.13, 1182.16, 1336.69, 1837.6. Most of these peaks detected in MS/MS spectra were not identified in GNPS molecular networking (Fig. 4.15) databases. Few peaks matched with Diketopiperazines, which was expected. The major components, which present in the spectra, except m/z 511.96 were not identified in the library even in analogue search option. De-replication using GNPS shows peak m/z 511.96 as 6-[5, 7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4-oxochromen-3-yl]oxy-3,4,5-trihydroxyoxane-2-carboxylic acid.

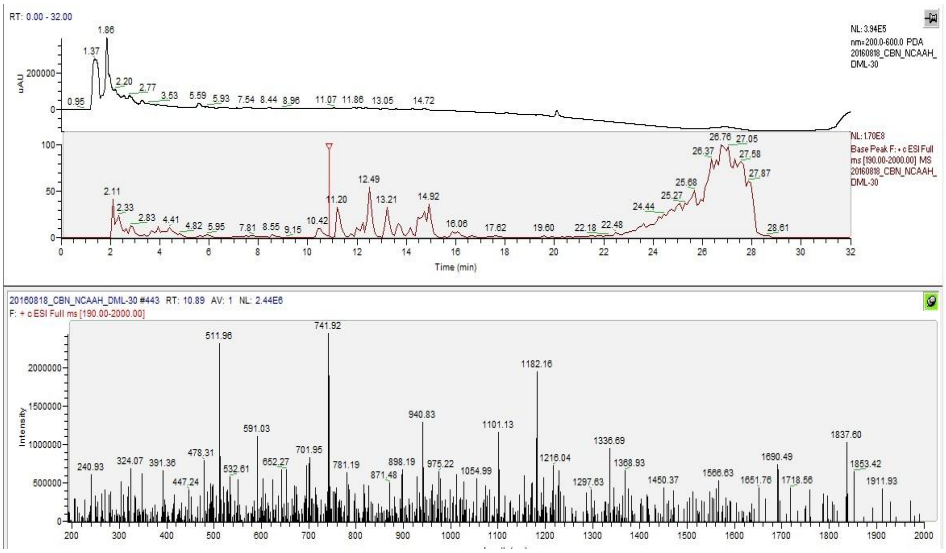


Fig. 4.14: LC-PDA-MS chromatogram of *Streptomyces* sp. MCCB246 crude extract

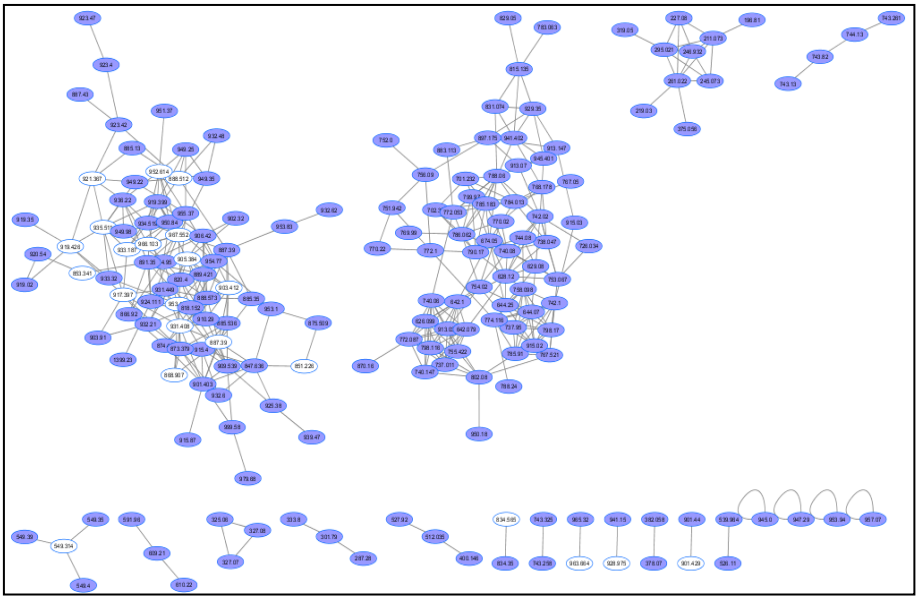


Fig. 4.15: LC-MS/MS based GNPS molecular networking of *Streptomyces* sp. MCCB246 crude extract. Nodes have been labeled with their MS parent ion, and many self-loop nodes (single peak clusters) were abridged here for brevity. White nodes are peaks detected in background file generated by injecting acetonitrile blank by the same method before blank.

4.3.2.3 Chemical dereplication efforts of *Pseudonocardia* sp. MCCB 268 by LC-PDA-MS/MS and molecular networking

After C18 SPE clean-up step, the crude ethyl acetate extract of *Pseudonocardia* sp. MCCB 268 was analyzed by positive ionization on LC-MS/MS analysis. After analysis seventeen major chemical components were found to be present in the crude extract (Fig.4.17). These molecules had mass spectra indicating protonated mass ions of m/z 211.09, 227.35, 590.57, 645.57, 695.45, 763.50, 809.33, 859.42, 1102.16, 1245.92, 1274.42, 1312.11, 1497.44, 1578.54, 1641.14, 1737.57 and 1811.67. Most of the dominant peaks detected in MS/Ms spectra were not identified in GNPS molecular networking databases even after analogue search option (Fig.

4.18). One of the dominant peaks 211.09 found as cyclo-(L-Leu-L-Pro), is a Diketopiperazine. Some other small peaks also showed similarity with other Diketopiperazine.

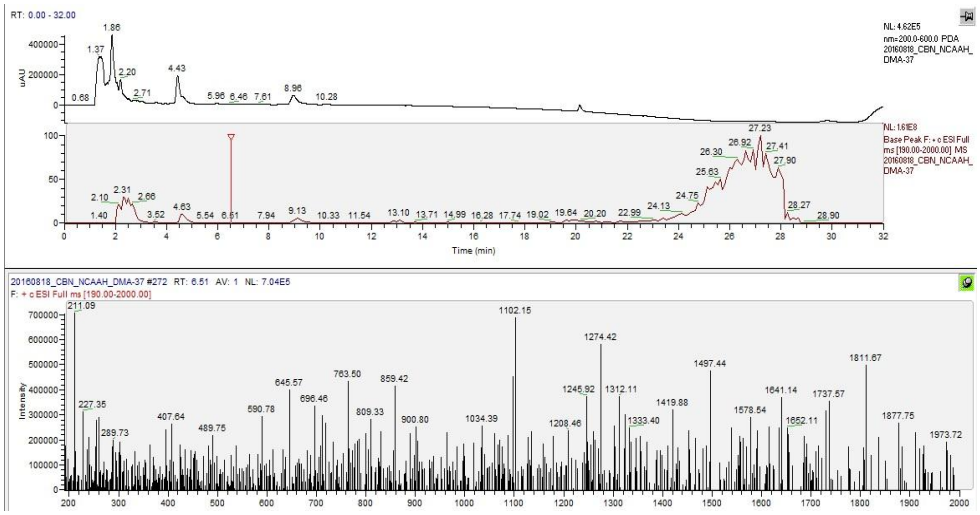


Fig. 4.17: LC-PDA-MS chromatogram of *Pseudonocardia* sp. MCCB 268 crude extract

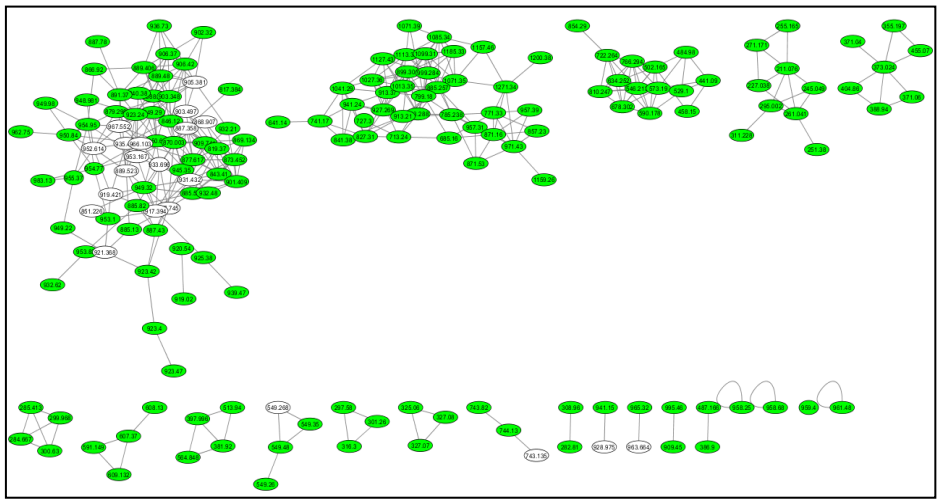


Fig. 4.18: LC-MS/MS based GNPS molecular networking of *Streptomyces* sp. MCCB 248 crude extract. Nodes have been labeled with their MS parent ion, scaled to size according to the intensity of their MS peak integration, and many self-loop nodes (single peak clusters) were abridged here for brevity.

4.3.2.4 Chemical de-replication efforts of *Streptomyces* sp. MCCB 248 by LC-PDA-MS/MS and molecular networking

The crude extract of *Streptomyces* sp. MCCB 248 was first subjected to a C18 SPE clean-up step, and then analyzed by positive ionization LC-MS/MS analysis. Four major chemical components were determined to be present in the crude extract. Peaks of interest were observed from 14 to 16 min. These four molecules had mass spectra indicating protonated mass ions of m/z 761, 827, 879, and 913, and appeared to possess a common UV chromophore (ν = 312 nm, broad absorption from 280-340 nm, and ν = 240 nm) (Fig. 4.19). These MS/MS spectra did not match with any library entry upon molecular networking (Fig. 4.20). However, they clustered together in a small molecular family, and upon library comparison with the analogue search option, were suggested to be structurally related to a polyhydroxy macrolide, such as bastimolide A (Shao et al. 2015). A number of minor metabolites were also detected in the chromatogram by MS or DAD UV, but were not analyzed further due to their low abundance.

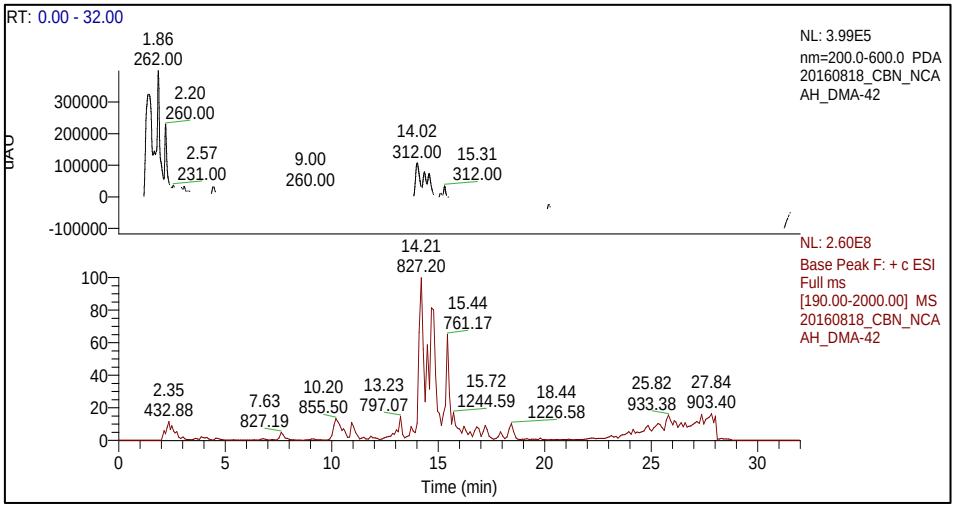


Fig. 4.19: LC-PDA-MS chromatogram of *Streptomyces* sp. MCCB 248 crude extract. Labels denote retention time (above) and maxima peak (below). Peaks of interest were observed from 14-16 min

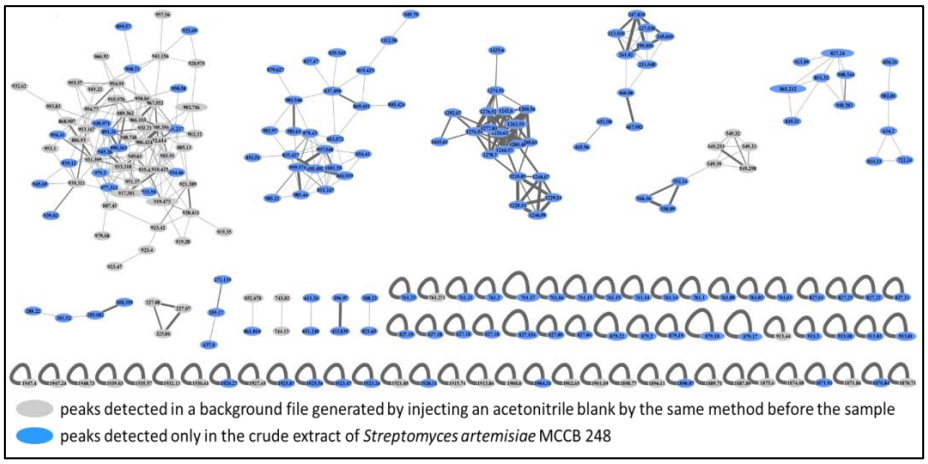


Fig. 4.20: LC-MS/MS based GNPS molecular networking of *Streptomyces* sp. MCCB 248 crude extract. Nodes have been labeled with their MS parent ion, scaled to size according to the intensity of their MS peak integration, and many self-loop nodes (single peak clusters) were abridged here for brevity.

4.3.3 Screening for biosynthetic genes involved in secondary metabolites production of actinomycetes

The biosynthetic potential of isolates were also analyzed by gene screening for different biosynthetic genes, viz. Type I polyketide synthase (PKS I), Type II polyketide synthase (PKS II), Nonribosomal peptide synthase (NRPS), Aminodeoxy isochorismate synthase (*phz*E) and Cytochrome P450 hydroxylase (CYP) genes. Details summarizing the presence of different biosynthetic genes are given in Table 4.3.

Table 4.3: Presence of biosynthetic genes in isolates

Actinomycetes isolate	PKS I	PKS II	NRPS	CYP	PhzE
Streptomyces sp. MCCB 267	+ KU556299	+ KU556300	–	–	–
Streptomces sp. MCCB246	-	+ MF405490	+ MF405492	+ MF405491	–
Pseudonocardia sp. MCCB 268	+ KT351404	+ KT351405	–	–	–
Streptomyces sp. MCCB 248	+ KT251042	–	+ KT277491	–	–

4.3.3.1 Screening for type I polyketide synthase gene (PKS I)

Type I polyketide synthase gene (PKS I) in selected isolates were screened by PCR amplification using degenerative primer. *Streptomyces* sp. MCCB 267, *Pseudonocardia* sp. MCCB 268, *Streptomyces* sp. MCCB 248 show amplification of PKS I gene (Fig.4.21 a-c), however *Streptomyces* sp. MCCB 246 did not showed amplification for PKS I. After NCBI GenBank blastx searching, the PKS I sequence of *Streptomyces* sp. MCCB 267 (GenBank accession number KU556299) showed 90% sequence similarity with its closest match *Streptomyces kanamyceticus*. Phylogenetic analysis of *Streptomyces* sp. MCCB 267 PKS I using translated nucleic acid sequences revealed that it formed a separate cluster in the tree (Fig 4.22).

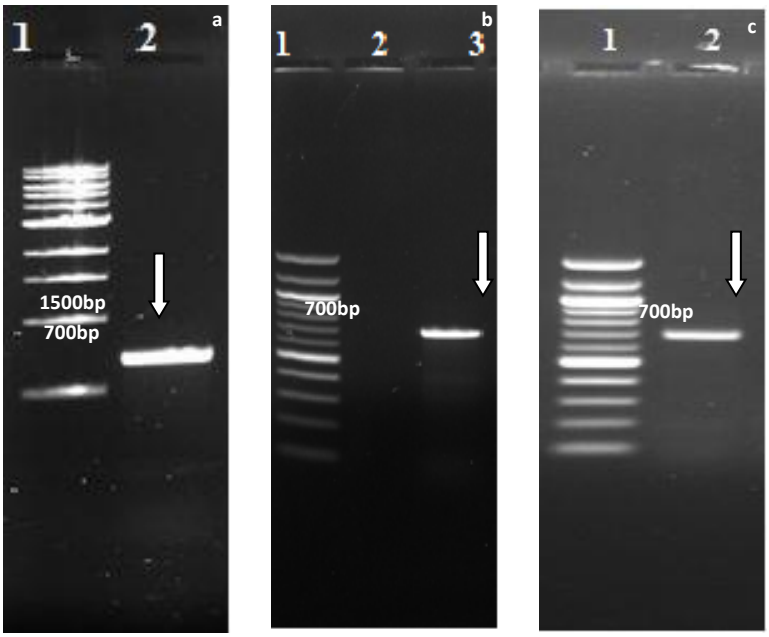


Fig. 4.21: PCR amplified product of PKS1 (arrow mark) (700bp) gene involved in secondary metabolites production of a) *Streptomyces* sp. MCCB 267 (Lane 2) b) *Pseudonocardia* sp. MCCB 268 (Lane 3) c) *Streptomyces* sp. MCCB 248 (Lane 2)

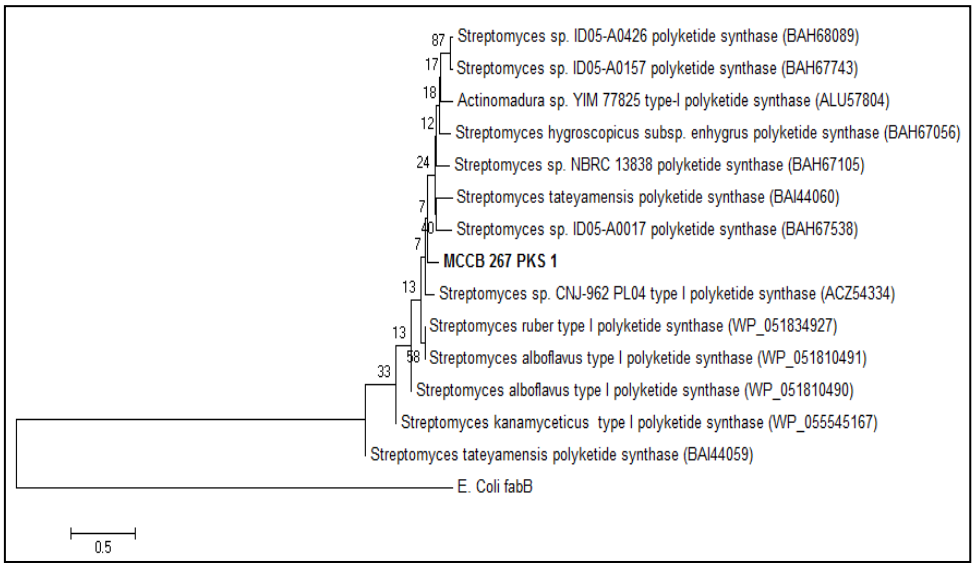


Fig. 4.22: Representative neighbour-joining phylogenetic tree of *Streptomyces* sp. MCCB 267 PKS1 amino acid sequences

PKS I gene sequence of *Pseudonocardia* sp. MCCB 268 (GenBank accession number KT351404) showed only very low sequence similarity (62%) after NCBI GenBank blastx search with closest match polyketide synthase of *Pseudonocardia* sp. HH130629-09. The sequence showed 80% sequence similarity with *Pseudonocardia autotrophica* strain KCTC9441 polyene biosynthetic gene cluster using blastn algorithm. In phylogenetic analysis of *Pseudonocardia* sp. MCCB 268 PKS I using translated nucleic acid sequence formed a separate branch with *Pseudonocardia spinosispora* type I polyketide synthase (Fig. 4.23).

PKS I nucleotide sequence of *Streptomyces* sp. MCCB 248 (GenBank accession number KT251042) had closest match with *Streptomyces* sp. ID05-A0179 polyketide synthase (83%) after NCBI GenBank blastx searching. Phylogenetic analysis of the *Streptomyces* sp. MCCB 248 PKS I using translated nucleic acid sequences revealed that it formed a separate

cluster along with *Streptomyces himastatinicus* ATCC 53653 and *Streptomyces aurantiacus* JA4570 (Fig. 4.24).

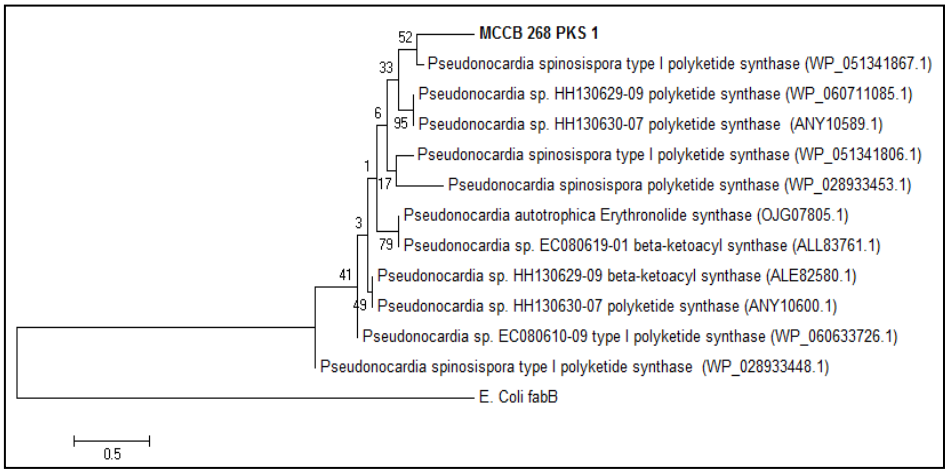


Fig. 4.23: Representative neighbour-joining phylogenetic tree of *Pseudonocardia* sp. MCCB 268 PKS1 amino acid sequences

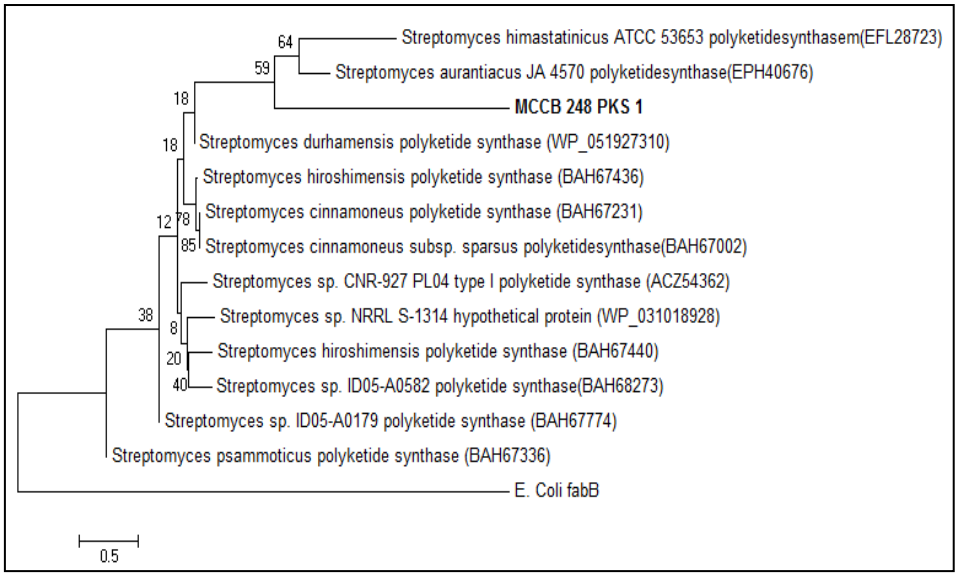


Fig. 4.24: Representative neighbour-joining phylogenetic tree of *Streptomyces* sp. MCCB 248 PKS1 amino acid sequences

4.3.3.2 Screening for type II polyketide synthase gene (PKS II)

After screening for type II polyketide synthase gene (PKS II), three of the isolates showed the presence of PKS II, which include *Streptomyces* sp. MCCB 267, *Streptomyces* sp. MCCB246 and *Pseudonocardia* sp. MCCB 268 (Fig. 4.25 a-b). However, *Streptomyces* sp MCCB 248 did not show any amplification for PKS II gene.

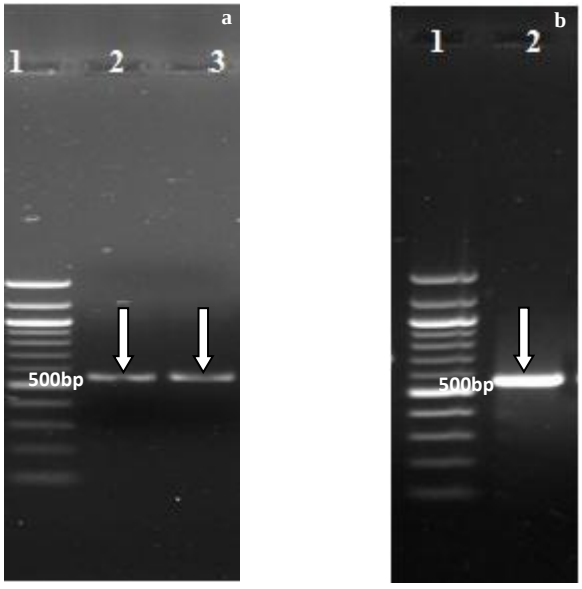


Fig. 4.25: PCR amplified product of PKS II gene (554bp) involved in secondary metabolites production of a) *Streptomyces* sp. MCCB 267 (Lane 2), *Pseudonocardia* sp. MCCB 268 (Lane 3) and b) *Streptomyces* sp. MCCB246 (Lane 2)

Streptomyces sp. MCCB 267 PKS II sequence (GenBank accession number KU556300) showed 92% sequence similarity to type II polyketide synthase of *Streptomyces scopuliridis* after NCBI blastx analysis. Phylogenetic analysis of the *Streptomyces* sp. MCCB 267 using translated nucleic acid sequences of PKS 2 revealed that it formed a separate branch in the tree (Fig.4.26).

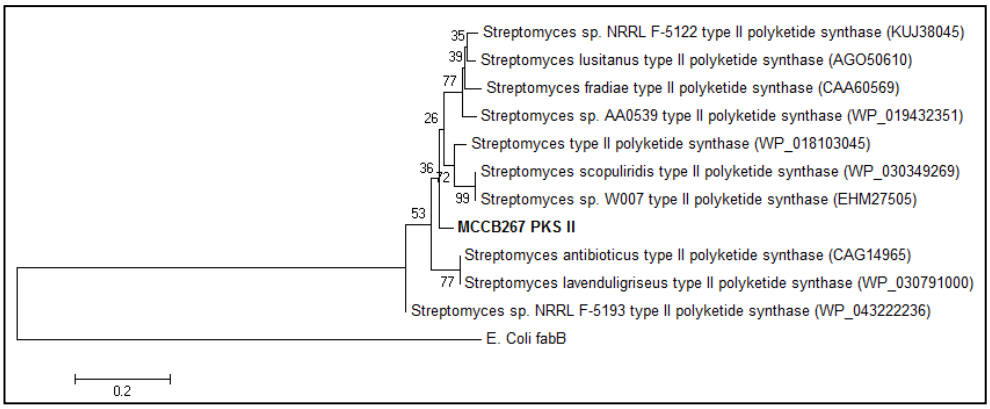


Fig. 4.26: Representative neighbour-joining phylogenetic tree of *Streptomyces* sp. MCCB 267 PKSII amino acid sequences

Similarly, *Streptomyces* sp. MCCB246 PKS II sequence (GenBank accession number MF405490) showed 98% sequence similarity with minimal PKS ketosynthase (KS/KS alpha) with *Streptomyces wuyuanensis* type strain and 93 % sequence similarity with *Streptomyces purpureus* and *Streptomyces* sp. CNT372. In phylogenetic tree *Streptomyces wuuyuanensis* MCCB246 formed a separate branch with *Streptomyces wuyuanensis* type strain (Fig.4.27).

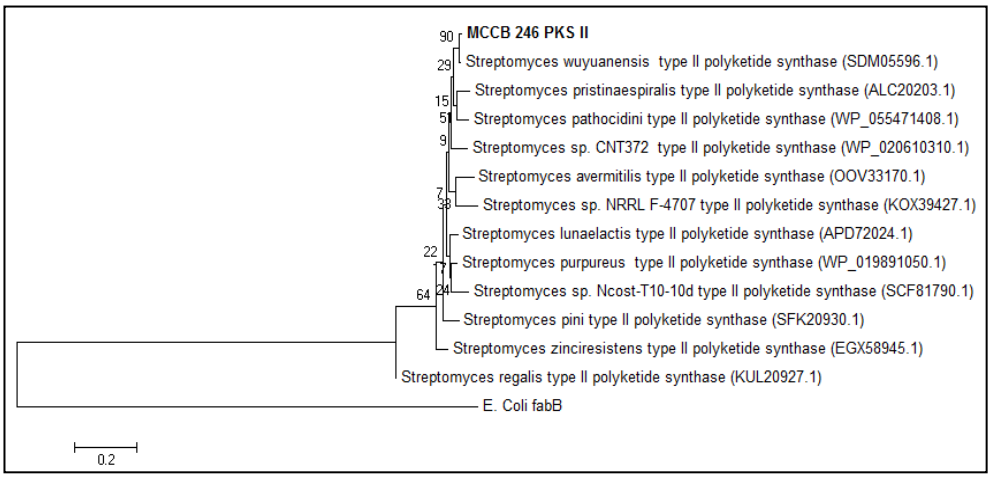


Fig. 4.27: Representative neighbour-joining phylogenetic tree of *Streptomyces* sp. MCCB246 PKSII amino acid sequences

PKS II nucleotide sequence of *Pseudonocardia* sp. MCCB 268 (GenBank accession number KT351405) showed 80% sequence similarity with type II polyketide synthase of *Pseudonocardia* sp. YIM 77571T after blastx search. In phylogenetic analysis using translated nucleotide sequences it formed a separate branch in the tree (Fig.4.28).

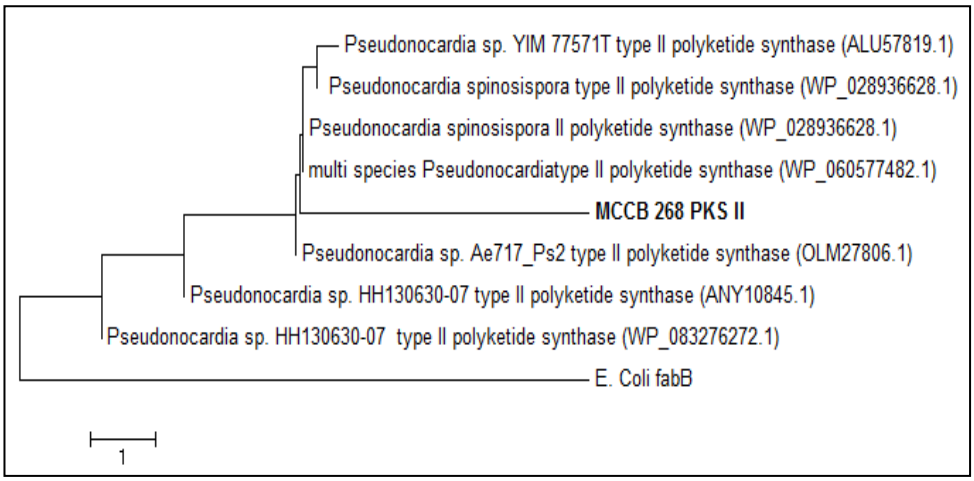


Fig. 4.28: Representative neighbour-joining phylogenetic tree of *Pseudonocardia* sp. MCCB 268 PKS II amino acid sequences

4.3.3.3 Screening for nonribosomal peptide synthetases gene (NRPS)

Streptomyces sp. MCCB246 and *Streptomyces* sp. MCCB 248 showed the amplification for NRPS gene after screening with degenerative primers (Fig.4.29 a-b). NRPS gene sequence of *Streptomyces* sp. MCCB246 (Genbank accession number MF405492) showed 99% sequence similarity with N-(5-amino-5-carboxypentanoyl)-L-cysteinyl-D-valine synthase of *Streptomyces* sp. CNT302 and 91% sequence similarity with N-(5-amino-5-carboxypentanoyl)-L-cysteinyl-D-valine synthase of *Streptomyces* sp. NRRL B-24891. In phylogenetic tree, NRPS of MCCB256 formed a separate branch with *Streptomyces* sp. CNT302 (Fig .4.30).

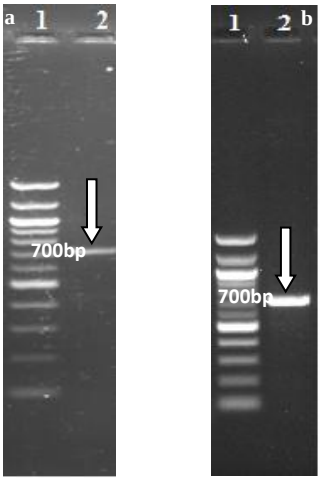


Fig. 4.29: PCR amplified product of NRPS (700bp) gene involved in secondary metabolites production of a) *Streptomyces* sp. MCCB246 (Lane 2) b) *Streptomyces* sp. MCCB 248 (Lane 2)

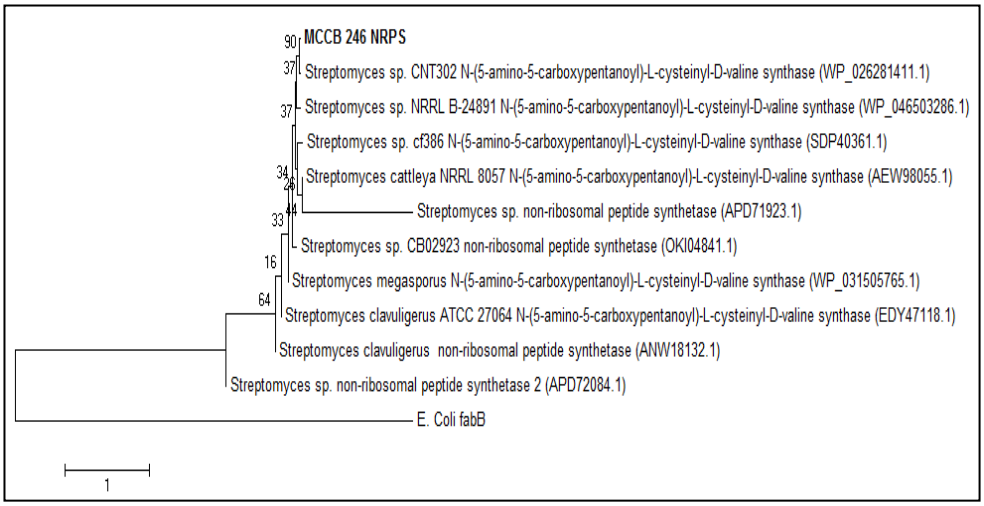


Fig. 4.30: Representative neighbour-joining phylogenetic tree of *Streptomyces* sp. MCCB246 NRPS amino acid sequences

Blastx analysis of the NRPS sequence from *Streptomyces* sp. MCCB248 (Genbank accession number KT277491) showed that it had closest similarity (83%) with one from *Streptomyces* sp. SCAU5132. Phylogenetic analysis of this NRPS using the deduced amino acid sequence

positioned *Streptomyces* sp. MCCB 248 as a distinct clade in the tree (Fig. 4.31).

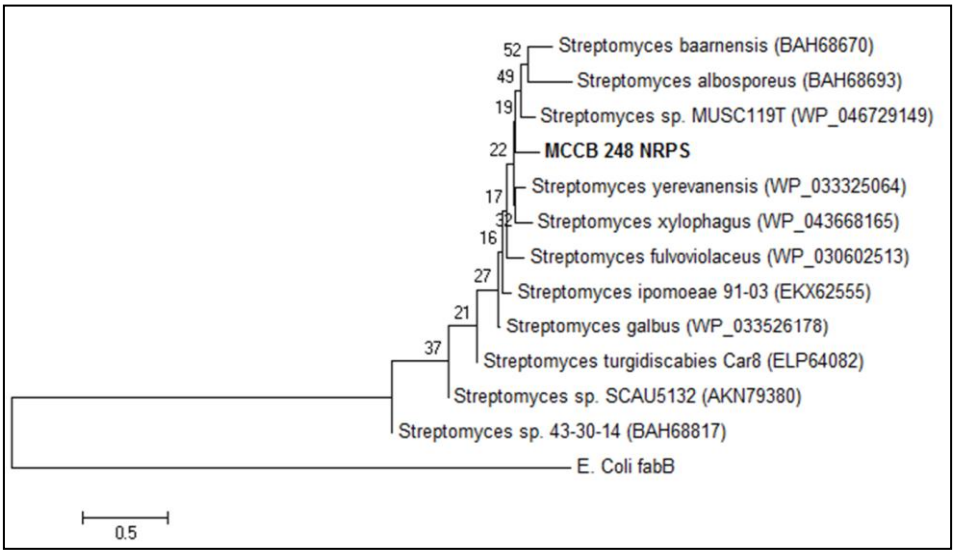


Fig. 4.31: Representative neighbour-joining phylogenetic tree of *Streptomyces* sp. MCCB 248 NRPS amino acid sequences

4.3.3.4 Screening for cytochrome P450 hydroxylase (CYP) genes

Among the four isolates screened for the presence of CYP genes, only *Streptomyces* sp. MCCB246 (Fig. 4.32) showed the presence of the gene. After NCBI GenBank BLASTX searching, the CYP nucleotide sequence of *Streptomyces* sp. MCCB246 (GenBank accession number MF405491) showed 98% sequence similarity with its closest matches with *Streptomyces hydrogenans* (Liu et al. 2017), *Streptomyces* sp. SM, *Streptomyces sp.* CNQ431, *Streptomyces* sp. KE1, *Streptomyces Igra*MP-1, and *Streptomyces* sp. FR-008, whereas, in phylogenetic analysis using translated nucleotide sequences, *Streptomyces* sp. MCCB246 formed a separate clade in the tree (Fig. 4.33).

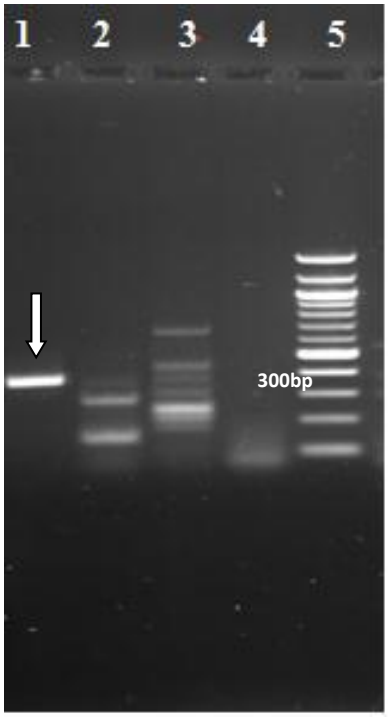


Fig. 4.32: PCR amplified product of CYP (350bp) gene involved in secondary metabolites production of *Streptomyces* sp. MCCB246 (Lane 1)

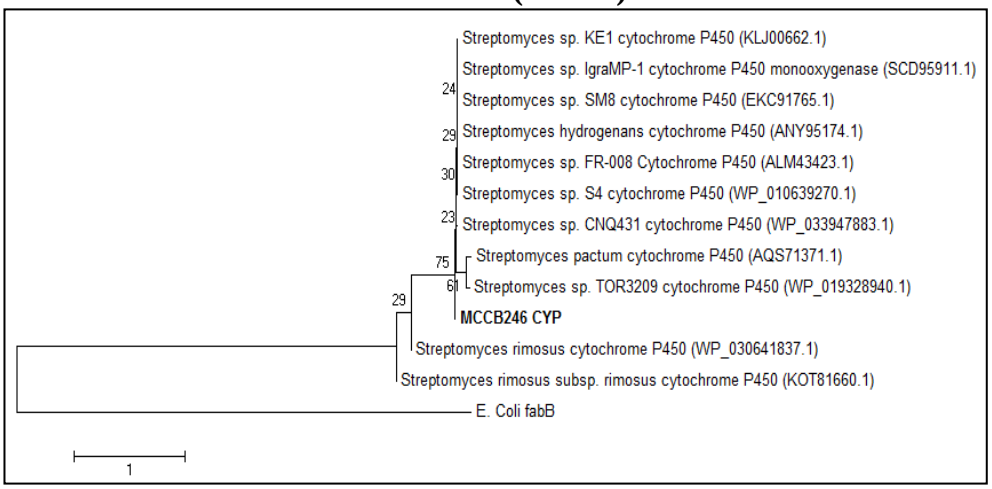


Fig. 4.33: Representative neighbour-joining phylogenetic tree *Streptomyces* sp. MCCB246 CYP amino acid sequences

4.4 Discussion

Actinomycetes are excellent sources of novel bioactive metabolites with potential pharmaceutical applications (Lam 2006). In the present study, all the four isolates showed promising IC_{50} values on NCI-H460 cells. When NCI-H460 human non-small cell lung cancer cells were treated with the active extracts of all the four selected actinomycetes, they showed nuclear condensation and fragmentation as evident from Hoechst 33342 staining. This is similar to what was observed with the known anticancer agent doxorubicin (Xin et al. 2012). A significant number of anticancer agents display cytotoxicity by damaging DNA, leading to apoptosis (Johnstone et al. 2002). Shrinkage of the cell and nucleus as well as condensation of nuclear chromatin were considered as the morphological hallmark of apoptotic cell death (Saraste 2000), and same thing was observed with application of active crude extracts. The DNA fragmentation observed in the TUNEL assay confirmed apoptotic cell death in cells treated with the extracts. Further, a time-dependent increase in Annexin V positive cells implied more cells had undergone a flip-flop of PS to the outer leaflet of the plasma membrane, another indicator of apoptosis. These results demonstrated that cytotoxicity induced by all the four extracts were mediated through apoptosis and but not through necrosis and hence, these results justify the selection of these four actinomycetes for bioprospecting of anticancer metabolites.

Apart from confirming the potential anticancer activity, the active isolates were studied further to evaluate their biosynthetic potentials to produce bioactive compounds. Screening of genes associated with biosynthesis of secondary metabolite production is a useful method to

evaluate the genetic makeup of an isolate to produce biologically active metabolites. Recently, Ayuso et al. (2005) reported a novel actinomycete strain de-replication approach based on the diversity of polyketide synthase and nonribosomal peptide synthetase biosynthetic pathways. Together with de-replication efforts using LC-MS data and the confirmation of biosynthetic potential at genome level would help in the selection of promising isolates for bioprospecting work. A wide range of bioactive polyketide and peptide compounds having applications in medicine, agriculture, and biochemical research are synthesized by type-I polyketide synthases (PKS-I) and nonribosomal peptide synthetases (NRPS). These metabolites include antibiotics (e.g., penicillins, vancomycin, and erythromycin), antifungals (e.g., nystatin), antitumor agents (e.g., ansamitocin, bleomycins), anthelmintics (e.g., avermectin) and immunosuppressive agents (e.g., rapamycin and FK506). Polyketide synthases (PKSs) are large multifunctional enzymes that are responsible for the biosynthesis of macrolides and other macrocyclic polyketides whose members have diverse structural and pharmacological properties (Katz 1997). Extensive studies of PKS-I and NRPS biosynthetic systems have been described in actinomycetes (Metsä-Ketelä et al. 1999; Ayuso-Sacido and Genilloud 2005; Abdelmohsen et al. 2014b) myxobacteria (Beyer et al. 1999) and cyanobacteria (Christiansen et al. 2001), among other bacterial taxa, and in filamentous fungi (Bingle et al. 1999). Though enormous progress has been made in the discovery and identification of the genetic organization and mechanism of biosynthesis of numerous compounds of commercial interest, very little is known about the distribution of these biosynthetic systems in other actinomycetes, or even in other microbial taxa.

In the current study, *Streptomyces* sp. MCCB 267 and *Pseudonocardia* sp. MCCB 268 showed the presence of Type I polyketide synthase gene (PKS I) and Type II polyketide synthase gene (PKSII) . *Streptomyces* sp. MCCB 246 showed the presence of PKS II, CYP and NRPS whereas PKS I and PhzE were not present. In *Streptomyces* sp. MCCB 248 Type I polyketide synthase gene (PKSI) and NRPS were detected. Liu et al. (2017) studied biosynthetic potential of cultivable actinomycetes associated with lichen symbiosis and found a high proportion of positive PCR amplification of NRPS (55.4%), PKS I (51.6%), and PKS II (39.9%) among the isolates, whereas CYP sequences were only 4.2%, of the strains screened in study. The compounds produced by these isolates may be the product of a single biosynthetic gene cluster or a hybrid product of different biosynthetic gene clusters.

Genomic sequence analysis of potential actinomycetes will help to reveal previously unrecognized biosynthetic potential and diversity of the organism and this approach is known as "Genome mining"(Hornung et al. 2007). In this approach, the genes that encode tailoring enzymes from natural product biosynthesis pathways will serve as indicator genes for the identification of isolates that have the genetic potential to synthesize natural products of interest. Nowadays, the computational mining of genomes has become an important part in the discovery of novel natural products. Larger number of tools are available to enable researchers to computationally mine genetic data and link them to known secondary metabolites(Ziemert et al. 2016). Scientists noticed the unexplored potential hidden in bacterial genomes, when they performed full genome sequences of two well studied natural product producing strains *Streptomyces coelicolor* (Bentley et al. 2002) and

Streptomyces avermitilis (Ikeda et al. 2003). On an average a *Streptomyces* genome contains about 30 secondary metabolite gene clusters.

Bioassay guided fractionation with the de-replication strategy of LC-MS/MS molecular networking has been successfully used as a tool for targeted isolation of a natural product possessing both a novel chemical structure and a desired biological activity (Naman et al 2017).

The GNPS molecular networking of *Streptomyces* sp. MCCB 267 revealed that, apart from some minor peaks, dominant MS/MS spectra did not match with any library entry. However, one of the dominant peaks 507.689 formed a cluster with polycyclic tetramate macrolactam compounds (PTMs), which are complex biologically active small molecules sharing a macrocyclic lactam ring with an embedded tetramic acid ring along with a variable set of stereochemically rich carbocyclic rings (Zhang et al. 2014). De-replication result indicates that the active compounds produced by the *Streptomyces* sp. MCCB267 could be a member of PTM compounds. However, beyond this conclusion, no exact information about the compound could be deducted out from this analysis. Moreover, PCR based screening for biosynthetic genes revealed the presence of PKS I and II in this isolate. PKS I nucleotide sequence of *Streptomyces* sp. MCCB 267 showed highest sequence similarity (90%) with PKS I of *Streptomyces kanamyceticus*, which produces aminoglycoside family of antibiotic kanamycin (Okami et al. 1959). PKS II of *Streptomyces* sp. MCCB 267 showed more sequence similarity with *Streptomyces scopuliridis*, a deep sea marine sediment isolate from South China Sea. Desotamides B, C and D together with a known Desotamide A were isolated from *Streptomyces scopuliridis* SCSIO

ZJ46 (Song et al. 2014). Considering all these it is concluded that the isolate *Streptomyces* sp. MCCB 267 could be a potential strain to study further.

De-replication and the screening for biosynthetic gene results indicate that *Streptomyces* sp. MCCB246 is a promising isolate to look for a promising secondary metabolites. The biosynthetic gene screening of *Streptomyces* sp. MCCB246 showed that the isolate contain PKS II, NRPS and CYP genes, and most of the peaks detected in MS/MS spectra were not identified after dereplication using GNPS molecular networking. PKS II of type strain *Streptomyces wuyuanensis* showed highest sequence similarity (98%) with PKS II of MCCB 246, but the type strain is not reported for any compound production till now. Another marine-derived *Streptomyces* sp. strain CNT-372 from Fiji, showed 99% sequence similarity with NRPS and 93% sequence similarity with PKS II of *Streptomyces sp.* MCCB 246, and is reported to have producing sesquiterpenoid nucleoside ethers Farnesides A and B. The farnesides are rare class of microbial terpenoid nucleoside metabolites (Zafrir Ilan et al. 2013). Cytochrome P450 hydroxylase (CYP) sequence of *Streptomyces* sp. MCCB246 showed 98% sequence similarity with a few *Streptomyces* strains. Out of these, *Streptomyces* sp. strain CNQ431 and *Streptomyces* sp. SM8 are known to produce bioactive compounds. *Streptomyces* sp. strain CNQ431, is a producer of the cytokine inhibitor splenocin (Yu et al. 2015). Metabolomic profiling and genomic study of *Streptomyces* sp. SM8 showed the presence of hydroxylated saturated fatty acids as major components present in the antibacterial fractions and Antimycin compounds in the antifungal fractions. *Streptomyces* sp. SM8 was found to have multiple secondary metabolism gene clusters, including a gene cluster for the biosynthesis of the antifungal antimycin family of compounds (Viegelmann et al. 2014).

After GNPS dereplication efforts, most of the dominant peaks detected in MS/Ms spectra of *Pseudonocardia sp.* MCCB 268 were not identified even after analogue search option. The isolate showed the presence of PKS I and PKS II after screening for biosynthetic genes involved in secondary metabolites production. *Pseudonocardia* groups are considered as rare actinomycetes which are well known for their ability to produce bioactive secondary metabolites. However, relatively few bioactive compounds have been described from these groups. Comparatively very few studies were reported on the biosynthetic genes of *Pseudonocardia* (Kim et al. 2009; Holmes et al. 2016). PKS I amino acid sequence of *Pseudonocardia* sp. MCCB 268 showed very less sequence similarity with other deposited PKS I sequences of *Pseudonocardia* genus, whereas nucleotide sequence showed 80% sequence similarity *with Pseudonocardia autotrophica* strain KCTC9441 polyene biosynthetic gene cluster, which produces a novel polyene compound NPP (Kim et al. 2015). The PKS II sequence of *Pseudonocardia* sp. MCCB 268 showed more similarity with *Pseudonocardia* sp. YIM 77571T, while this isolate does not have any reported bioactivity.

The presence of PKS I and NRPS genes in *Streptomyces* sp. MCCB 248 suggests that it may produce bioactive secondary metabolites belonging to these two classes of natural products, or a hybrid of both. Relatively low sequence similarity of the PKS and NRPS gene sequences with those available in GenBank indicates the potential of this isolate for the production of novel compounds. On phylogenetic analysis of PKS I using the deduced amino acid sequence, it formed a separate branch along with *Streptomyces himastatinicus* ATCC 53653 and *Streptomyces aurantiacus. Streptomyces aurantiacus* has been reported for the production of

resistomycin, as an anticancer compound isolated and characterized (Vijayabharathi et al. 2011). The HPLC–PDA-MS/MS chemical analysis and dereplication efforts for the crude extract of *Streptomyces* sp. MCCB 248 suggested some key molecular features associated with the major components of this mixture. The MS and MS/MS spectra of the major four metabolites in the extract showed repeated losses of 18 mass units, which is typical of polyols that show neutral ion losses of water. Furthermore, the UV–Vis spectrum associated with these compounds was suggestive of a tetraene moiety in each, due to the typical absorption band from 280 to 340 nm with clear maxima at 312 nm. Taken together, these data suggest the presence of tetraene polyols in the extract, which would be in-line with the production of PKS or hybrid PKS natural products by similar actinomycetes such as the novonestmycins, separacenes, bahamaolides and marinisporolides (Kwon et al. 2009; Kim et al. 2012; Bae et al. 2013; Wan et al. 2015). However, the parent masses observed in this study were not found to correlate with any known molecules of this class in the MarinLit or Dictionary of Natural Products databases. Additionally, the molecular networking performed using GNPS failed to yield any matches against spectra present in the available MS/MS library databases for the four major metabolites present in the extract, but did suggest that, these compounds could be analogs of the reported polyhydroxy macrolide natural product known as bastimolide A (Shao et al. 2015). On the other hand, of the lower abundance molecules in the extract, it was possible to identify several known diketopiperazines.

In conclusion, four actinomycetes isolated and identified as, *Streptomyces* sp. MCCB 267, *Streptomces* sp. MCCB246 , *Pseudonocardia* sp. MCCB 268, and *Streptomyces* sp. MCCB 248 from different marine

environment showed promising cancer cell toxicity towards NCI-H460 cells *in vitro*. From the cell-based assays, it could be concluded that this activity likely results in the induction of apoptotic pathways leading to cell death. Chemical analysis, dereplication efforts and biosynthetic gene comparisons for these isolates suggested that all the four organisms produce potentially bioactive secondary metabolites.

Molecular networking studies revealed that *Streptomyces* sp. MCCB 267 contain active compounds belonging to group of PTMs and the isolate also showed the presence of PKS I and II genes. Being a rare and new actinomycetes *Pseudonocardia* sp. MCCB 268 is also a potential isolate for further exploration. As a result, among the four promising isolates which showed promising activity two isolates viz, *Streptomyces* sp. MCCB 267 and *Pseudonocardia* sp. MCCB 268 were selected for further study, including the purification and characterization of the active compounds as well as mode of action of such anticancer compound(s).

Chapter 5

ISOLATION AND STRUCTURAL ELUCIDATION OF CYTOTOXIC COMPOUNDS FROM *STREPTOMYCES* SP. MCCB 267

Contents

5.1 Introduction

Marine bioprospecting has yielded considerable number of drug candidates in the last few decades. One of the best strategies to isolate the lead compound for biomedical development is bioactivity-guided isolation or bioassay guided fractionation, purification and identification of active secondary metabolites. The usual procedure starts with extraction using a suitable solvent and the extract obtained will be separated into different fractions by liquid–liquid partitioning followed by various chromatographic separation techniques including thin layer chromatography, column chromatography (CC) and preparative high-performance liquid

chromatography. Isolation of active compound responsible for bioactivity is usually followed by bioactivity assays (antimicrobial, cytotoxic assays, antioxidant assays etc.) which finally yield active compounds. Structure elucidation of active compound is achieved through spectroscopic techniques, including 1D and particularly 2D NMR and High resolution mass spectrometry analysis.

This chapter describes the isolation and structural elucidation of cytotoxic compounds from the potential isolate *Streptomyces* sp. MCCB 267.

5.2 Materials and Methods

The overall procedure involved in the separation of active compound is given as a flow chart below (Fig. 5.1).

5.2.1 Mass production of *Streptomyces* sp. MCCB267

Streptomyces sp. MCCB267 was mass cultured for the production of secondary metabolites. A loopful of actinomycetes spore was inoculated into a seawater-based seed medium (100 mL, 35ppt) (Beef extract 3g/L, peptone 5g/L). The flasks were incubated at 28 ºC for 48 h on a rotary shaker set at 150 rpm. Subsequently, the content was used to inoculate seawater based fermentation medium in a Erlenmeyer flasks (a total of 10 liters of 1 liter each) (Yang et al. 2013) (soybean meal 3g/L, yeast extract 3g/L, proline 1g/L, beef extract 3g/L, glycerol 6mL/L, K_2HPO_4 0.5g/L, $MgSO_4 \cdot 7H_2O$ 0.5g/L, $FeSO_4 \cdot 7H_2O$ 0.5g/L, $CaCO_3$ 2g/L, pH 7.4) and incubated at 28 ºC for 8 days on a rotary shaker at 150 rpm. After incubation, the broth was centrifuged at 8000 rpm for 5 minutes to separate

mycelial cake from fermentation broth and the supernatant was extracted three times with an equal volume of ethyl acetate. The extract was concentrated under reduced vacuum on a rotary evaporator at 35 ºC and this crude was used for further separation.

5.2.2 Sephadex LH-20 column chromatographic separation

Initially, the crude of *Streptomyces* sp. MCCB267 was fractionated by Sephadex LH-20 (50 x 4 cm) column chromatography to yield 12 fractions. Fractions were eluted using chloroform-methanol solvent system (1:1$CHCl_3$/MeOH). The content and purity of the fractions were examined by TLC (Merck, 10% methanol in $CHCl_{3)}$, UV detection, Vanillin spraying agent) and then combined by their similarity. All the fractions were subjected to bioactivity test using sulforhodamine B (SRB) assay as described elsewhere.

5.2.3 HPLC separation and activity testing of active fractions

All sephadex LH-20 fractions which showed potent cytotoxicity were combined and separated by gradient reversed phase HPLC using RP-C8 column (Waters, COSMOSILC-8 column, 20:80 acetonitrile/0.1% aq. TFA). HPLC separation yielded 5 fractions and which were subjected to cytototoxicity evaluation using sulforhodamine B (SRB) assay. Final purification of the active fractions were done by Semipreparative reversed phase HPLC (C-8, 20: 80 ACN/0.1% aq. TFA), over 35 min and yielded a four pure compound (1- 4).

5.2.4 Characterization of the anticancer molecules from *Streptomyces* sp. MCCB 267

^{1}H and 2D NMR spectroscopic data of the pure compounds were recorded at 500 MHz in $CDCl_3$+ CD_3OD (Varian, USA). ^{13}C- NMR spectra were acquired at 100 MHz (Varian System spectrometer). The structure of the isolated compounds was established by analyses of the 1D and 2D NMR spectra, high-resolution mass spectroscopy, UV spectra, IR spectra, optic rotation (α_D) and comparison of the NMR and MS data with published data (Jomon et al. 1972; Cao et al. 2010; Lacret et al. 2014).

5.2.5 Cytotoxicity evaluation of the fractions using sulforhodamine B (SRB) assay

Cytotoxicity evaluations of the fractions were performed using the sulforhodamine B (SRB) (Sigma, USA) colorimetric assay on 96-well culture plates. The NCI-H460 cell line, obtained from National Centre for Cell Science (NCCS, Pune, India) was maintained in RPMI-1640 (Himedia, India) supplemented with 10% fetal bovine serum (FBS) (Himedia, India) at 37 ºC in CO_2 incubator with 5% carbon dioxide. Aliquots of 190 µL cell suspension with a density of 1.9 x 10^4 cells were pipetted into 96 well micro titer plates. Two fold serial dilutions of each fraction to have a concentration ranging from 1 mg/mL to 15.625 µg/mL in 10% DMSO were prepared and a sample of 10µl of this was added to cells. SRB assay was performed to evaluate cytototoxicity as per the protocol mentioned in Chapter 4 (4.2.1). IC_{50} value was determined by probit analysis (Brownlee et al. 1952).

5.3 Results

5.3.1 Bioassay-Guided Isolation

The secondary metabolites of *Streptomyces* sp. MCCB 267 kept for mass production for 8 days was extracted using ethyl acetate (Fig. 5.2). The metabolites extracted in ethyl acetate were concentrated under vacuum. Dried crude extract yielded ≈1 g and was used for further separation. Bioassay-guided fractionation details and yield of each fractions was given in flow chart (Fig. 5.1).

Initially, the crude ethyl acetate extract was separated into 12 fractions by sephadex LH-20. Fractions 4-6, Fraction 7-9 and Fraction 10-12 were found containing similar types of compounds in TLC. Similar fractions were pooled, concentrated under vacuum and subjected to SRB analysis. IC_{50} value shown by each fraction was tabulated in Table 5.1. Among these 6 fractions, Fr.3 and Fr.4-6 showed IC_{50} values of 9.2 µg/ mL and 1.64 µg/ mL, respectively and these active fractions were pooled and subjected to HPLC separation using RP-C8 column. HPLC separation yielded 5 fractions which were subjected to bioactivity testing. After cytotoxicity analysis Fr.2-5 showed promising IC_{50} of 16.21, 8.11, 1.72, 2.5 µg/ mL, respectively (Table 5.2). Further HPLC separation of these fractions yielded 4 compounds (**1-4**) (Fig. 5.3). The structures of the isolated pure compounds were established by analyses of the 1D and 2D NMR spectra, high-resolution mass spectroscopy, UV spectra, IR spectra, optic rotation (α_D) and comparison of the NMR and MS data with published data (Jomon et al. 1972; Lacret et al. 2014). The compounds were identified as ikarugamycin (**1**, IK, 20 mg, R.T 26.5 min), 30-oxo-28-*N*-methylikarugamycin (**2**, OI, 13.2 mg, R.T 14.9 min), 30-oxo-ikarugamycin (clifednamide) (**3**, CF, 8.6

mg, R.T 13.6 min), and 28-*N*-methylikarugamycin (**4**, MI, 4 mg, R.T 31.5 min).

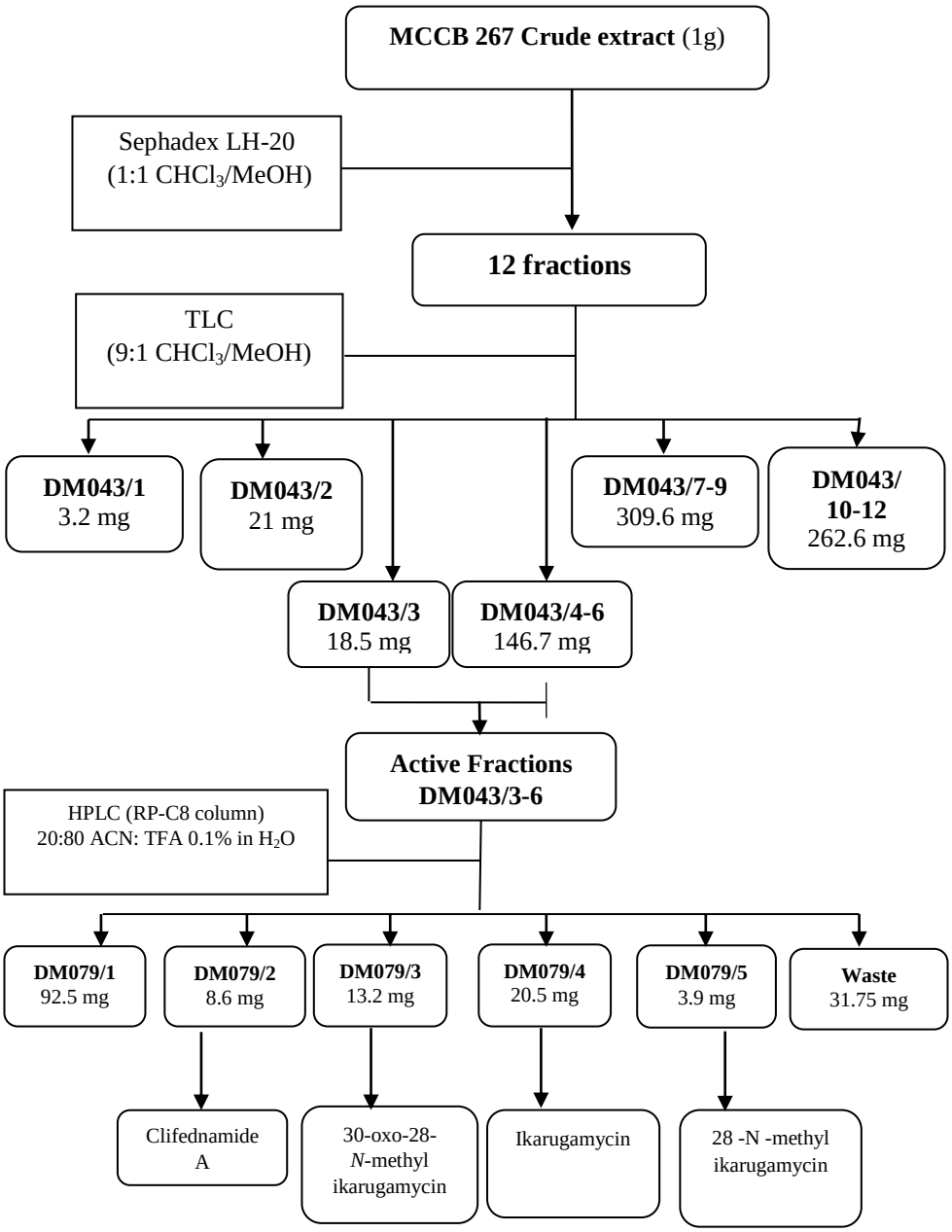


Fig. 5.1: Schematic representation of separation of active compound

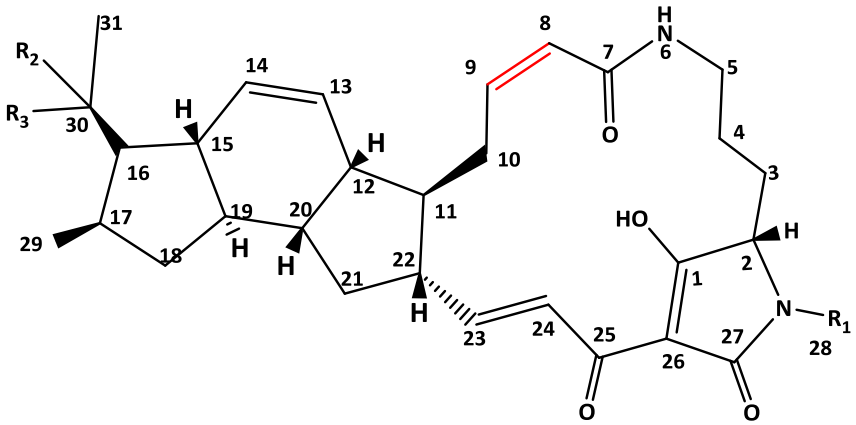
Fig.5.2: a) Fermentation b) Ethyl acetate extraction of *Streptomyces* sp. MCCB 267

Table 5.1: Quantity of each fraction and IC_{50} value shown by each fraction after Sephadex LH 20 chromatography

Fraction	Fraction weight (mg)	IC_{50}
DM043/1	3.2 mg	> 50 µg/ mL
DM043/2	21 mg	Insoluble in DMSO
DM043/3	18.5 mg	9.2 µg/ mL
DM043/4-6	146.7 mg	1.64µg/ mL
DM043/7-9	309.6 mg	> 50 µg/ mL
DM043/10-12	262.6 mg	> 50 µg/ mL

Table 5.2: Quantity of each fraction and IC_{50} value shown by each fraction after RP-HPLC

Fraction name	Fraction weight (mg)	Cytotoxicity IC_{50} (µg/mL)
DM079/1	92.5	26.95
DM079/2	8.6	16.21
DM079/3	13.2	8.11
DM079/4	20.5	1.72
DM079/5	3.9	2.5



R1=H R2=R3=H Ikarugamycin (**1**)

R1=CH_3 R2=R3=O 30-oxo-28-N- methyl ikarugamycin (**2**)

R1=H R2=R3=O Clifednamide A (**3**)

R1=CH_3 R2=R3=H 28-N- methyl ikarugamycin (**4**)

Fig. 5.3: Compounds isolated from culture broth of *Streptomyces* sp. MCCB 267

5.3.2 Structural Determination of the Compound 1

Compound **1** was isolated as a white amorphous solid. The ESI-TOF spectrum of this compound displayed molecular ion at m/z 501.2729 (calc. for [M + H] +501.2720) corresponding to a molecular formula of $C_{29}H_{38}N_2O_4$. The ^{1}H NMR spectrum (Table 5.3and Fig. 5.4) showed the presence of six olefinic protons at δH 7.04 (1H, d, J = 15.0 Hz, H-24), 6.70 (1H, dd, J = 10.0, 15.0 Hz, H-23), 5.86 (1H, brd, J = 10.0 Hz, H-14), 5.61 (1H, m, H-13), 5.98 (1H, dt, J = 3.0, 11.0 Hz, H-9), 5.72 (1H, d, J = 11.0 Hz, H-8). On the other hand, two methyl groups in the aliphatic region at δ 0.79 (3H, d, J = 7.0 Hz, H-29) and 0.85 (3H, t, J= 7.0 Hz, H-31) could also be distinguished. The ^{13}C NMR spectrum exhibited 29 signals and the multiplicity edited HSQC spectra suggested the presence of 5 quaternary, 15 methine, 7 methylene and 2 methyl carbons (Fig. 5.5). The NMR data indicated that compound **1** is ikarugamycin and the molecular formula further confirmed that this compound was ikarugamycin.

Table 5.3: NMR Data of DM079/4 (Ikarugamycin) in $CDCl_3$ + CD_3OD

Position	δ_C [b]	δ_H, multiplicity, *J*(Hz)
1	197.0	
2	61.4	3.77 m
3	26.9	1.70 brd (14.0), 1.95 m
4	20.7	1.08 m, 1.46 m
5	38.7	2.52 dt (3.0, 12.0), 3.48 dt (3.0, 12.0)
6-*N*H		5.76 m
7	166.9	

Cont… Table 5.3

Position	δ_C [b]	δ_H, multiplicity, *J*(Hz)
8	123.5	5.72 d (11.0)
9	141.0	5.98 dt (3.0, 11.0)
10	25.1	2.30 m, 3.38 dt (6.0, 11.0)
11	47.0	1.48 m
12	42.7	2.43 m
13	127.8	5.61 m
14	131.3	5.86 brd (10.0)
15	48.7	1.49 m
16	46.8	1.28 m
17	32.8	2.18 m
18	38.2	0.62 m, 2.02 m
19	48.3	1.09 m
20	41.6	2.01 m
21	36.5	1.16 m, 2.06 m
22	49.3	2.42 m
23	152.6	6.70 dd (10.0, 15.0)
24	121.9	7.04 d (15.0)
25	173.4	
26	100.5	
27	175.4	
28-*N*H		
29	17.4	0.79 d (7.0)
30	21.4	1.28 m, 1.38 m
31	13.0	0.85 t (7.0)

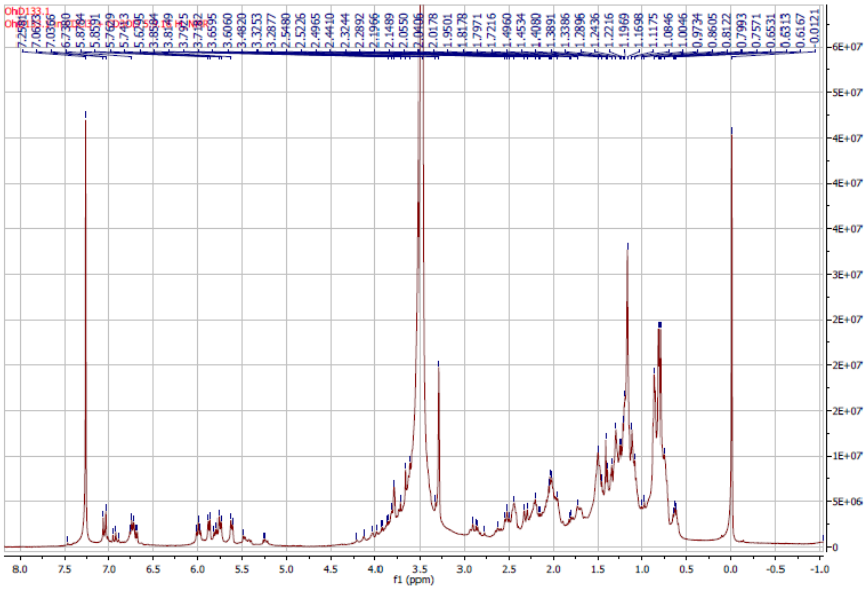


Fig. 5.4: ^{1}H NMR of Ikarugamycin (1)

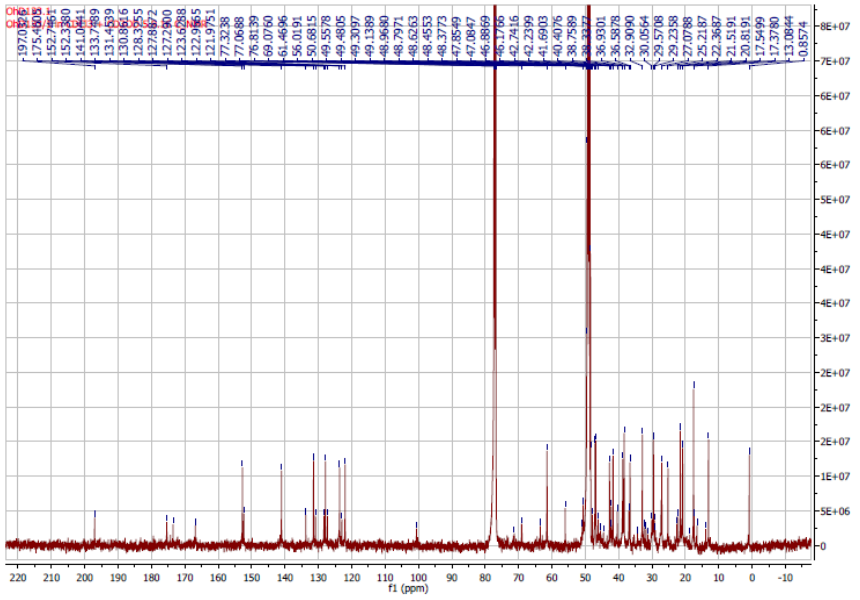


Fig. 5.5: ^{13}C NMR of Ikarugamycin (1)

5.3.3 Structural Determination of the Compound 2

The molecular formula of compound **2** was established as $C_{30}H_{38}N_2O_5$ by ESI-TOFMS (*m*/*z* 507.2859, calc. for [M + H] + 507.2854). Its ^{1}H NMR spectrum (Table 5.4, Fig. 5.6) was very similar to that of compound **4**. The most significant difference between the two spectra was the chemical shift and multiplicity of the signals due to protons of 31-C one of the methyl groups at δ 2.16 (3H, s). NMR data suggested that these two compounds differed in the presence of one carbonyl group in **2**, which must be located at C-30. The ^{13}C NMR spectrum contained 30 signals: 3 methyl, 6 methylene, 15 methine and 6 quaternary carbons, according to spectrum. Four of the quaternary carbons at δ 166.3, 172.4, 173.4 and 210.1 corresponded to carbonyl groups (Fig. 5.7). On the basis of these data the structure suggested for this compound was 30-oxo-28-*N*-methylikarugamycin (Lacret et al. 2014).

Table 5.4: NMR Data of DM079/3 (30-oxo-28-N-methylikaarugamycin) in $CDCl_3$ + CD_3OD

Position	δ_C [b]	δ_H, multiplicity, *J*(Hz)
1	196.0	
2	66.3	3.71 m
3	24.9	1.80 d (12.5), 2.13 m
4	20.6	1.12 m, 1.46 m
5	38.8	2.62 m, 3.70 m
6-*N*H		5.84 m
7	166.3	
8	123.9	5.82 d (10.0)

Cont… Table 5.4

Position	δ_C [b]	δ_H, multiplicity, *J*(Hz)
9	140.8	6.06 t (10.0)
10	25.1	2.35 m, 3.50 m
11	48.1	1.55 m
12	42.5	2.52 m
13	128.7	5.70 t (10.0)
14	130.0	5.73 t (10.0)
15	43.0	2.38 m
16	58.8	2.69 t (10.5)
17	33.7	2.64 m
18	38.7	0.82 m, 2.16 m
19	47.7	1.22 m
20	41.1	2.14 m
21	36.5	1.25 m, 2.16 m
22	49.3	2.52 m
23	151.4	6.72 dd (10.0, 15.0)
24	122.4	7.13 d (15.0)
25	172.4	
26	100.8	
27	173.4	
28	26.3	2.93 s
29	18.7	0.86 d (6.5)
30	210.1	
31	31.4	2.16 s

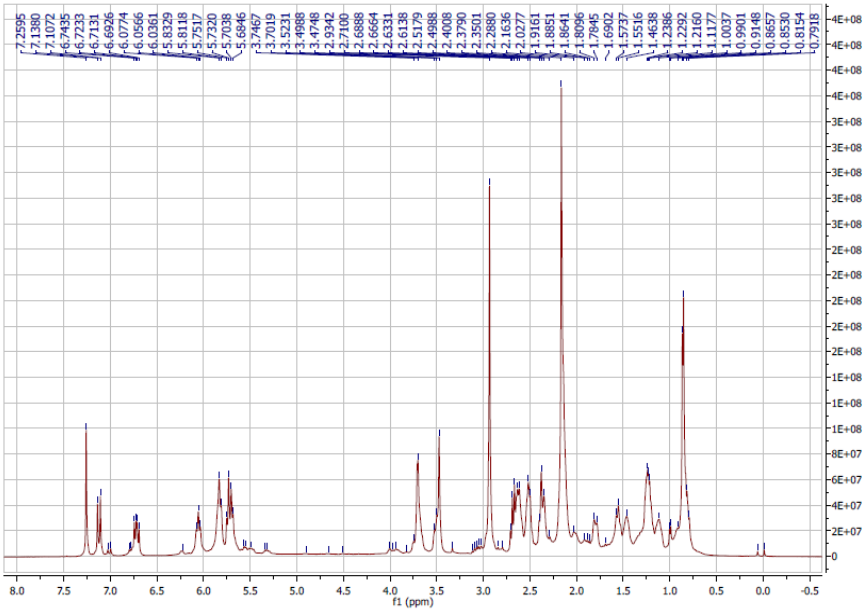


Fig. 5.6: ^{1}H NMR of 30-oxo-28-N- methyl ikarugamycin (2)

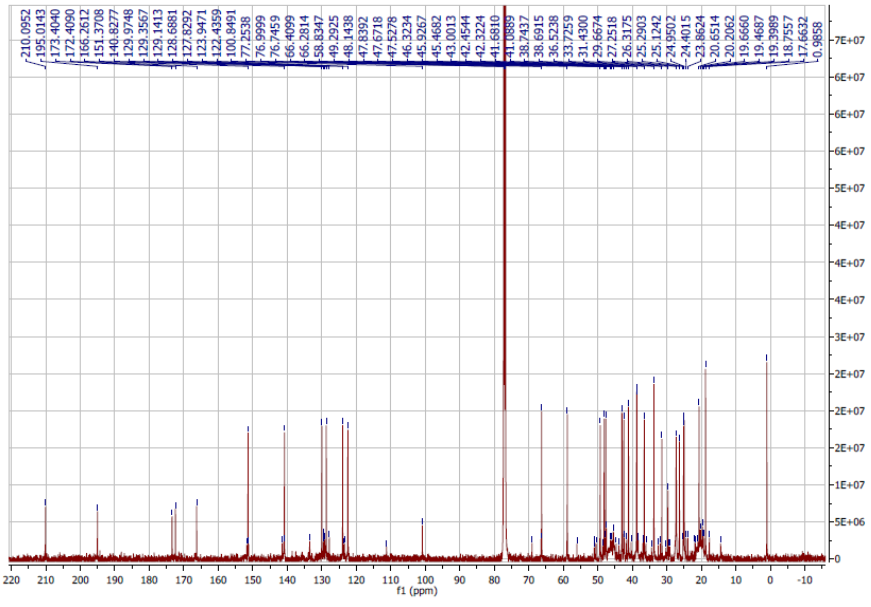


Fig. 5.7: ^{13}C NMR of 30-oxo-28-N- methyl ikarugamycin (2)

5.3.4 Structural Determination of the Compound 3

Compound **3** was isolated as white amorphous solid. The molecular formula of compound was established as $C_{29}H_{36}N_2O_5$ by ESI-TOFMS m/z 493.2697 $[M + H]^+$(calculated for 493.2702). Four of the quaternary carbons at δ 166.8, 173.4, 175.4 and 211.2 corresponded to carbonyl groups. The 28 NH was hidden. The ^{1}H NMR and ^{13}C NMR spectra of 3 (Table 5.5 and Fig. 5.8-9) were very similar to those of 1 (Table 5.3), indicating **3** as derivative of ikarugamycin, and identified as Clifednamide A. The difference between the two compounds was the presence of carbonyl group in C-30 position.

Table 5.5: NMR Data of DM079/2 (Clifednamide A) in $CDCl_3$ + CD_3OD

Position	δC b	δH, multiplicity, J(Hz)
1	197.0	
2	61.4	3.78 brs
2	26.9	1.70 brd (13.0), 1.94 m
4	20.7	1.08 m, 1.45 m
5	38.7	2.52 m, 3.50 m
6-NH		Hidden
7	166.8	
8	123.7	5.75 d (10.5)
9	140.7	5.98 dt (1.5, 10.5)
10	24.9	2.28 t (10.5), 3.43 m
11	48.4	1.48 m

Cont… Table 5.5

Position	δC b	δH, multiplicity, J(Hz)
12	42.3	2.45 m
13	128.6	5.64 t (10.0)
14	129.7	5.65 t (10.0)
15	42.8	2.28 m
16	58.8	2.63 t (10.5)
17	33.6	2.57 m
18	38.5	0.73 m, 2.10 m
19	47.4	1.14 m
20	41.0	2.07 m
21	36.4	1.16 m, 2.08 m
22	49.3	2.43 m
23	**152.0**	6.69 dd (10.0, 15.5)
24	122.2	7.06 d (15.5)
25	173.4	
26	100.6	
27	175.4	
28-NH		
29	18.5	0.78 d (7.0)
30	211.2	
31	31.2	2.10 s

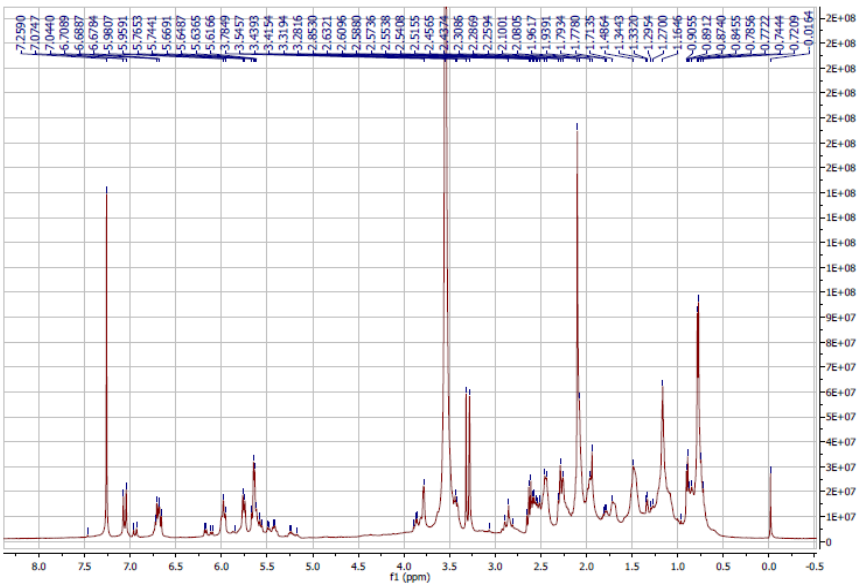


Fig. 5.8: ^{1}H NMR of Clifednamide A (3)

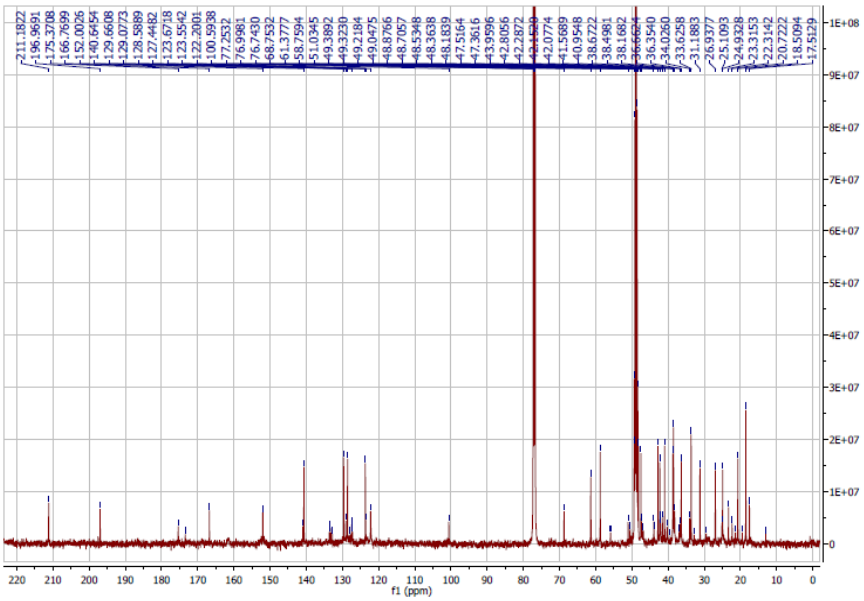


Fig. 5.9: ^{13}C NMR of Clifednamide A (3)

5.3.5 Structural Determination of the Compound 4

Compound **4** was obtained as a white amorphous solid. Its molecular formula was assigned as $C_{30}H_{40}N_2O_4$ by ESI-TOFMS (*m*/*z* 493.3063, calc. for [M + H] + 493.3061). The ^{1}H NMR and ^{13}C NMR spectra of **4** (Table 5.6 and Fig. 5.10-11) indicated that it was a derivative of ikarugamycin **(1).** The singlet at δ 2.94 (3H, s) was assigned to an *N*-methyl group, being the major difference with respect to the spectrum of ikarugamycin, indicating that compound **4** should be an *N*-methyl polycyclic tetramic acid macrolactam with ikarugamycin skeleton, and identified as 28-N- methyl ikarugamycin. The ^{13}C NMR spectrum exhibited 30 signals and the multiplicity confirmed the presence of 5 quaternary, 15 methine, 7 methylene and 3 methyl carbons.

Table 5.6: NMR Data DM079/5
(28-N- methyl ikarugamycin) in $CDCl_3$ + CD_3OD

Position	δ_C	δ_H, multiplicity, *J*(Hz)
1	195.0	
2	66.3	3.71 d (3.5)
3	25.0	1.81 brd (15.0), 2.14 m
4	20.7	1.12 m, 1.48 m
5	38.8	2.62 brd (12.0), 3.70 m
6-*N*H		5.78 d (6.0)
7	166.3	
8	123.9	5.82 d (12.0)
9	141.1	6.07 dt (2.5, 12.0)
10	25.3	2.40 dd (2.5, 17.5), 3.47 m

Cont… Table 5.6

Position	δ_C	δ_H, multiplicity, *J*(Hz)
11	48.2	1.57 t (11.0)
12	42.8	2.52 m
13	128.0	5.70 td (2.5, 10.0)
14	131.5	5.95 d (10.0)
15	47.2	1.57 m
16	47.0	1.37 m
17	33.0	2.28 m
18	38.4	0.70 td (7.0, 12.0) 2.02 m
19	48.8	1.15 m
20	41.7	2.08 m
21	36.7	1.24 m, 2.11 m
22	49.4	2.52 m
23	151.9	6.74 dd (15.0, 10.0)
24	122.3	7.13 d (15.0)
25	172.6	
26	100.8	
27	173.5	
28	26.3	2.94 s
29	17.7	0.88 d (7.0)
30	21.6	1.37 m, 1.47 m
31	13.2	0.94 t (7.0)

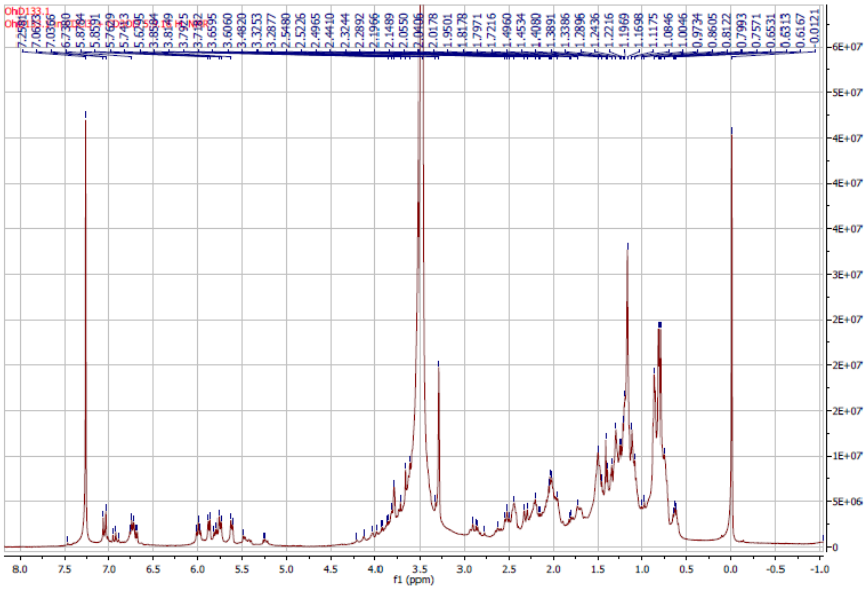


Fig. 5.10: ^{1}H NMR of 28-N- methyl ikarugamycin (4)

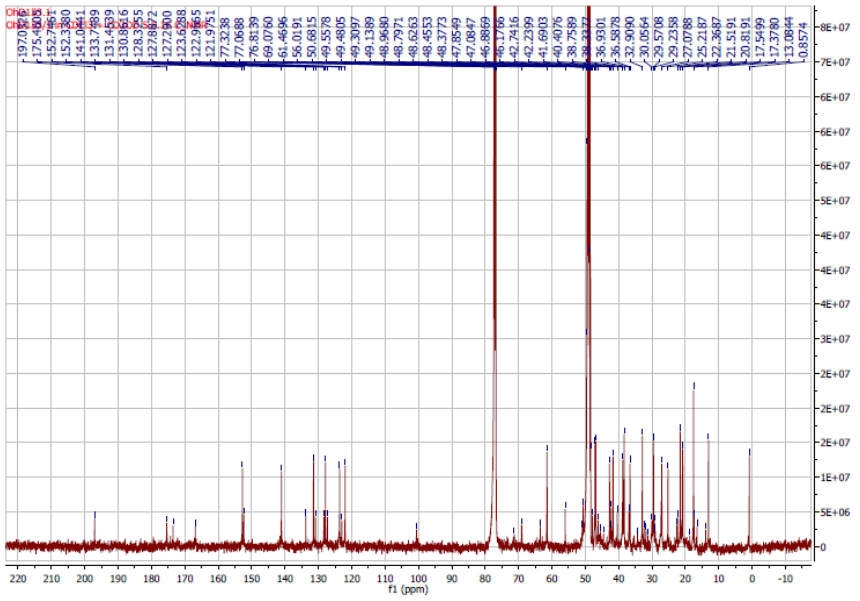


Fig. 5.11: ^{13}C NMR of 28-N- methyl ikarugamycin (4)

5.4 Discussion

The bioassay guided separation of the *Streptomyces* sp. MCCB267 extract yielded four potential anticancer compounds belonging to Ikarugamycin family of polycyclic tetramate macrolactams (PTMs). After molecular networking, it was found that one of the major chemical groups in *Streptomyces* sp. MCCB267 was PTMs. Isolation of these PTMs, supports and validates the GNPS molecular networking analysis about the presence of PTMs like compounds in the extract. It is also important to note that the Ikarugamycine and its analogouese spectra were not annotated in the GNPS mass spectral databases and that is why the GNPS molecular networking did not showed the Ikarugamycine or it's analogous during the analysis.

Polycyclic tetramate macrolactams (PTMs) contain one tetramic acid and a polycyclic system (usually two to three rings) fused to macrolactams. PTMs exhibit a wide range of biological activities such as antiprotozoal (Jomon et al. 1972), antiviral (Luo et al. 2001), antibacterial (Bertasso et al 2003) and antitumor properties (Jomon et al. 1972; Popescu et al. 2011). Few PTMs were already isolated directly from marine sponges or from its symbionts (Royles 1995). Discodermide is an cytotoxic PTM isolated from a marine sponge *Discodermia dissolute* (Gunasekera et al. 1991). A cytotoxic PTM Cylindramide was isolated from the marine sponge *Halichondria cylindrata* (Kanazawa et al. 1993). Alteramide A was isolated from a bacterium *Alteromonas* sp. associated with the marine sponge *Halichondria okadai* (Shigemori et al. 1992). PTMs were also isolated from many free living bacteria including diverse genera *Streptomyces*, *Alteromonas*, *Stenotrophomonas*, and *Lysobacter*. *Stenotrophomonas maltophilia* produces an antifungal PTM compound called Maltophilin

(Jakobi et al. 1996). *Lysobacter enzymogenes* produces a heat stable antifungal factor HSAF which is a PTM class of compound with a novel mode of action (Yu et al. 2007; Lou et al. 2011). Apart from these bacterial genera *Streptomyces* itself produces different PTM compounds which include ikarugamycin, dihydromaltophilin, frontalamides etc (Jomon et al. 1972; Graupner et al. 1997; Blodgett et al. 2010).

The identification of the frontalamide biosynthetic locus facilitated systematic searches for similar PTM loci (Blodgett et al. 2010). Such genome-mining efforts revealed highly conserved frontalamide-like biosynthetic clusters in the genomes of phylogenetically different bacteria ranging from proteobacteria to actinomycetes, particularly within the *Streptomyces* genus. Notably, almost all of the identified bacteria were not previously recognized as PTM producers. Later, PCR based screen to detect putative PTM biosynthetic loci was developed to probe environmental isolates, leading to identification of numerous isolates harbouring putative PTM clusters (Cao et al. 2010).

Ikarugamycin (IK) was first isolated from *Streptomyces phaeochromogenes* sub-sp. *ikaruganensis*, which was first reported for its strong antiprotozoal activity (Jomon et al. 1972). Later, ikarugamycin was isolated from other bacteria such as *Streptomyces zhaozhuensis*, *Streptomyces harbinensis*, *Micromonospora* sp. K310 etc. (Liu et al. 2013; Kyeremeh et al. 2014; Lacret et al. 2014). Ikarugamycin was reported to induce cytotoxicity in wide ranges of tumor cell lines but do not show any selective anticancer activity (Popescu et al. 2011). Recently, Lacret et al. (2014) reported 30-oxo-28-*N*-methyl ikarugamycin (OI), 28-*N*-methyl ikarugamycin (MI) from *S. zhaozhuensis* and reported their antifungal and

antibacterial properties. Clifednamide A (CF) was recently reported from an environmental *Streptomyces* sp. using a targeted PCR based screening approach (Cao et al. 2010). However, apart from ikarugamycin, the other three compounds were not reported or tested for anticancer activity. Also the bioactivity of Clifednamide A (CF) has not been reported sofar.

Biological activity of Ikarugamycin and related analogues are quite interesting. Ikarugamycin induces apoptosis in leukemia cells through genotoxicity and activation of caspases 9, 8 and 3. Caspase activation was in part correlated to an increase in intracellular calcium concentration and the activation of p38 MAP kinase (Popescu et al. 2011). Ikarugamycin was also found to inhibit oxidized low-density lipoprotein (LDL)-induced accumulation of cholesteryl ester in macrophage J774 at very low concentration. The studies indicate that IK inhibits cholesteryl ester accumulation in macrophage J774 by specifically inhibiting the uptake of oxidized LDL (Hasumi et al. 1992). Ikarugamycin also shows antiviral activities by inhibiting Human immunodeficiency virus type I Nef-induced CD4 cell surface downregulation (Luo et al. 2001). Recently, Elkin et al. (2016) verified the inhibitory effect of IK on clathrin-mediated endocytosis (CME) and found that long-term incubation with IK has cytotoxic effects, whereas short-term inhibitory effects on CME are reversible. Thus, it is possible to use IK as a useful tool for probing routes of endocytic trafficking.

This is the first time that all the four members of PTMs antibiotic viz. clifednamide A (CF), 30-oxo-28-*N*-methyl ikarugamycin (OI), ikarugamycin (IK) and 28-*N*-methyl ikarugamycin (MI) have been isolated from the same natural source. PTM compounds are structurally and

pharmacologically unique natural products. PTMs exhibit unique structural diversity by fusing the macrolactam ring with a different set of carbocyclic rings. Ikarugamycin (IK) and clifednamides (CF) have a 5-6-5 set of carbocyclic rings, but majority of other PTMs has either 5-5-6 (discodermide and frontalamides) or 5-5 (alteramide and cylindramide) ring arrangements. The post-modifications of carbocyclic ring and the macrolactam ring would yield many structural analogues of Ikarugamycin. The analogues of Ikarugamycin are probably the products of post-modification of parent IK and the mechanism of this biotransformation of IK to these analogues is not well studied. Clifednamides (30-oxo ikarugamycin) could be the product of oxidation of the side chain at C-30 and methylation at N-28 of the macrolactam ring gives rise to 28-*N*-methyl Ikarugamycin. However, methylation of clifednamide at N-28 yields 30-oxo-28-*N*-methyl Ikarugamycin. PTMs produced by the *Streptomyces* sp. could be the products of a single gene cluster and it is significant to note that an actinomycete harbouring PTM gene cluster could be potentially capable of producing diverse types of PTMs, if activated appropriately. Recently, Blodgett et al (2010) reported that the PTM gene cluster is highly conserved in phylogenetically diverse bacterial strains and could remain silent or cryptic.

In short, this study reports the isolation of four PTMs compounds, Ikarugamycin, Clifednamides, 28-*N*-methyl Ikarugamycin, and 30-oxo-28-*N*-methyl Ikarugamycin from a single isolate of *Streptomyces* sp. MCCB 267.

Chapter 6

IN VITRO EVALUATION OF ANTICANCER ACTIVITY OF IKARUGAMYCIN AND ITS ANALOGUES ISOLATED FROM *STREPTOMYCES* SP. MCCB 267 ON NON SMALL CELL LUNG CANCER CELL LINES

Contents

6.1 Introduction

Chemotherapy is a type of cancer treatment that uses drugs to kill cancer cells. Chemotherapy was introduced in to clinic for more than fifty years ago for the treatment of cancers (Johnstone et al. 2002). It is very successful for tumors such as testicular cancer and certain leukemias, whereas success for the treatment of common epithelial tumors of the breast, colon, and lung has been comparatively less. Most of the anticancer drugs used in chemotherapy kill cancer cells by damaging DNA, which leads to programmed cell death; the apoptosis. It is characterized by morphological

and physiological hall marks including shrinkage of cells, DNA fragmentation and membrane blebbing.

In the present chapter ikarugamycin and its analogues isolated from *Streptomyces* sp. MCCB 267 were analyzed for mode of anticancer activity. Their ability to bind DNA by molecular docking and molecular dynamic simulation were studied. Validation of the *in silico* modelling results were done by *in vitro* cell based bioassay experiments and elucidation of mode of anticancer activity was attempted.

6.2 Materials and Methods

6.2.1 Molecular Docking

Ikarugamycin and analogues were studied for their interaction and binding affinity with DNA by molecular docking studies.

The B form DNA model (5'-CGCGAATTCGCG-3') was generated with structure build tool in UCSF chimera molecular visualization package (Goddard et al. 2007). The ikarugamycin (IK) derivatives were designed and their geometry optimized using Marvin Sketch software (ChemAxon 2013). Docking studies were done using AutoDock 4.2 (Norgan et al. 2011) to accurately predict the orientation of the drug derivatives in the DNA binding groove. Docking was performed by calculating the energy grids of molecular interaction in a cubic box size 34 X 34 X 34 Å; grid spacing 0.375 Å centered around the minor groove of the DNA target. The internal degrees of freedom of the DNA were held fixed whereas the drug molecules were made flexible. Charges were computed by the Gasteiger-Marsili method (Gasteiger and Marsili 1980). Lamarkian Genetic Algorithm was employed to carry out the estimation of the position of each drug with

random starting position and conformation with respect to the DNA energy grids and standard parameters (Morris et al. 1998) applied. The final docked conformations were ranked according to their binding free energy and clustered using 3 Å root mean square deviations (RMSD). The graphical representation of the results was carried on Pymol program (DeLano 2002).

6.2.2 Molecular Dynamic Simulations

To verify robustness of the docking affinity results, molecular dynamic (MD) simulations were performed for IK and derivatives complexed with DNA. Molecular Dynamic (MD) simulations for DNA bound with IK and derivatives were performed using GROMACSv5.1.4 (Berendsen et al. 1995) with Amber ff99sb force field (Pérez et al. 2007). The topology for the ligands were generated using PRODRG (Schüttelkopf and van Aalten 2004) with charges kept full and no energy minimization done. The complex was solvated with SPC water molecules added to a dodecahedron box with periodic boundary conditions. To neutralize the system counter-ions (Na+) were added into the simulation box. Particle Mesh Ewald (PME) (Petersen 1995) method was used for electrostatic calculations with coulomb cutoff 10A, van der Waals at 14A and Fourier spacing 1.6. The covalent bonds were constrained by the Linear Constraint Solver (LINCS) algorithm (Hess et al. 1997). The system is energy minimized with steepest descent algorithm with a tolerance value of 1000 kJ mol^{-1} nm^{-1}. Further the system was equilibrated with NPT (constant number of particles, pressure, and temperature) ensemble followed by NVT (constant number of particles, volume and temperature) at 300K for 5ns. Finally, the production of MD simulations was performed for 20ns duration for all the simulations. The binding energy of DNA-ligand interactions were

estimated using Molecular Mechanics Poisson-Boltzmann surface area (MMPBSA) (Kollman et al. 2000). G_mmpbsa(Kumari et al. 2014) tool was used to calculate the MMPBSA calculation using the snapshots achieved from last 5ns of the system trajectories.

6.2.3 Evaluation cytotoxicity of compounds and IC_{50} determination using MTT assay

Cytotoxicity evaluations of the compounds were performed on NCI-H460 lung cancer cell line as well as on a cell line derived from normal epithelial kidney cells from the African green monkey (BS-C-1) using MTT cell viability assay. The NCI-H460 cell line was obtained from the National Centre for Cell Science (NCCS, Pune, India) and was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37 ºC in an incubator with 5% carbon dioxide. Two-fold serial dilutions of the compounds were prepared to obtain concentrations ranging from 1 mg/mL to 15.625 µg/mL in 10% aqueous (aq.) DMSO. A 10 µL aliquot of each concentration was added to the wells containing 190 µL NCI-H460 cell suspensions with a density of 1.9 x 10^4 cells/well, in 96 well micro titer plates. Aqueous DMSO (10%) was used as control. All assays were performed in triplicate. The plates were incubated for 48 h at 37 ºC in a CO_2 (5%) incubator. After incubation period the culture medium was discarded and replaced with 200µL fresh medium and further treated with 50µl (1 mg/ml) MTT solution. Treated samples were incubated in dark for further 4 h at 37 ºC. After that both medium and MTT reagents were discarded and 200µl of DMSO was added to each well to dissolve the formazan crystals and the optical density (OD) was recorded at 570 nm in a microplate reader (Tecan, Switzerland). Percentage of cell-growth inhibition (GI) was calculated according to the following equation:

Percentage of viability = (Mean OD_{sample}- Mean OD_{blank})/ (Mean $OD_{control}$- Mean OD_{blank}) *100

Percentage of Growth Inhibition = 100 - Percentage of viability

IC_{50} values were determined based on probit analysis (Brownlee et al. 1952).

6.2.4 Hoechst 33342 staining for scoring apoptosis

NCI-H460 cells were seeded in a chamber slide at a concentration of 1.9×10^4 cells per well. Compounds were added to NCI-H460 cells at its log phase at a concentration of their IC_{50} value and incubated for 24 h at 37 °C with 5% CO_2. After 24 h of treatment, the culture medium was removed and cells were washed twice with PBS and stained with DNA specific Hoechst 33342 dye (2 µg/mL in PBS, Sigma Chemicals, USA) for 10 min at 37°C. At the end of staining, cells were observed under a fluorescence microscope for apoptotic features. Doxorubicin (Sigma, USA) was used as a positive control and DMSO was used as a negative control.

6.2.5 *In situ* detection of DNA damage (TUNEL assay) for the evaluation of apoptosis

In situ labelling of the 3′-OH end of the DNA fragments generated during apoptosis was performed using *In Situ* Cell Death Detection Kit (TUNEL) (Roche Diagnostics, Switzerland). Briefly, the NCI- H460 cells at its log phase were treated with the compounds at their IC_{50} value for 24 h in chamber slide (Millicell EZ slide, Millipore Corporation, US) and incubated at 37 °C in a CO_2 incubator at 5% CO_2. At the end of the treatment, culture medium was removed and the cells were washed with 1X PBS and were air dried. The cells were then fixed with freshly prepared 4% paraformaldehyde

for 1 h at 25º C. The slide was rinsed with PBS and was incubated in permeabilisation solution (0.1% Triton X-100 in 0.15% sodium citrate) for 2 minutes on ice. Slides were washed twice with PBS and 50µL of TUNEL reaction mixture was added in each well. The cells were incubated in dark for 60 minutes at 37ºC in a humidified atmosphere. Subsequently, cells were washed three times with PBS and observed under fluorescent microscope for DNA strand breaks.

6.2.6 Annexin V-Propidium iodide (PI) double staining for confirming apoptosis

AnnexinV-Propidium iodide (PI) staining was performed as per the manufacturer's instructions (Roche Diagnostics,Switzerland). Briefly, cells grown in chamber slides (Millicell EZ slide, Millipore Corporation, US) were treated with compounds at their IC_{50} value, i.e Clifednamide (CF, 16.3 µg/mL), 30-oxo-28-*N*-methyl ikarugamycin (OI, 6 µg/mL), ikarugamycin (IK, 1.5 µg/mL) and 28-*N*-methyl ikarugamycin (MI, 1.8 µg/mL). After adding the compounds, the cells were counted and observed under a fluorescent microscope for apoptotic features at regular intervals of treatment (0, 4, 12 and 24 h). A minimum of three different microscopic fields were counted and the average calculated and expressed as a percentage of the total cell population. Cells treated with 10% aqueous DMSO was used as a negative control. The results represent the data of three replicates (means ± SD) (* $P< 0.05$, ** $P < 0.01$ and *** $P < 0.005$ **** $P < 0.001$ versus the control).

6.2.7 Cell cycle Analysis using Flow cytometry

Cell cycle analysis was performed using flow cytometer (BD *FACSCalibur*™, USA). Briefly, cells were cultured in 6 well microtiter

plate (4× 10^5 cells/well) for 24 h and was then treated with the compounds at their IC_{50} values for 24 h. After incubation, cells were trypsinized, centrifuged and re-suspended in 1 mL cold Ca^{++} and Mg^{++} free PBS (GIBCO) and added into 3mL chilled absolute ethanol. Fixed cells were washed with 1 mL of ice cold Ca^{++} and Mg^{++} free PBS and 1 mL of Propidium iodide (PI) staining solution (2.5 mg PI, 4 mg RNAase, 30 µL tween20 in 100 mL Ca & Mg free PBS) was added to pellet and analyzed the percentage of cells in Sub G1, G0-G1, S and G2-M phase by FACS machine.

Similarly, the percentage of Sub-G1 peak, indicative of DNA fragmentation and a measure of apoptosis, was determined using flow cytometry by treating NCI-H460 cells with three different concentrations of drugs (13 µg/mL, 16.3µg/mL and 19 µg/mL of clifednamide (CF); 6 µg/mL, 7.2 µg/mL and 9 µg/mL of 30-oxo 28-*N*-methyl ikarugamycin (OI); 1 µg/mL, 1.5 µg/mL and 2µg/mL ikarugamycin (IK) and 1 µg/mL, 1.8 µg/mL and 2.5µg/mL of 28-N-methyl ikarugamycin (MI) for 24h. Data represent the mean of independent experiment (means ± SD). * P< 0.05, ** P < 0.01 and *** P < 0.005 **** P < 0.001 as compared to control cells.

6.2.8 Statistical analysis

Significance of results was performed by t-test or one-way ANOVA with GraphPad Prism 7.03 software (SPSS Inc., USA). Graph figures represent mean ± SD of at least three independent experiments.

6.3 Results

6.3.1 Molecular Docking

Docking studies were carried out using AutoDock, which allowed to estimate the interaction energies between the ligands and DNA target. IK

and derivatives were docked to the minor groove of the DNA in order to understand the binding process at atomic scale. A blind docking was performed in the binding pocket to predict the possible binding modes of the ligands. Through clustering analyses, the docked conformations of the docked derivatives were ranked in the order of their increasing energies. The docked energies reported by AutoDock include the intermolecular, intramolecular interaction and de-solvation energies. The docking energies were calculated to be -7.971, -7.149, -5.580 and -3.689 kcal/mol for IK, OI, MI and CF complexes respectively. The predicted docking score of derivatives are listed in Table 6.1. From this docking analysis, it was concluded that the structural derivatives of IK affect its binding affinity and gives a hypothetical assessment of binding mechanism of the ligands under study.

Table 6.1: Comparison of the docking scores of Ikarugamycin and derivatives. E_{stat}- denotes electrostatic interactions, vdw- van der Waals, hb-hydrogen bonds, dsolv-desolvation energies

dsolv	vdw	hb	E_{stat}	Docking energy (kcal/mol)	Ligand
3.0116	-11.8319	-0.4591	-0.4815	-7.9711	IK
2.5053	-11.4414	0	0.2955	-7.1491	OI
2.136	-9.105	-1.179	-0.1167	-5.58	MI
1.09	-3.7331	-0.8371	-1.1041	-3.6893	CF

6.3.2 Molecular Dynamic Simulation Analysis

The low energy structures obtained from docking were used as starting conformation for MD study. In order to measure the structural stability, the energy of the system has been measured in relative to time. The overall energy of the system fluctuated around the mean energy at 300K

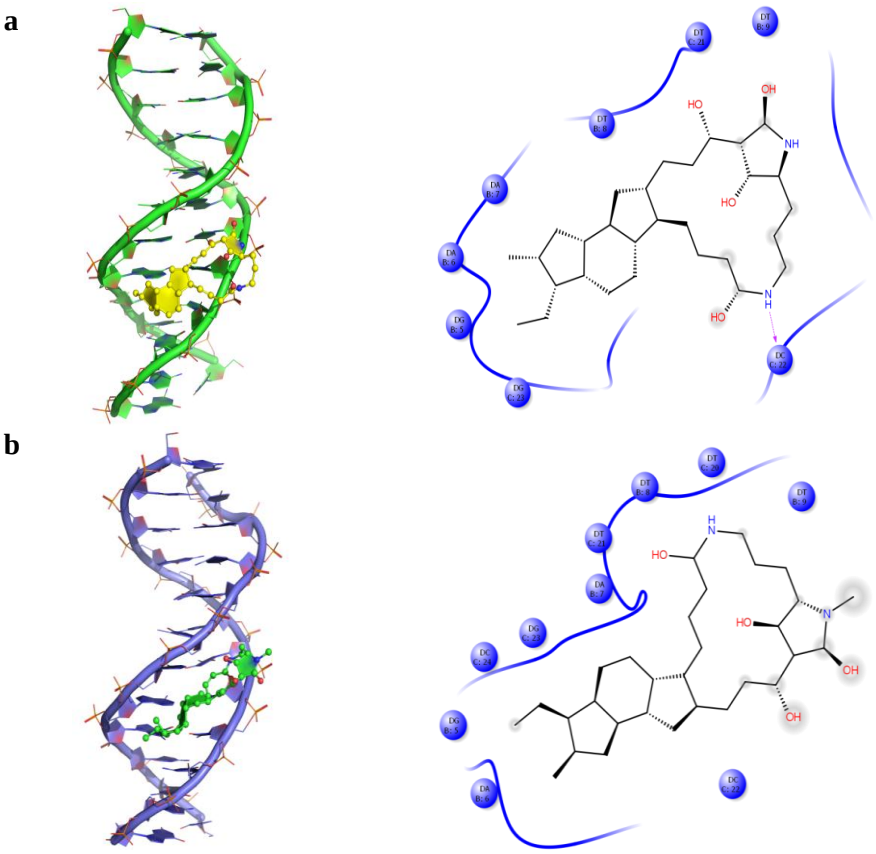
temperature and one atmosphere pressure. The initial and final structures of the DNA-ligand complexes retrieved from the trajectories are shown in the Fig. 6.1. Based on the production trajectory, the MM-PBSA based binding energy (ΔE) was calculated for the last 5ns for the DNA versus ligands using g_mmpbsa tool. The binding free energy of the ligands in the minor binding groove of DNA and the contribution of each component were summarized in Table 6.2.

Table 6.2: The calculated binding free energy components of IK, MI, OI and CF based on MM-PBSA. vdw: van der Waals; SASA: Solvent Accessible Surface Area

MM-PBSA energy terms (KJ/mol)					Ligand
$\Delta E_{binding}$	ΔE_{SASA}	$\Delta E_{polar\ solvation}$	$\Delta E_{electrostatics}$	ΔE_{vdw}	
-248.965 ± 11.114	-19.685 ± 2.605	301.456 ± 8.725	-480.248 ± 4.734	-50.452 ± 3.424	IK
-84.827 ± 7.233	-39.303 ± 3.112	105.019 ± 6.177	-45.168 ± 1.977	-105.624 ± 4.634	MI
-35.856 ± 6.833	-42.898 ± 2.968	116.618 ± 8.148	0.561 ± 0.994	-110.144 ± 4.891	OI
4.502 ± 10.507	-20.880 ± 2.301	106.088 ± 10.721	-28.063 ± 1.576	-52.282 ± 2.468	CF

Table 6.2 reports the $\Delta E_{binding}$ for IK and derivatives bound to target DNA. The predicted $\Delta E_{binding}$ report the most favorable binding energy for IK compared to MI, OI and CF. The result shown as $\Delta E_{binding}$ was a summation of different energy terms, and each energy terms were analyzed individually. Large positive polar solvation energy indicates a transition of charged/polar residues to a higher hydrophobic situation. Interestingly, the IK has large $\Delta E_{electrostatic}$ and low ΔE_{vdw} energy providing a favorable gain only for IK and contributed towards its affinity. The complex formation of DNA-IK was mainly compensated by the favorable electrostatic energy and

unfavorable polar solvation energy, while such stabilization arising from polar interactions were not observed in MI and OI. A large contribution of ΔE_{SASA} suggesting more solvent accessibility in MI and OI that might affect the possibility of interaction between these derivatives with minor groove of DNA target and substantially decreased their affinity. Furthermore, the unfavorable polar solvation energy was neither compensated by vdw energy, electrostatic interactions and SASA predicting essentially the least $\Delta E_{binding}$ (positive) for CF. Subsequently SASA results revealed that the change hydrophobic to hydrophilic regions in the derivatives had also affected their DNA binding. The initial and final structure of the DNA-ligand complexes captured from the trajectories was shown in Fig.6.1.



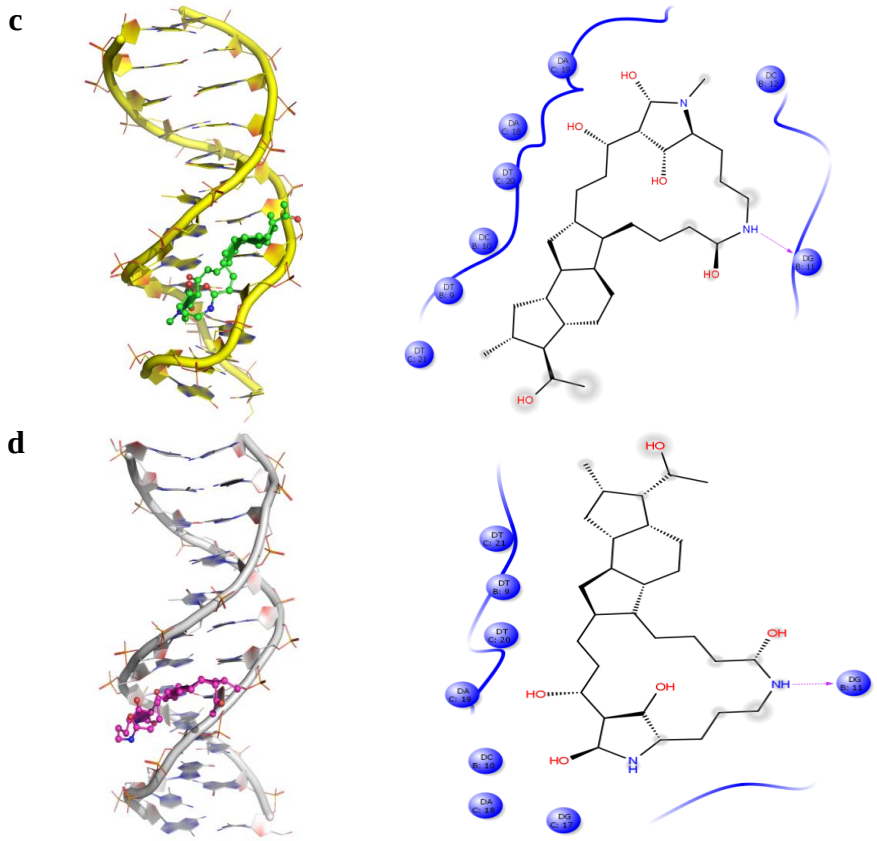


Fig. 6.1: A diagrammatic representation of the DNA molecule with the ligands highlighted in ball and stick in the minor binding groove (Left). DNA-ligand interaction map representing the nucleotide residues in the minor groove bound to the ligands, Hydrogen bonds are shown as dotted lines (Right) (a) IK (b) MI (c) OI and (d) CF

6.3.3 Evaluation for potential antitumor activity on NCI-H460 cells *in vitro* using MTT assay

The antitumor activity and IC_{50} values of the compounds were evaluated by MTT assay. The IC_{50} values of compounds CF, OI, IK and MI were found to be 16.29, 7.17, 1.43,1.78 µg/mL, respectively in NCI-H460 cells and16.33, 7.39, 3.27 and 8.25 µg/mL, respectively in BS-C-1 normal cells (Table 6.3). All the compounds showed growth inhibition in a

concentration dependent manner (Fig. 6.2). Probit regression line for the determination of IC_{50} for all the four compounds are shown in Fig. 6.3 and Fig.6.4

Table 6.3: IC_{50} of IK and its analogues using probit analysis

Sl. No.	Compound	IC_{50} (µg/mL) NCI-H460	IC_{50} (µg/mL) BS-C-1
1	Clifednamide A	16.29	16.33
2	30-oxo-28-N-methyl ikarugamycin	7.17	7.39
3	Ikarugamycin	1.43	3.27
4	28-N-methyl ikarugamycin	1.78	8.25

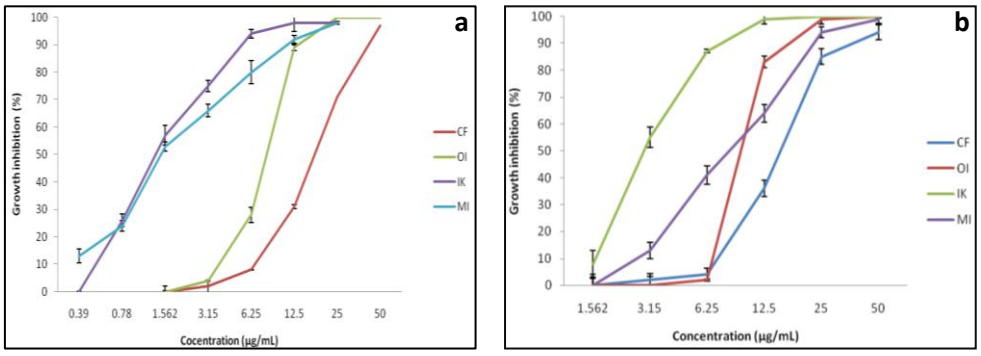


Fig. 6.2: Growth inhibitory effect of compounds on a) NCI-H460 cells and b) BS-C-1cells after 48 h incubation determinations (Results were shown as means ± SD of at least triplicate observations)

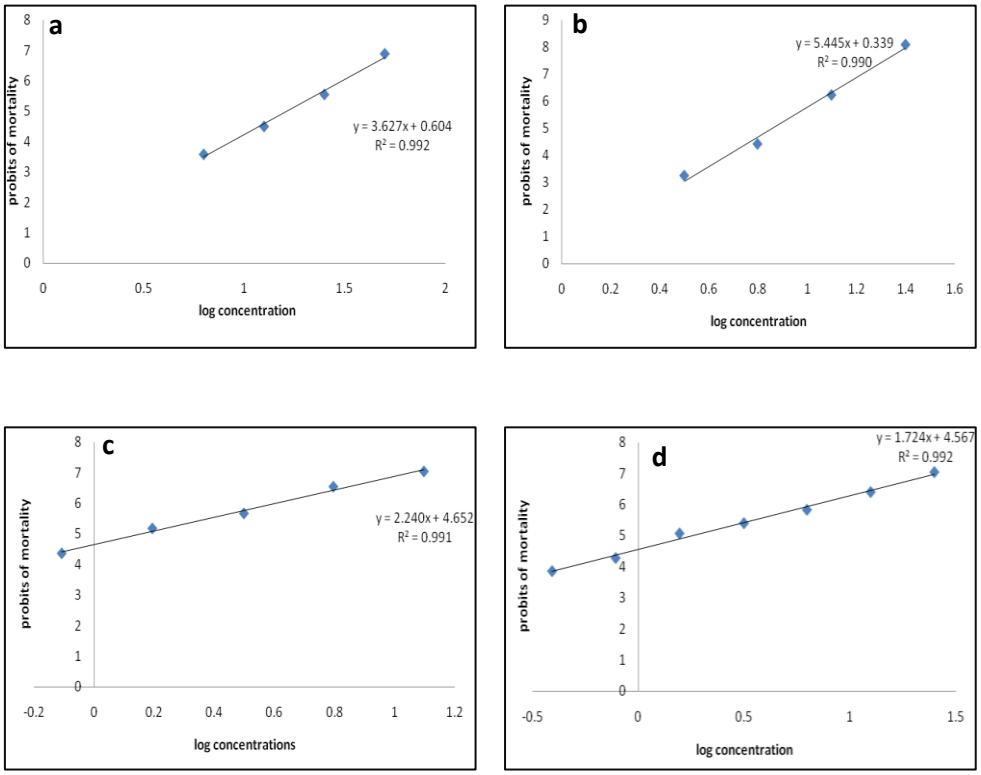
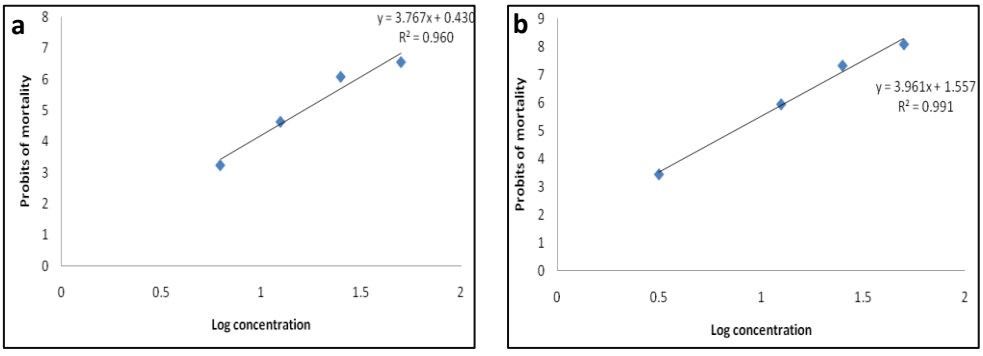


Fig. 6.3: Probit regression line for the determination of IC_{50} of a) CF b) OI c) IK and d) MI on NCI-H460 cells



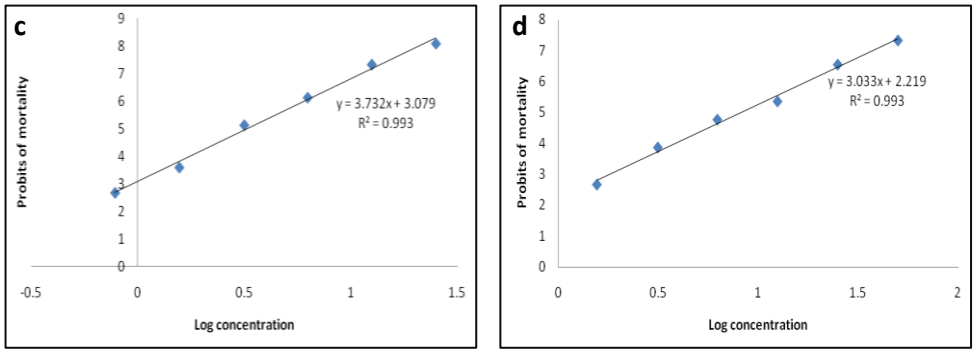
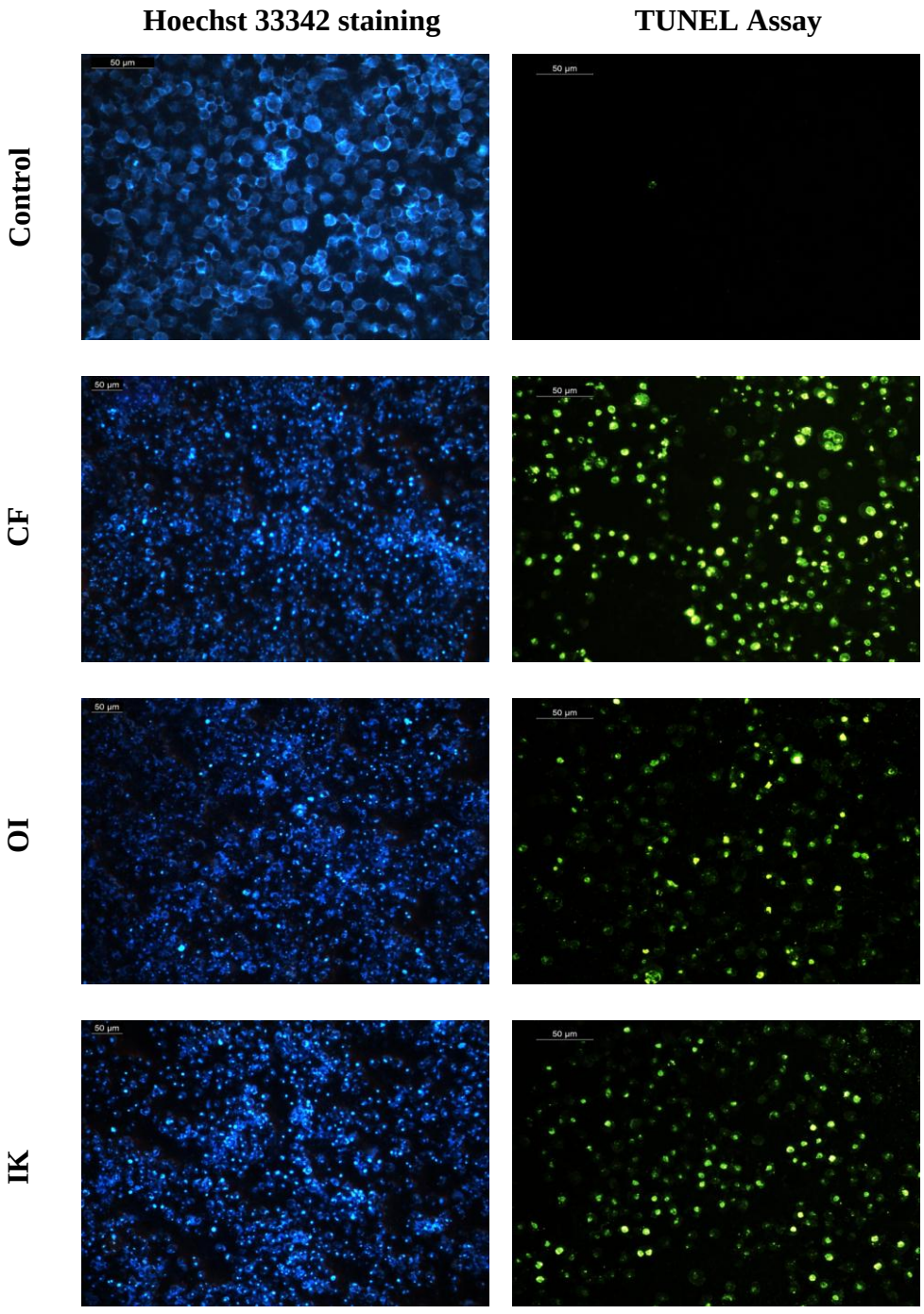


Fig. 6.4: Probit regression line for the determination of IC_{50} of a) CF b) OI c) IK and d) MI on BS-C-1 normal cells

6.3.4 Compounds induced morphological changes

To evaluate morphological changes induced by the anticancer compounds, NCI-H460 cells were stained with Hoechst 33342 and observed for characteristics of apoptosis under a fluorescence microscope. At the end of 24 h of treatment, all the four compounds showed typical features of apoptosis such as shrinkage and condensation of cellular nuclei with nuclear fragmentation whereas the control cells were uniformly stained and showed round and intact nuclei (Fig. 6.5). Further, TUNEL assay confirmed the nuclear DNA fragmentation induced by the compounds, which is an important hallmark of apoptosis. All the four compounds treated at its IC_{50} value showed condensed TUNEL positive chromatin within the cell nuclei, whereas control cells where negative for TUNEL reaction (Fig. 6.5). DNA fragmentation results of the compounds corroborate the Hoechst 33342 assay results.


Hoechst 33342 staining
TUNEL Assay
Control
CF
OI
IK
50 µm

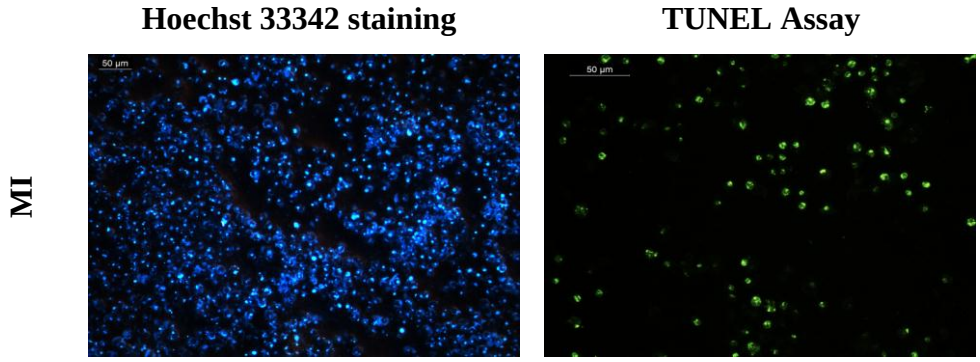


Fig. 6.5: Compounds induced morphological changes in NCI-H-460 cells after 24 h of drug treatment at IC_{50}

6.3.5 Confirmation of induction of apoptosis by the compounds

A time dependent increase in apoptotic cells were evident in the Annexin V assay which indicates that cell death induced by the compounds were mediated through apoptosis and not by necrosis (Fig. 6.6 and Fig. 6.7). At the beginning of the treatment (0h), the majority of cells were negative for both Annexin V and PI staining. At the end of 4 h, cells treated with CF 24% cells turned Annexin V positive implies early apoptosis and only 3% cells were in late apoptosis (both Annexin V and PI positive). After 10 h of treatment, both early and late apoptotic cells increased to 44.5% and 28 % respectively. At the end of 24 h, more cells turned to late stages of apoptosis (66%), which is indicated by a time dependent increase in apoptotic cells. In case of cells treated with OI, the assay showed 55% (4 h), 36% (10 h) and 30% (24 h) of the early apoptotic cells, whereas the percentages of the late apoptotic cells were 16, 59 and 70% at 4, 10 and 24h respectively. In case of IK, 40% cells were early apoptotic and 11% cells were in late apoptotic stage after 4h of treatment. After 10 h of treatment, 57% cells became early apoptotic and 38% turned late apoptotic. More cells turned to late apoptotic stage (67%), after 24h of treatment. In case of treatment with MI, 20% cells

showed early apoptotic signals and only 7% cells turned to late apoptotic stage. After 10h, more cells become early apoptotic (72%) and comparatively less number of cells becomes (27%) late apoptotic. After 24h of treatment, more cells turned to late apoptotic (53%) and percentage of early apoptotic cells decreased gradually (47%).

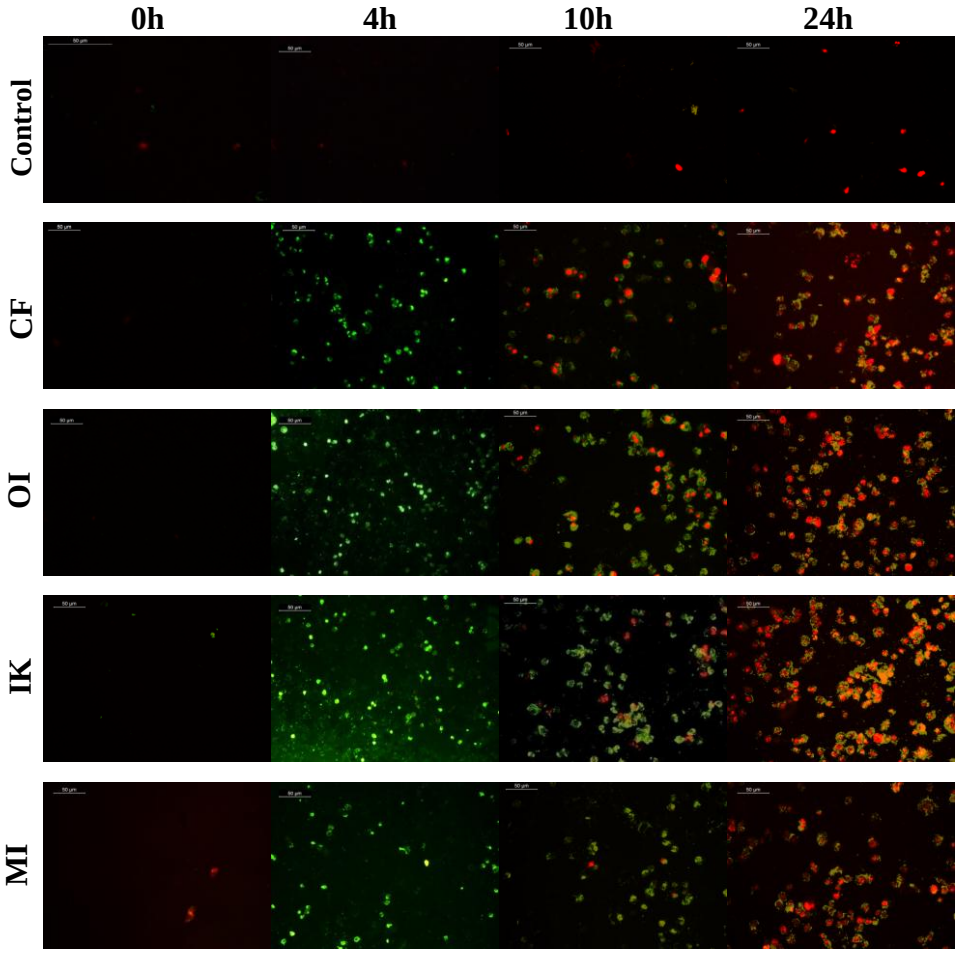


Fig. 6.6: Annexin-V/PI double staining microscopic image of NCI-H460 cells after the treatment with compounds. Cells were analyzed after 0, 4, 10 and 24 h of treatment.

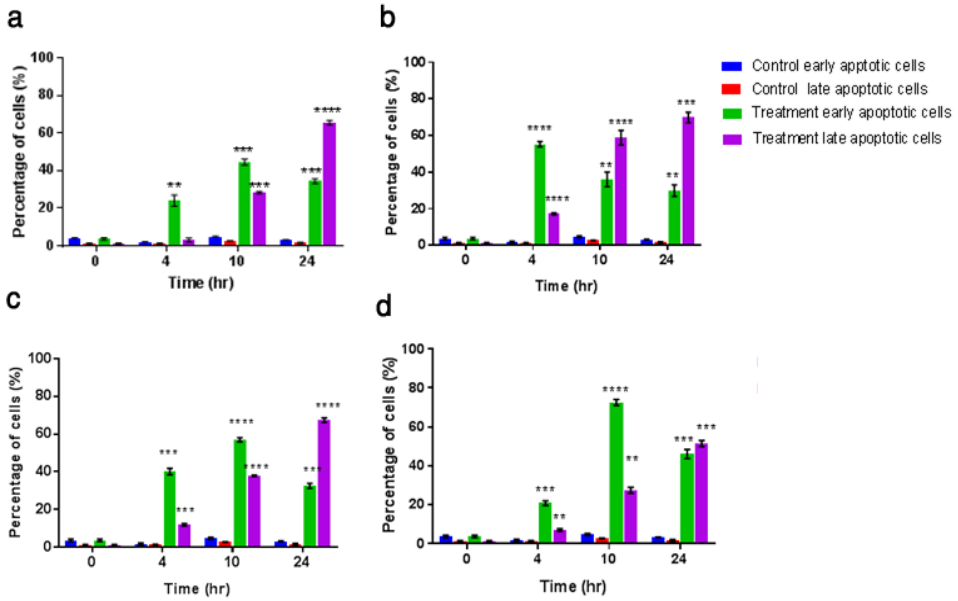


Fig. 6.7 : Percentage of cells showing apoptosis in NCI-H460 cells after treatment with a) CF; b) OI; c) IK; d) MI after 0, 4, 10 and 24 h of treatment (Data shown are mean of four independent observations and its standard deviation) * P< 0.05, ** P < 0.01 and *** P < 0.005 **** P < 0.001 as compared to control cells

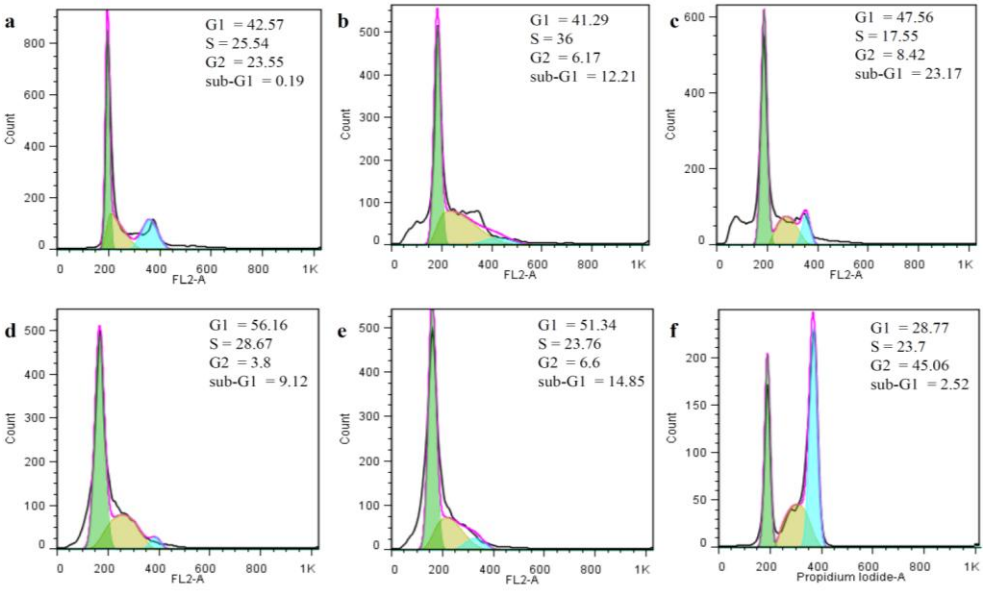


Fig. 6.8 : Cell cycle analysis by flow cytometry after being treated with compounds for 24h (a) Control (b) CF (c) OI (d) IK (e) MI (f) doxorubicin (positive control)

6.3.6 Flow cytometry for Cell cycle distribution

To study the effect of compound on cell cycle, flow cytometric analysis was carried out (Fig. 6.8). When NCI-H460 cells treated with 16.3 µg/mL of CF, the cells showed accumulation of cells in S phase (36%) with a significant decrease in G2M phase, when compared to untreated control cells. Whereas, OI treatment showed a higher percentage of cells in G1 phase (47.56%) while both S and G2 M phase showed significantly less population, when compared with untreated control cells. Similar observations were made in the case of other ikarugamycin type compounds as IK showed 56.14% cells in G1 phase, while MI showed 51.34% cells in G1 phase when compared to control cells (42.57%).

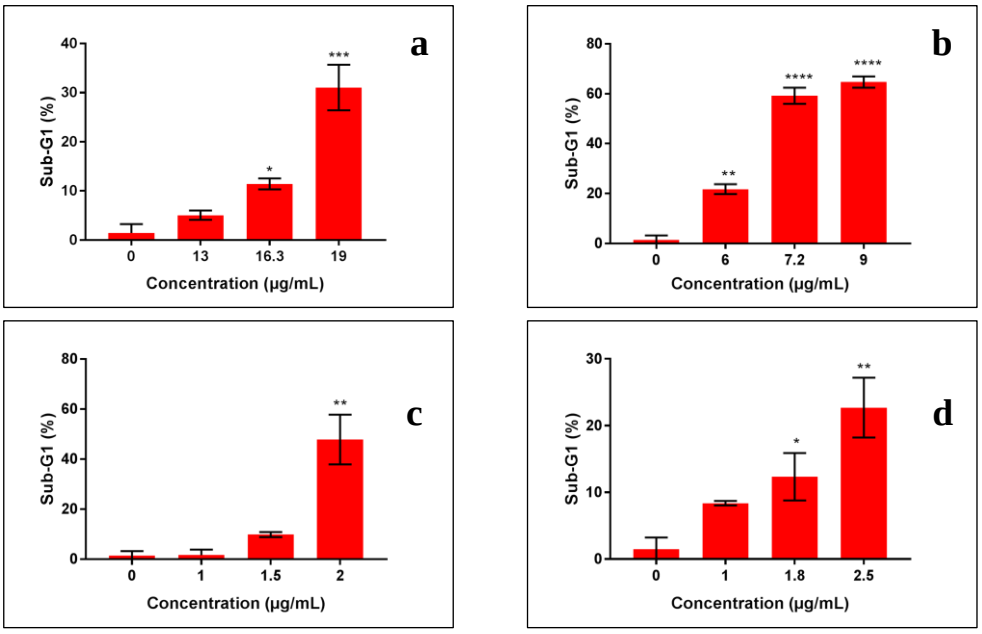


Fig. 6.9: Sub–G1 peak indicating fragmented DNA of NCI-H460 cells treated at various concentrations of compounds for 24h. Cells treated with a) CF (0,13, 16.3 and 19 µg/mL) b) OI (0,6, 7.2, 9 µg/mL) c) IK (0,1, 1.5 and 2µg/mL) d) MI (1, 1.8 and 2.5 µg/mL) * P< 0.05, ** P < 0.01 and *** P < 0.005 **** P < 0.001 as compared to control cells.

The sub-G1 (apoptosis) peaks analysis by flow cytometric assay for all four compounds showed a dose-dependent increase in the percentage of Sub-G1 cells indicating DNA fragmentation, a hallmark of apoptosis (Fig. 6.9)

6.4 Discussion

Polycyclic tetramate macrolactams (PTMs) exhibits a wide range of biological activities such as antiprotozoal (Jomon et al. 1972), antiviral, (Luo et al. 2001; Yi et al. 2010), anti-HIV and antitumor properties (Jomon et al. 1972; Popescu et al. 2011). Most of the macrocyclic tetramic acids exhibits significant cytotoxicity on various cancer cell lines increasing their importance as potential cancer therapeutics (Popescu et al. 2011)The compounds studied in this chapter comes under this class of compounds and which include Ikarugamycin(IK), 30-oxo-28-N-methyl ikarugamycin (OI), 28-N-methyl ikarugamycin (MI) and Clifednamide (CF). Ikarugamycin is reported for its cytotoxic activities in MCF-7 breast carcinoma, HMO2 gastric adenocarcinoma, Hep G2 hepatocellular carcinoma, HL-60 human promyelocytic leukemia cells and Huh 7 hepatoma cells (Bertasso et al. 2003; Popescu et al. 2011).

Many chemotherapeutic anticancer drugs interact with DNA directly or interfere with the replication process by preventing the proper relaxation of DNA (through the inhibition of topoisomerases), which indicates the significance of DNA-binding compounds as anticancer substances (Palchaudhuri and Hergenrother 2007). DNA–Drug interactions can be classified into two major categories, intercalation and groove binding. DNA intercalation involves the insertion of a planar compound between DNA base pairs, which results in a decrease in the DNA helical twist and

lengthening of the DNA (Lerman 1961). Unlike intercalation, groove binding do not induce large conformational changes in the DNA and may be considered similar to standard lock-and-key models for ligand–macromolecular binding. Groove binders are usually crescent-shaped molecules that bind to the minor groove of DNA (Chaires 1997). Grove binder compounds have proven clinical utility as anticancer and antibacterial agents, an important example being mitomycin (Bischoff and Hoffmann 2002).

Molecular docking studies revealed that ikarugamycin derivatives bind to minor groove of DNA. The estimation of docking scores predicted by Autodock showed approximately similar docking energies between IK and OI, however MI and CF was found considerably less specific to DNA. It is further elucidated that inhibition potential of DNA-ligand complexes obtained from docking by MD simulations and MM-PBSA free energy calculations. On the basis of data obtained from MD simulations, it can be concluded that all these compounds have the potential to be used as lead molecule for anticancer treatment with IK being the better candidate. MM-PBSA based calculations established that electrostatic interactions contribute towards the interaction energy and polar solvation energy being the stabilization factor of IK. In the case of OI, MI and CF, van der Waals interaction is found to be more compared to electrostatic interaction while polar solvation energy contributes to their total binding energy.

Most of the anticancer drugs presently available in market have been shown to induce apoptosis in susceptible cells (Hickman 1992). Even though, the active compound interacts with different targets, induced cell death with some common features (cell shrinkage, endonucleolytic cleavage

of DNA, changes in chromatin condensation) suggests that the cytotoxic property of the compound is determined by its ability to induce 'programmed' cell death or apoptosis. The fundamental mechanisms for commencement of an apoptotic response upon cytotoxic chemotherapy may depend on the individual stimulus and have often not exactly been identified. Though, the damage to DNA or to any other critical molecules is considered to be a initial event which then proceeds to apoptosis (Rich et al. 2000). In *in vitro* evaluation of the Ikarugamycine and related analogues for cytotoxic activities were validated in the molecular docking and simulation studies. Anticancer activity of these compounds was comparable with standard anticancer drugs. Apoptosis is an significant factor leading to cellular suicide (Komatsu et al. 2006) and all the *in vitro* assays (Hoechst33342, TUNEL, Annexin V-PI, quantification ofsub-G1 peaks by flow cytometry) substantiate the potential of the compounds to induce apoptosis. The anticancer activity of these compounds except IK, were not reported earlier. Also there are not many studies about cell cycle arrest caused by polycyclic tetramate macrolactam compounds.

In this study results of the cell cycle analysis suggest that OI, IK, MI showed cell cycle arrest in G1 phase of NCI-H460 cells whereas, CF induced cell arrest at S phase. In a previous study, Popescu et al.(2011) reported that IK induces cell death in HL-60 cell without any particular specific cell cycle arrest, when studied in 400nM of IK in two different time intervals (8h and 24h). In this study, IK showed a clear increase in G1 phase of NCI-H460 cells, when treated with 1.5µg/mL for 24h. G1-phase arrest is usually caused by DNA damage and S phase arrest is caused by either DNA damage or problems associated with replication. At G1 phase, cells grow and synthesize mRNA and proteins in preparation to duplicate their DNA in

S phase. Cyclins and cyclin dependent kinases (CDK) are key players in cell cycle regulations (Shapiro and Harper 1999). DNA damage induced by G1 arrest of cells can be either dependent or independent of p53. While p53 dependent cell cycle arrest is induced by the stabilization of p53 (tumor suppressor protein) leading to expression of the CDK inhibitor, p21. The p53 independent G1 arrest induction is mediated by proteolysis of cyclin D1, which ultimately leads to the release of p21 (Agami and Bernards 2000). Unlike other PTMs, CF induces cell arrest at S phase. In contrast to G1 arrest, p53 or p21 have no specific roles to play in the intra-S-phase check point control and S phase arrest. S phase check point is controlled and manifested by decreased rate of DNA synthesis in the presence of DNA damaging agents (Bartek and Lukas 2001). However, how the S-phase DNA damage checkpoint slows replication is still unclear (Willis and Rhind 2009). Recently, Ding et al. (2016) reported a new polycyclic tetramate macrolactam antibiotioc Alteramide B isolated from *Lysobacter enzymogenes* which cause cell cycle arrest at the G2/M phase in *Candida albicans.*

Recently, Yu et al. (2017) identified antipancreatic cancer polycyclic tetramate macrolactams (PTMs) from a mangrove-derived *Streptomyces xiamenensis*. They have isolated three known compounds Ikarugamycin, Capsimycin A and Capsimycin B along with two new compounds, Capsimycin C and Capsimycin D. Compounds Ikarugamycin, Capsimycin and Capsimycin B, exhibited anti-proliferative activities against pancreatic carcinoma with IC_{50} values of 1.30–3.37 μM. In another study, Bertasso et al. (2003) reported a new PTM compound Ripromycin, along with Ikarugamycin and a new derivative of it, Ikarugamycin epoxide.

Compounds showed antibiotic activities against gram-positive bacteria and cytostatic effects to various human tumor cell lines.

Nowadays lots of studies are coming up in the field of evaluation of cytotoxic potential of marine derived compounds. Cytotoxic effect of Fascaplysin, the natural product of a marine sponge against small cell lung cancer cell lines, was reported by Hamilton, (2014). It showed very high cytotoxicity against small cell lung cancer cells and induced cell cycle arrest in G1/0 at lower and S-phase at higher concentrations, respectively. Dicitrinone B, another compound isolated from a marine-derived fungus, *Penicillium citrinum* inhibits the proliferation of different tumor types. Among them, A375 (human malignant melanoma) cell, was confirmed to be the most sensitive. Morphologic evaluation, apoptosis rate analysis and cell cycle arrest results showed that dicitrinone B significantly induced A375 cell apoptosis (Chen et al. 2014).

In last decades, the isolation of marine actinomycetes has been a great source of new compounds with potential bioactivity especially as anticancer compounds (Olano et al. 2009). These anticancer compounds belong to several structural classes such as anthracyclines, enediynes, indolocarbazoles, isoprenoides, macrolides and non-ribosomal peptides. An important member in this group is Salinosporamide A, which is a highly cytotoxic proteasome inhibitor from a novel marine actinomycetes identified as *Salinospora* (Feling et al. 2003). It exhibits potent cell cytotoxicity and appears to exert its cytotoxic effects through inhibition of the 20S proteasome. Salinosporamide A displayed very promising *in vitro* cytotoxicity against HCT-116 human colon carcinoma with very low IC_{50} value. This compound also displayed potent and highly selective activity in

the NCI's 60-cell-line panel (Feling et al. 2003). Xin et al. (2012) reported a novel capoamycin-type antibiotics and polyene acids from marine *Streptomyces fradiae* PTZ0025. Among them, Fradimycins B was the most active agent and significantly inhibited cell growth of colon cancer and glioma. Further studies confirmed that Fradimycin B arrested the cell cycle at the G0/G1 phase and induced apoptosis in tumor cells. Two new cytotoxic quinones of the angucycline class, Marmycins A and B were isolated from the culture broth of a marine sediment-derived *Streptomyces*. Among them, Marmycin A was a very potent compound, and it induced modest apoptosis and arrested the G1 phase of the cell cycle (Martin et al. 2007).

In conclusion, a marine actinomycete *Streptomyces* sp. MCCB 267 isolated from a sponge yielded multiple cytotoxic compounds belonging to polycyclic tetramate macrolactams (PTMs) family of natural products. Molecular simulation study showed the DNA binding ability of these compounds and subsequent *in vitro* evaluation confirmed the anticancer potential of the isolated PTM compounds. However, there exists considerable difference in the cell cycle distribution profile of tested compounds, wherein clifednamide A induced S phase arrest whereas ikarugamycin and other analogues induced G1 phase arrest of the cells.

Chapter 7

ISOLATION AND STRUCTURAL ELUCIDATION OF CYTOTOXIC COMPOUNDS FROM *PSEUDONOCARDIA* SP. MCCB 268

Contents

7.1 Introduction

The genus *Pseudonocardia* is considered as rare group of actinobacteria and studies exploring these group of organisms for discovering new bioactive compounds were comparatively less in literature (Lazzarini et al. 2000). Li et al. (2011) and Tian et al. (2013) described Pseudonocardians A–C from deep-sea actinomycete *Pseudonocardia antitumoralis*, and these are some of the well studied compounds from this genus. These compounds exhibited very potent cytotoxic activities against three tumor cell lines (SF-268, MCF-7 and NCI-H460) and showed very low IC_{50} values. Recently, Braña et al. (2017) reported two new antibiotics, Branimycins B and C from the marine actinobacterium *Pseudonocardia carboxydivorans* M-227,which exhibited antibacterial activity.

This chapter gives details of the investigation on bio-assay guided fractionation, isolation, purification and characterization of active anticancer compound from *Pseudonocardia* sp. MCCB 268 isolated from marine sediment collected from Arctic fjord. Further, *in vitro* anticancer assays were performed to validate the anticancer activity and the mode of cytotoxicity was analyzed.

7.2 Materials and Methods

Schematic representation of separation of active compound from *Pseudonocardia* sp. MCCB 268 was given in flow chart (Fig.7.1)

7.2.1 Mass production of *Pseudonocardia* sp. MCCB 268

Pseudonocardia sp. MCCB 268 which showed potent anticancer activity (Chapter 2) was mass cultivated for the production of secondary metabolites. A loopful of actinomycetes spore was inoculated into a seawater-based seed medium (100 mL) (beef extract 3g/L, peptone 5g/L). The flasks were incubated at 28 ºC for 48 h on a rotary shaker set at 150 rpm. Subsequently, the content was used to inoculate 10 Erlenmeyer flasks containing 1L each seawater based fermentation medium (Yang et al. 2013) (Soybean meal 3g/L, Yeast extract 3g/L, Proline 1g/L, Beef extract 3g/L, Glycerol 6mL/L, K_2HPO_4 0.5g/L, $MgSO_4 \cdot 7H_2O$ 0.5g/L, $FeSO_4 \cdot 7H_2O$ 0.5g/L, $CaCO_3$ 2g/L, pH 7.4) and incubated at 28 ºC for 8 days on a rotary shaker at 150 rpm. After incubation, the broth was centrifuged at 8000 rpm for 5 min to separate mycelial cake from fermentation broth, and the supernatant was extracted three times with an equal volume of ethyl acetate. The extract was concentrated under reduced vacuum on a rotary evaporator at 35 ºC and used for separation.

7.2.2 Flash Chromatographic separation of crude extract

Initially crude ethyl acetate extract (1.79 gm) of *Pseudonocardia* sp. MCCB 268 was fractionated using Reverse phase flash chromatographic separation (COMBIFlash, Toledyne ISCO, USA). Gradient elution with mobile phase Water (A) and Methanol (B) for 35 min was performed. Nine fractions were collected, concentrated and weight of each fraction was noted, and bioactivity tested.

7.2.3 Semiprep HPLC separation of active fraction

After flash chromatography, active fraction (Fr.7) was purified by reversed-phase HPLC (Phenomenex Luna, C18, 250 × 10.0 mm, 2.5 mL/min, 5 μm) using a gradient solvent system from 10% to 99% acetonitrile (0.1% TFA) over 30 min to afford 6 subfractions (Fr.7.1 to Fr.7.6). All sub-fractions were concentrated and weight of each fraction was recorded and further subjected to bioactivity testing.

7.2.4 Characterization of the anticancer molecules from *Pseudonocardia* sp. MCCB 268

Fraction Fr.7.6 showed bioactivity and yielded one pure compound and designated as Compound **5**. ^{1}H and ^{13}C NMR spectroscopic data were recorded at 600 MHz in Methanol solution on a Varian System spectrometer. The structure of the isolated compound was established by analyses of the NMR spectra, high-resolution mass spectroscopy, UV spectra, and comparison of these data with published data (Shin et al. 2010a).

7.2.5 Evaluation of cytotoxicity of compound and IC_{50} determination by MTT assay

Cytotoxicity evaluations of the compound **5** was performed on NCI-H460 lung cancer cell line as well as to a cell line derived from normal epithelial kidney cells from the African green monkey (BS-C-1) using MTT cell viability assay. Both cell lines were procured from NCCS, Pune and maintained at NCAAH on RPMI-1640 (Himedia, India), supplemented with 10% fetal bovine serum (FBS) (Himedia, India) and used for the assay. Two-fold serial dilutions of the compounds were prepared to obtain concentrations ranging from 1 mg/mL to 15.625 µg/mL in 10% aqueous (aq.) DMSO. A 10 µL aliquot of each concentration was added to the wells containing 190 µL NCI-H460 cell suspensions with a density of 1.9 x 10^4 cells/well, in 96 well microtiter plates. Aq. DMSO (10%) was used as control. All assays were performed in triplicate. The plates were incubated for 48 h at 37 ºC in a CO_2 (5%) incubator. After incubation period the culture medium was discarded and replaced with 200µL fresh medium and further treated with 50µl (1 mg/ml) MTT solution. Treated samples were incubated in dark for a further period of 4 h at 37 ºC. Subsequently, both medium and MTT reagents were discarded, 200µl of DMSO added to each well to dissolve the formazan crystals, and the optical density (OD) was read at 570 nm in a microplate reader (Tecan, Switzerland). Percentage of cell-growth inhibition (GI) was calculated according to the following equation:

Percentage of viability = (Mean OD_{sample}- Mean OD_{blank})/ (Mean $OD_{control}$-Mean OD_{blank}) *100

Percentage of Growth Inhibition = 100 - Percentage of viability

IC_{50} values were determined based on probit analysis (Brownlee et al. 1952).

7.2.6 Hoechst 33342 staining for scoring apoptosis

NCI-H460 cells were seeded in a chamber slide at a concentration of 1.9×10^4 cells per well. Compound **5** was added to NCI-H460 cells at its log phase at a concentration of their IC_{50} value and incubated for 24 h at 37 °C with 5% CO_2. After 24 h of treatment, the culture medium was removed and cells washed twice with PBS and stained with DNA specific Hoechst 33342 dye (2 μg/mL in PBS, Sigma Chemicals, USA) for 10 min at 37°C. At the end of staining, cells were observed under a fluorescence microscope for apoptotic features. Doxorubicin (Sigma, USA) was used as a positive control, and DMSO was used as a negative control.

7.2.7 *In situ* detection of DNA damage (TUNEL assay) for the evaluation of apoptosis

In situ labeling of the 3′-OH end of the DNA fragments generated during apoptosis was performed using *In Situ* Cell Death Detection Kit (TUNEL) (Roche Diagnostics, Switzerland). Briefly, the NCI- H460 cells at its log phase were treated with the compound **5** at its IC_{50} value for 24h in a chamber slide (Millicell EZ slide, Millipore Corporation, US) and incubated at 37°C in a CO_2 incubator at 5% CO_2. At the end of the treatment, culture medium was removed and the cells were washed with 1X PBS, and air dried. The cells were then fixed with freshly prepared 4% paraformaldehyde for 1 h at 25º C. The slide was rinsed with PBS and incubated in permeabilisation solution (0.1% Triton X-100 in 0.15%sodium citrate) for 2 min on ice. Slides were washed twice with PBS, and 50μL of TUNEL reaction mixture added in to each well. The cells were incubated in dark for

60 min at 37ºC in a humidified atmosphere. Subsequently, cells were washed three times with PBS and observed under fluorescent microscope for DNA strand breaks.

7.2.8 Annexin V–Propidium iodide (PI) double staining for confirming apoptosis

Annexin-V-FLOUS/Propidium iodide (PI) staining was performed as per the manufacturer's instructions (Roche Diagnostics,Switzerland). Briefly, cells grown in chamber slides (Millicell EZ slide, Millipore Corporation, US) were treated with compound **5** at its IC_{50} value. After addition of the compound, cells were observed and counted under a fluorescent microscope for apoptotic features at regular intervals of treatment (0, 4, 12 and 24 h). A minimum of three different microscopic fields was counted and the average calculated and expressed as a percentage of the total cell population. Cells treated with 10% Aq. DMSO were used as a negative control. The results represent the data of four replicates (means ± SD) (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ **** $P < 0.001$ versus the control)

7.2.9 Statistical analysis

Graph figures represent mean ± SD of at least three independent experiments. Significance of results was performed by t-test or one-way ANOVA with GraphPad Prism 7.03 software (SPSS Inc., USA).

7.3 Results

7.3.1 Bioassay-Guided Isolation

Pseudonocardia sp. MCCB 268 was kept for 10L mass production for 2 weeks and the secondary metabolites produced were extracted with ethyl

acetate (Fig. 7.2). The crude extract was concentrated under reduced vacuum. Bioassay-guided fractionation details and yield of each fractions was given in flow chart (Fig. 7.1).

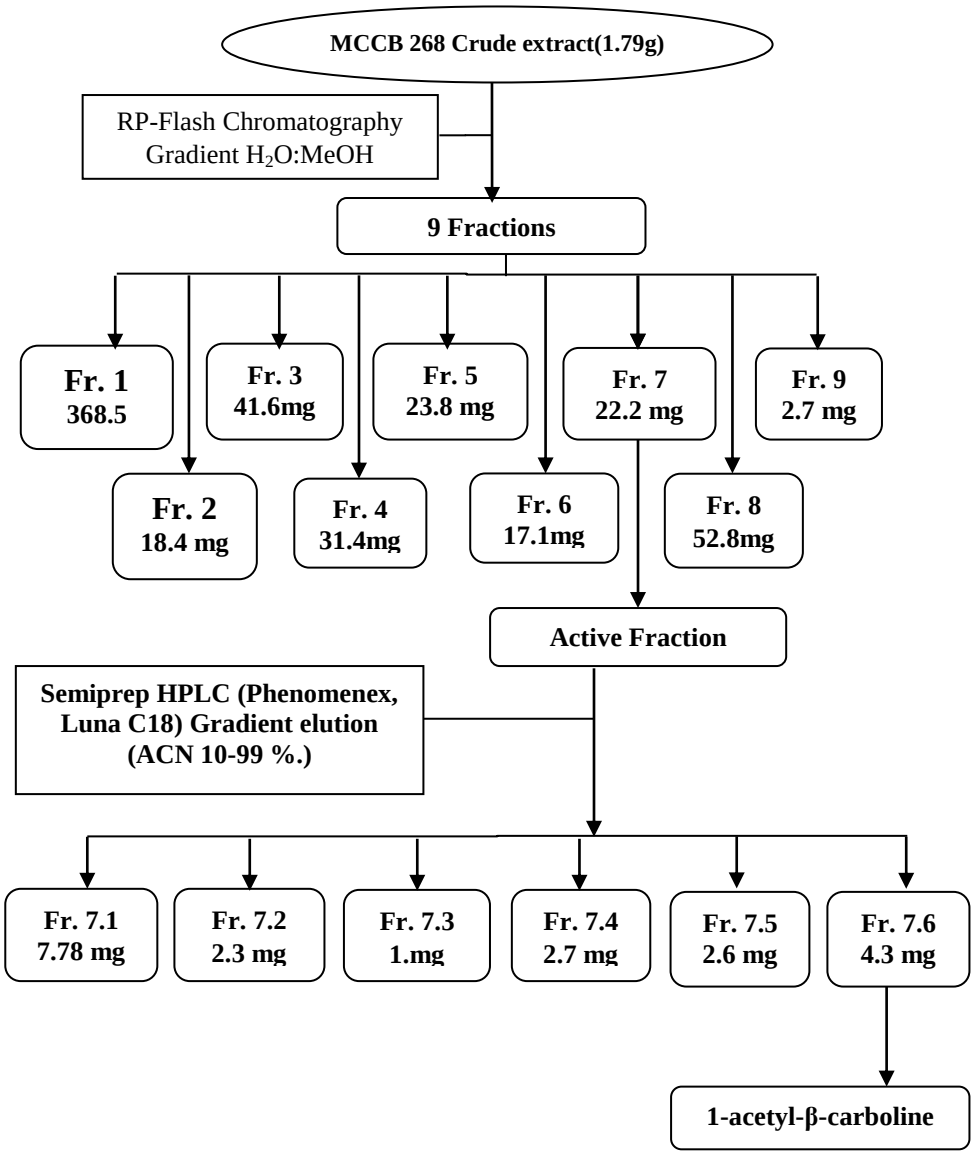


Fig. 7.1: Schematic representation of separation of active compound from MCCB268

Fig. 7.2: a) Mass production and b) Ethyl acetate extraction of *Pseudonocardia* sp. MCCB 268

Initially, the crude extract was fractioned into nine fractions by reverse phase flash chromatography using gradient elution with mobile phase Water (A) and Methanol (B) (Fig. 7.3). The fractions were subjected to bioactivity and Fr. 7 showed modest activity. Active fraction Fr.7 was further subjected to Semiprep HPLC (Phenomenex -Luna C18) in Gradient elution (35 min, ACN 10-99 %.) using Water: Acetonitrile as mobile phase for isolation of active compound (Fig. 7.4). 6 peaks were observed, collected (Sub fraction Fr7.1 to Fr7.6) and subjected to activity analysis. Sub Fraction Fr. 7.6 showed modest activity and yielded one pure compound which was subsequently identified as 1-acetyl-β-carboline (Compound **5**).

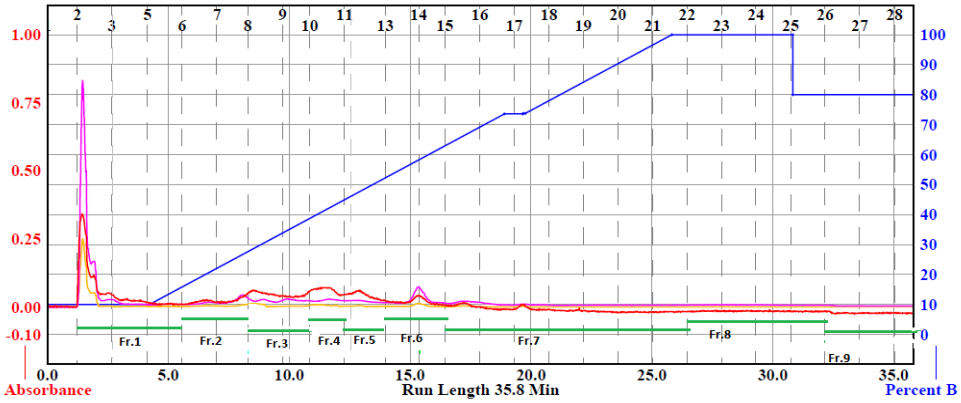


Fig. 7.3: Flash Chromatographic separation of crude *Pseudonocardia* sp. MCCB 268 extract

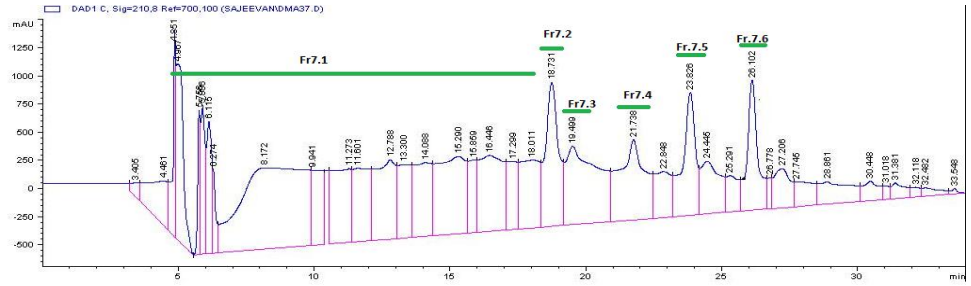


Fig. 7.4: Semiprep HPLC of Fr. 7 for isolation of active compound

7.3.2 Structural Determination of the Compound

In LC-MS/MS the compound showed a mass of m/z 211.1 $[M+H]^+$ (Fig. 7.5 b). The UV spectrum of purified compound exhibited characteristic absorption peaks at 216, 298, and 375 nm (Fig. 7.5 a), diagnostic features of *β*-carboline compounds (Sun et al. 2004; Wang et al. 2008). This was supported by the AntiBase Database and SciFinder search for compounds with a mass of 210. ^{1}H NMR data revealed resonances for 6 aromatic protons (δ 8.45, δ 8.30, δ 8.21, δ 7.70, δ 7.59, δ 7.31). ^{13}C NMR exhibited resonances for 11 olefinic carbons. Together with the H and C-NMR

chemical shifts, the identity of the compound was confirmed as 1-acetyl-β-carboline with the molecular formula ($C_{13}H_{10}N_2O$), exact mass, 210.0793. The structural elucidation of 1-acetyl-β-carboline were reported earlier (Shin et al. 2010).

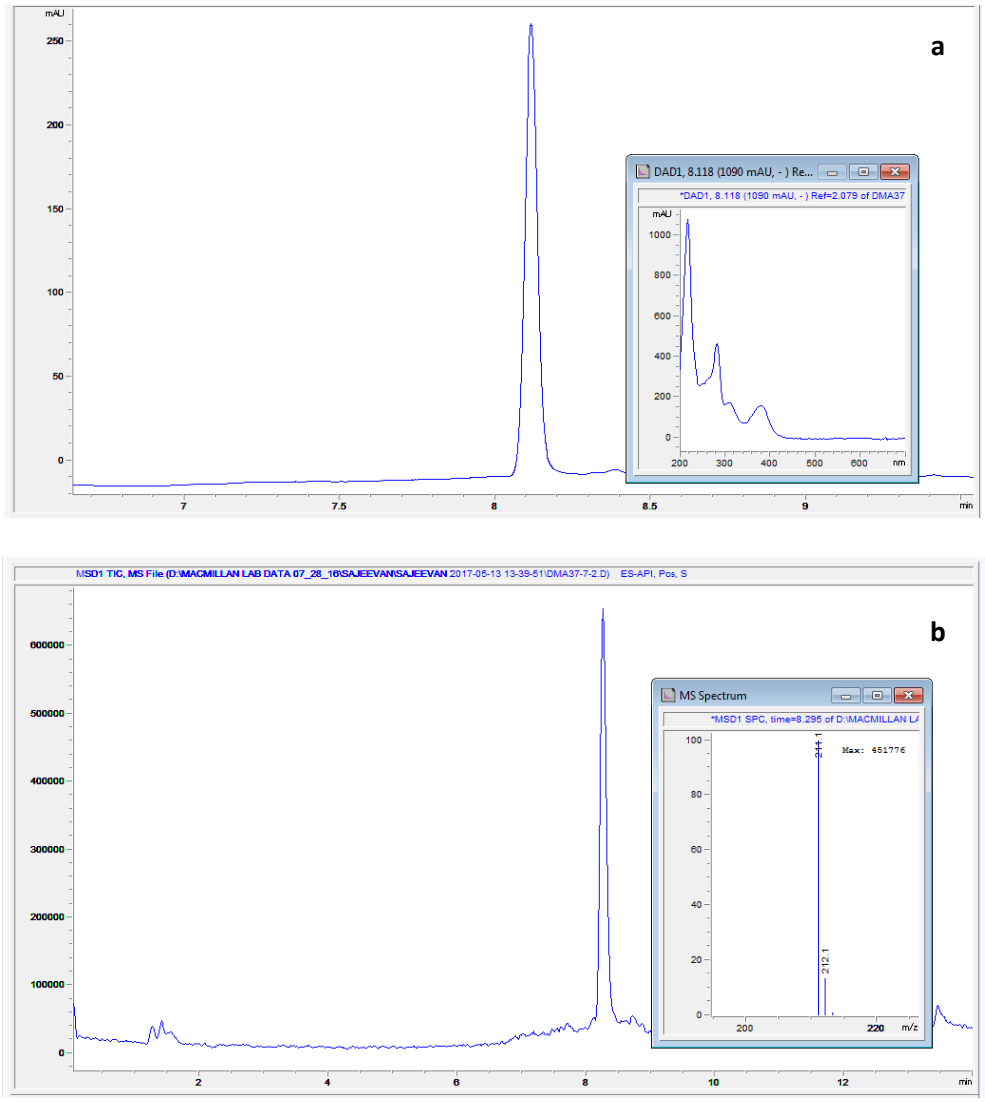
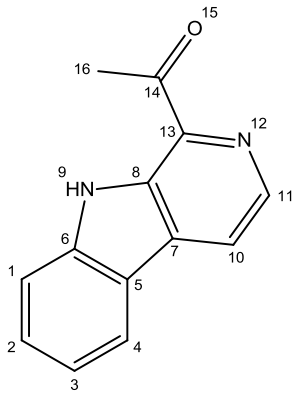


Fig. 7.5: a) UV profile of pure compound Fr.7.6 showing characteristic absorption bands at 216, 298, and 375 nm and b): Mass spectra of compound (Fr.7.6) showing a mass peak of m/z 211.1 $[M + H]^+$



$C_{13}H_{10}N_2O$
Exact Mass: 210.0793

Fig. 7.6: Structure of 1-acetyl-β-carboline

Table 7.1: ^{1}H and ^{13}C NMR Data of 1-acetyl-β-carboline in CD_3OD-d4.

No	Δ_H	Mult (*J* in Hz)	δ_C
1			137.4
2			
3	8.45	d (5.4)	138.8
4	8.30	d (5.4)	120.4
4a			133.4
5a			121.8
5	8.21	d (7.8)	122.8
6	7.31	td (7.8, 1.0)	121.8
7	7.59	td (7.8, 1.0)	130.5
8	7.7	d (7.8)	113.6
8a			143.6
9			
9a			136.3
10			203.4
11	2.82	s	26.2

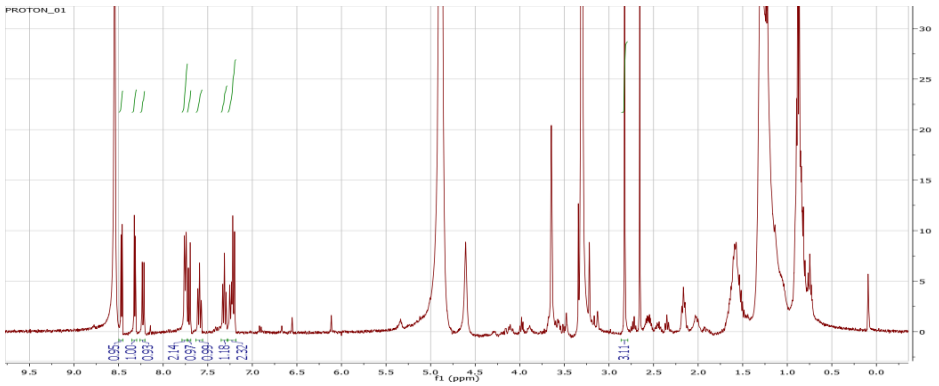


Fig. 7.7: ^{1}H NMR of 1-acetyl-β-carboline

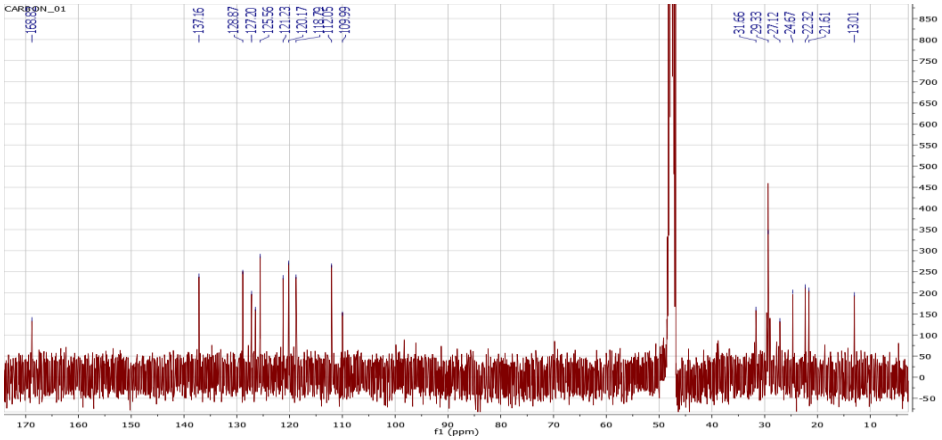


Fig. 7.8: ^{13}C NMR of 1-acetyl-β-carboline

7.3.3 Evaluation cytotoxicity of 1-acetyl-β-carboline by MTT assay on NCI-H460 and BS-C-1 cells

1-acetyl-β-carboline showed an IC_{50} value of 18.73µg/mL in NCI-H460 lung cancer cells, and 20.69 µg/mL in BS-C-1 normal cells. Growth inhibition curve and probit regression line of 1-acetyl-β-carboline on NCI-H460 and BS-C-1 cells are given in Fig. 7.9 and 7.10.

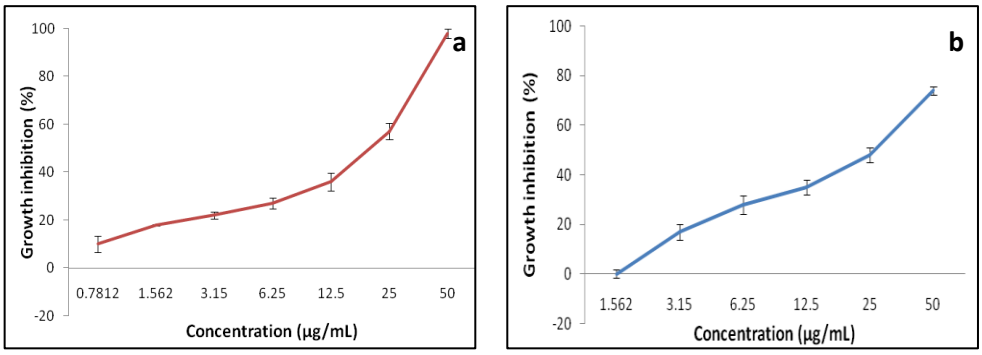


Fig. 7.9: Growth inhibition of 1-acetyl-β-carboline on a) NCI-H460 and b) BS-C-1 cells after 48 hrs incubation (Results were shown as means ± SD of at least triplicate observations)

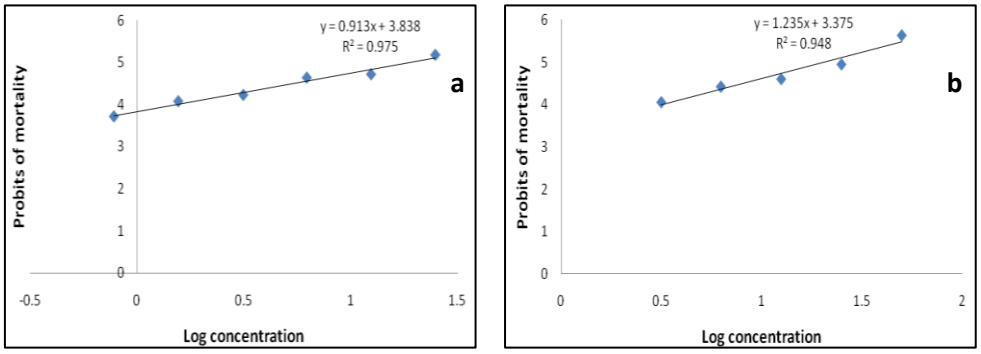


Fig. 7.10: Probit regression line for the determination of IC_{50} of 1-acetyl-β-carboline on a) NCI-H460 and b) BS-C-1 cells after 48 hrs incubation

7.3.4 Evaluation of mode of anticancer activity

Nuclear morphological change induced by 1-acetyl-β-carboline was studied by Hoechst 33342 staining and TUNEL assay after exposing NCI-H460 cells to the purified compound. In Hoechst 33342 staining, when NCI-H460 cells were exposed to 1-acetyl-β-carboline at its IC_{50}, the treated cells showed characteristic apoptotic morphology, such as shrinkage of cell nuclei, chromatin condensation and nuclear fragmentation. However, control cells showed round and intact cellular nuclei (Fig. 7.11). TUNEL

assay also confirmed nuclear fragmentation by 1-acetyl-β-carboline (Fig. 7.12). Cells treated with the compound showed TUNEL positive chromatin, whereas control cells showed TUNEL negative reaction.

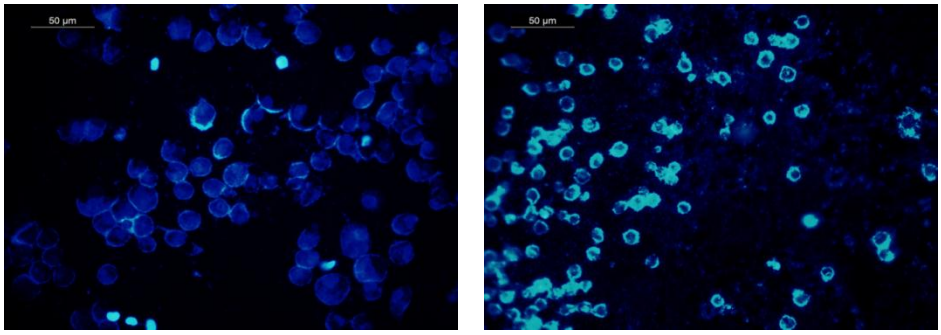


Fig. 7.11: Fluorescent microscope image of Hoechst 33342 stained NCI-H460 cells; a) control cells, and b) 1-acetyl-β-carboline treated cells

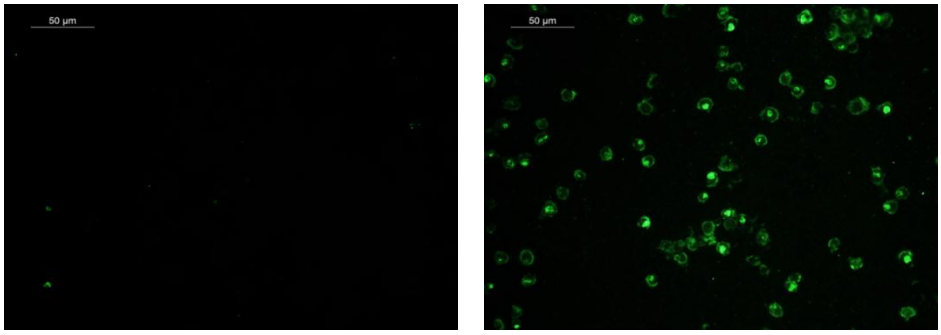


Fig. 7.12: Fluorescent microscope image of TUNEL assay of NCI-H460 cells; a) control cells, and b) 1-acetyl-β-carboline treated cells

In Annexin V –PI double staining, a time dependent increase of Annexin V positive cells were observed in NCI-H460 cells treated with 1-acetyl-β-carboline (Fig. 7.13).

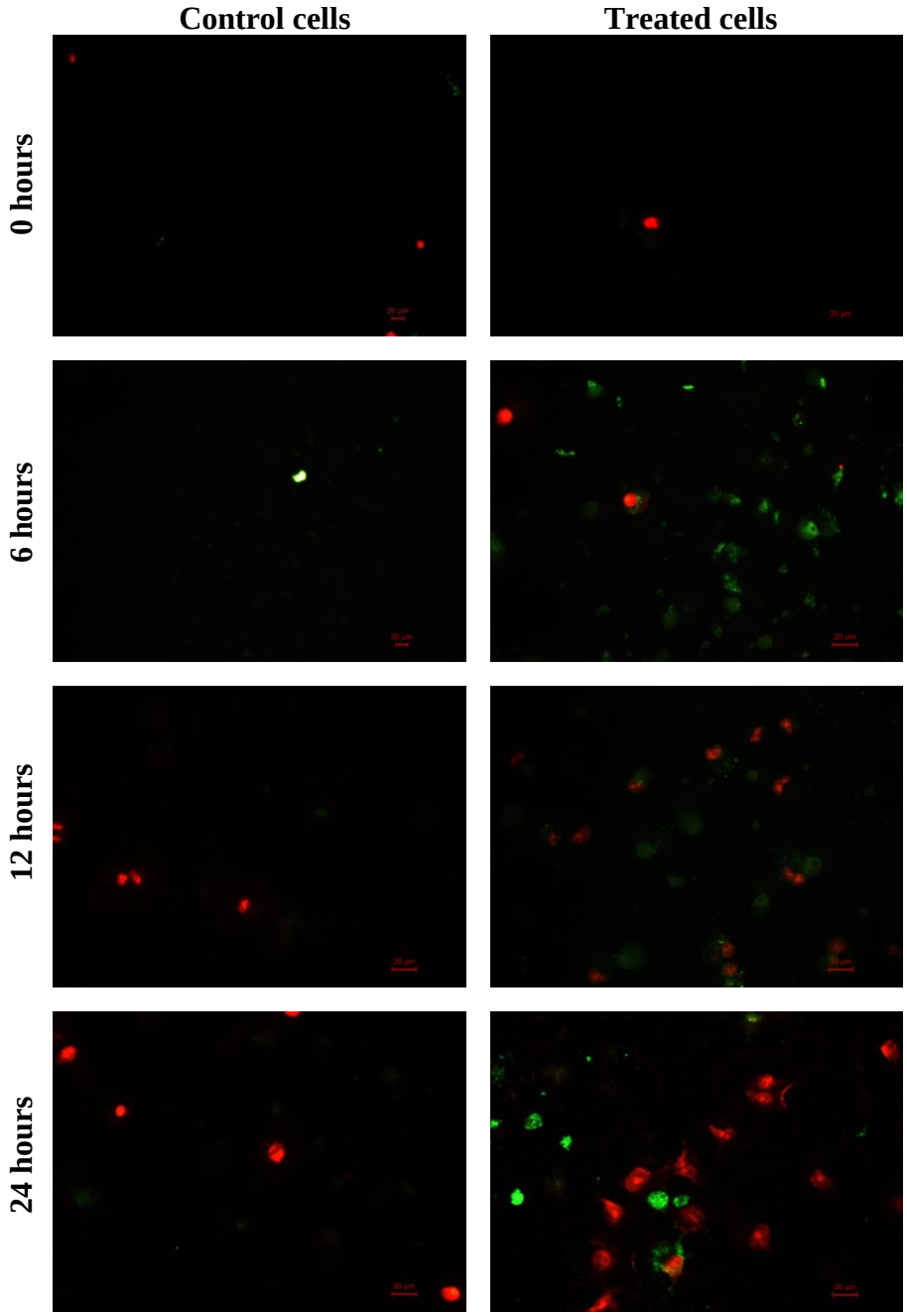


Fig. 7.13: Fluorescent microscope image of Annexin-V/PI double-staining assay. After treating with 1-acetyl-β-carboline, cells were stained with Annexin V-FITC and propidium iodide and analyzed after 0, 6, 12 and 24 h of treatment

At the beginning of the treatment only 4.3% of the total cells were positive to Annexin V which indicates early stages of apoptosis, and 3.5% cells were positive to both Annexin V and PI indicating later stages of apoptosis. After 6 h of treatment 34.5% cells turned to Annexin V positive (early stages apoptosis), whereas only 6.5% showed later stages of apoptosis (both Annexin V and PI positive). Similar to 6 h treatment, 12 h treatment results were also indicative of increase in Annexin V positive cells (57.5%) and 23.6 % cells were positive to both Annexin V and PI. After 24 h, 38% of cells were Annexin V positive and the percentage of Annexin V and PI positive cells gradually increased to 47%, indicative of the later stages of apoptosis, which implies more dead cells (Fig. 7.14).

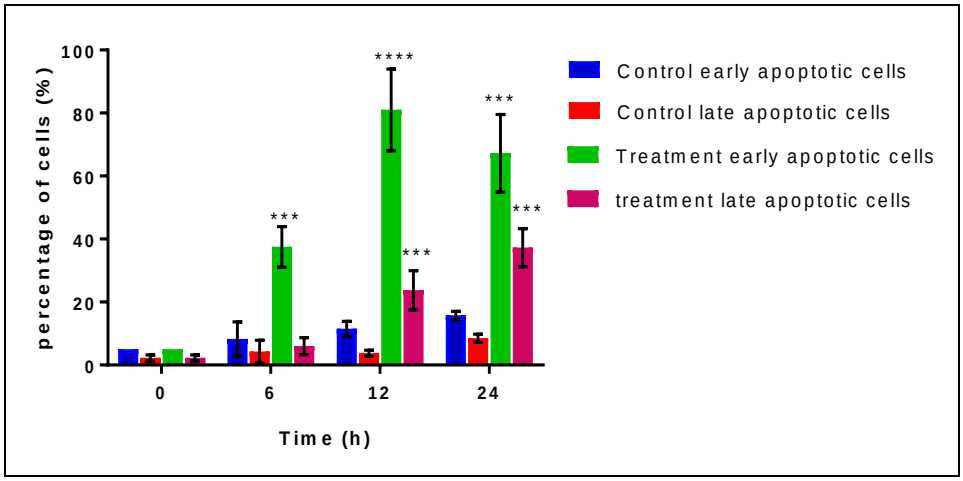


Fig. 7.14 Percentage of NCI- H-460 cells showing early and late apoptotic cell death at 0, 6, 12, 24 h after exposure to the 1-acetyl-β-carboline (data shown are mean of four independent observations and its standard deviation. * P< 0.05, ** P < 0.01 and *** P < 0.005 **** P < 0.001 as compared to control cells

7.4 Discussion

Bioassay guided fractionation of crude extract of *Pseudonocardia* sp. MCCB 268 yielded a cytotoxic compound **5** later identified as 1-acetyl-β-carboline. The identity of compound 1-acetyl-β-carboline was confirmed by interpreting the MS data as well as NMR, including both ^{1}H and ^{13}C NMR, spectroscopic data and comparing with those reported in the literature. 1-acetyl-β-carboline is a derivative of *β*-carboline alkaloids. β-Carboline comes under the group of indole alkaloids and consists of pyridine ring that is fused to an indole skeleton and some of which are widely distributed in nature (Abramovitch and Spenser 1964; Cao et al. 2007). β-Carboline compounds are of great interest due to their diverse biological activities. It was originally isolated from *Peganum harmala* (Zygophillaceae, Syrian Rue), which is being used as a traditional herbal drug as abortifacient in Middle East and North Africa (Moloudizargari et al. 2013). Apart from that *Peganum harmala* has been traditionally used to treat alimentary tract cancers and malaria in Northwest China (Chen et al. 2005). During the last decades, a large number of simple and complicated beta carboline alkaloids have been isolated and identified from various terrestrial plants (Tsuchiya et al. 1999), and marine invertebrates including marine sponges (Inman et al. 2010). They exhibited a broad spectrum of activities such as antitumor, anxiolytic, sedative, hypnotic, anticonvulsant, antiviral, antiparasitic as well as antimicrobial activities.

Though β-Carboline alkaloids are generally widespread in plants (Zhou et al. 1998; Chan et al. 2014) they are relatively rare in microorganisms (Allen and Holmstedt 1980). Among microorganisms, 1-acetyl-β-carboline was first isolated from the culture filtrate of *Streptomyces*

kasugaensis (Proksa et al. 1990). Later, it has been isolated from other actinobacteria such as *Streptomyces* sp. TN262 (Elleuch et al. 2010), *Marinactinospora thermotolerans* (Huang et al. 2011). Until now, there is no report of isolation 1-acetyl-β-carboline or any other beta carboline compounds from the Genus *Pseudonocardia.*

In this study bioassay guided fractionation of crude extract of *Pseudonocardia* sp. MCCB 268 yielded a pure compounds and identified as 1-acetyl-β-carboline. The *in vitro* anticancer activity evaluation of 1-acetyl-β-carboline was demonstrated and the compound was found to induce apoptosis in NCI-H460 lung cancer cells at IC_{50} value of 18.73 µg/mL. The compound induced nuclear morphological changes and DNA fragmentation after drug treatment, which was very evident in Hoechst 33342 and TUNEL assay. 1-acetyl-β-carboline induced a time dependent increase in Annexin positive cells which indicated that more phosphatidylserine (PS) were translocated into the outer plasma membrane as a result of early apoptotic cell changes. Annexin V-PI double staining revealed that cytotoxicity by 1-acetyl-β-carboline is NOT by necrosis, but through induction of apoptotic pathways.

Antimicrobial activity of 1-acetyl-β-carboline from *Streptomyces* sp. TN262 was reported by Elleuch et al. (2010). Shin et al. (2010) reported the anti-Methicillin-Resistant *Staphylococcus aureus* (anti-MRSA) activity of 1-acetyl-β-carboline isolated from the fermentation broth of a marine *Streptomyces.* They also studied the synergistic antibacterial activity of combination of 1-acetyl-β- carboline with ampicillin against MRSA and found this combination exhibited synergistic antibacterial activity against MRSA.

Biological activity of beta carboline compounds were found to be their ability to intercalate with DNA which ultimately leads to altered DNA replication or influence on enzymatic activities in DNA repair process (de Meester 1995; Taira et al. 1997; Cao et al. 2007). Beta carboline compounds Harman and Norharman were confirmed to induce DNA damage in a dose-dependent manner in SH-SY5Y human bone marrow neuroblastic cells (Uezono et al. 2001).

β-Carboline compounds are known to exhibit antitumor activities as well. Ishida et al. (1999) evaluated twenty-six beta-carbolines compounds for their *in vitro* cytotoxic activity on human tumor cell line panel. Among them, Harmine and β-carboline analogues exhibited significant activities against several human tumor cell lines including three drug-resistant KB cell lines with various resistance mechanisms. Xiao et al. (2001) studied a series of synthetic 3-substituted-β-carboline derivatives, its intercalating binding mode as well as their antitumor activity. The compounds showed cytotoxic activities against human tumor cell lines including HL-60, KB, HeLa and BGC. Their results indicated that β-Carboline compounds bind with DNA in an intercalative binding fashion and they showed high selectivity to the G–C base pair.

Numerous β -carboline derivatives bearing various substitutions at different positions of β-carboline nucleus were synthesized and evaluated for their antitumor activities *in vitro* and *in vivo* (Cao et al. 2005). Most of the compounds showed promising antitumor activities *in vitro* against a panel of human tumor cell lines including gastric carcinoma (BGC-823), liver carcinoma (HepG2 and Bel-7402), non-small cell lung carcinoma (PLA-801), cervical carcinoma (HeLa) and colon carcinoma (Lovo). Some

selected β-carboline derivatives showed potent antitumor activities against mice bearing Lewis lung cancer and Sarcoma 180 with the tumor inhibition (Cao et al. 2004; Cao et al. 2005). Boursereau and Coldham, (2004) synthesized and screened 1-amino substituted β –carbolines for their antitumor activities for the NCI 60 cell line panel. Among that 1-(N, N'-diethyl-propyl) amino- β -carboline showed the best activities against HOP-92 nonsmall cell lung cancer.

More recently, a series of novel hybrids β-carboline-4-thiazolidinones were synthesized and evaluated for their *in vitro* antitumor and antiviral activities. Out of the β-carboline-4-thiazolidinones, one bearing the 4-dimethylaminophenyl group at C-1 of β-carboline was selected for further study concerning cell death and cell cycle profile, focusing on the human renal adenocarcinoma cell line and showed very promising activity (Barbosa et al. 2016). Ling et al. (2017) synthesized a series of hydroxamic acid histone deacetylase (HDAC) inhibitors in which the β-carboline motif has been incorporated. Most of these synthesized compounds were found to show significant HDAC inhibitory effects and good antiproliferative activity at very low IC_{50} value.

In conclusion, bioassay guided fractionation as well as comparison with the chemical shifts from previous literature helped in the indention of the active compound in *Pseudonocardia* sp. MCCB 268 crude extract as 1-acetyl-β-carboline. *In vitro* evaluation of 1-acetyl-β-carboline on lung cancer NCI-H460 cells showed that it is a promising compound with antitumor activity, similar to other β-carboline compounds.

SUMMARY AND CONCLUSION

Cancer is considered as one of the deadliest diseases in the world, and its occurrence is increasing day by day because of various factors. Chemotherapy is currently the primary treatment modality in many tumors. However, the development of multidrug resistance (MDR) to chemotherapeutic drugs is a main obstacle for the successful treatment of malignant tumors. Therefore, development of novel chemotherapeutic agents would play a key role in the treatment of refractory or relapsing cancer patients. The natural environment is still the most important supply of novel drugs despite development of combinatorial chemistry, which can quickly generate thousands of new chemicals.

Marine environment constitutes rich and biologically productive region constituting most of the species of the world as a result of which there is a higher magnitude of scope for research and investigation to explore the potential of both marine organisms and marine microorganisms as producers of novel drugs. Of all the marine forms, marine actinomycetes remain the richest source of natural products since they have capabilities to produce compounds with interesting biological activities. Studies validate that marine actinomycetes are abundant in various ocean sediments. Hence, the present study was designed and directed towards discovery of antitumor

compounds from marine actinomycetes, which could be used as potential anticancer compounds.

Salient findings of this study are as follows:

- A total of 62 marine actinomycetes were isolated from different marine environment includes twenty nine isolates from the sediment samples collected from the South-West coast of Indian Ocean, three isolates from a marine sponge *Mycale* sp. and thirty isolates from Arctic Ocean sediment. All isolates were deposited in NCAAH microbial culture collection.
- Among the 5 different media tested for isolation of marine actinomyctes, Actinomycetes Isolation Agar (AIA) was found to be the most suitable medium for isolation of marine actinomyctes.
- In the preliminary *in vitro* anticancer screening assay with a single concentration (50 µg/mL), 19 isolates showed cytotoxicity. Among these positive isolates, 4 isolates were identified to be unique and showing complete *in vitro* growth inhibition were selected for further study.
- The four marine actinomycetes with potential anticancer activity, and identified using 16S rRNA gene sequence analysis, were designated as:
    - *Streptomyces* sp. MCCB 267
    - *Streptomyces* sp. MCCB 246
    - *Pseudonocardia* sp. MCCB 268
    - *Streptomyces* sp. MCCB 248
- *Pseudonocardia* sp. MCCB 268 isolated from arctic marine sediment, showed low sequence similarity (98%) with its closest neighbour

Pseudonocardia kongjuensis and it was selected for further detailed morphological, physiological and chemotaxonomic characterization along with DNA-DNA hybridisation with nearest neighbour *Pseudonocardia kongjuensis* (DSM 44525). In DNA-DNA hybridisation study *Pseudonocardia* sp. MCCB 268 exhibited low DNA–DNA relatedness (41.5 %) to its closest relative which was well below the cut of value of 70%, and could be designated as a novel isolate.

- *Streptomyces* sp. MCCB 267, *Streptomyces* sp. MCCB 246 and *Streptomyces* sp. MCCB 248 were closely related to their nearest neighbours, *Streptomyces zhaozhuensis*, *Streptomyces wuyuanensis* and *Streptomyces artemisiae* respectively, but showed distinct physiological and biochemical characteristics (especially spore structure, Carbon utilization, FAME analysis) in detailed analysis, suggesting that they might be previously undescribed strains of these species.
- IC_{50} determination of the crude extracts of selected Actinomycetes on NCI-H460 was performed and cytotoxicity was confirmed. Hoechst 33342 assay, TUNEL assay and Annexin V –PI double staining proved that cytotoxicity is NOT by necrosis but through induction of apoptotic pathways.
- GNPS molecular networking based chemical dereplication of *Streptomyces* sp. MCCB 267, was performed and analysis revealed the presence of a dominant mass ions of m/z 507.689 suggestive of the presence of polycyclic tetramate macrolactam (PTM) group.
- The biosynthetic potential of the isolates were analyzed by PCR based gene screening for different biosynthetic genes, viz. Type I polyketide

synthase (PKS I), PKS II, Nonribosomal peptide synthase (NRPS), Aminodeoxyisochorismate synthase (phzE) and Cytochrome P450 hydroxylase (CYP) genes. After screening, all the selected isolates were found to contain at least two to three biosynthetic genes responsible for bioactive secondary metabolites production.

- Bioassay guided fractionation of the crude ethyl acetate extract of *Streptomyces* sp. MCCB 267 yielded four active potential anticancer compounds belonging to polycyclic tetramate macrolactam (PTM) family. Active compounds were identified as:
  - Ikarugamycin
  - Clifednamide
  - 30-oxo-28-*N*-methyl ikarugamycin
  - 28-N-methyl ikarugamycin
- All the four compounds (clifednamide A (CF), 30-oxo-28-N-methyl ikarugamycin (OI), ikarugamycin (IK), and 28-N-methyl ikarugamycin (MI)) were initially evaluated and confirmed for their cytotoxic potential using MTT assay on NCI-H460 and BS-C-1 cells. The IC_{50} values of compounds IK, OI, MI, and CF were found to be 1.43, 7.17, 1.78, and 16.29 µg/mL, respectively on NCI-H460.
- Hoechst33342 staining and TUNEL assays revealed all four PTMs induced nuclear morphological changes on NCI-H 460 lung cancer cell lines. Compounds were also studied for apoptotic induction using Annexin V-PI double staining.
- In Cell cycle analysis Clifednamide A induced S phase arrest whereas ikarugamycin and other analogues induced G1 phase arrest of the cells. Apart from that all the compounds were found to show

concentration dependent increase in Sub G0 population of cells indicating DNA fragmentation, a hallmark of apoptosis.

- Molecular docking (MD) and Molecular dynamic simulations studies were done to understand interaction between IK and its derivatives with DNA at molecular level. Molecular Dynamic (MD) Simulations results showed IK, and its analogues induced DNA damage by bind with the DNA minor groove. MD results were validated by *in vitro* evaluation of cytotoxicity.
- Bioassay guided fractionation of crude extract of *Pseudonocardia* sp. MCCB 268 yielded 1-acetyl-β-carboline as the active compound. *In vitro* anticancer evaluation of 1-acetyl-β-carboline confirmed apoptosis induction by the compound.

Scope of further research

- Ikarugamycin type polyketides and 1-acetyl-β-carboline were found to activate apoptotic mode of cell dealth. However, it is not yet clear which apoptotic pathway was stimulated by all these compounds. Hence, future studies with the expression profiling of genes involved in apoptosis will help to find out which signal transduction pathways are getting modulated and leading to the apoptosis by these compounds.
- The other two potential isolates viz *Streptomyces* sp. MCCB246 and *Streptomyces* sp. MCCB 248 could be explored further for isolation and characterisation of anticancer compounds.
- Targeted delivery of Ikarugamycin by Nanoformulation can be attempted to reduce toxicity of the compounds on non-targeted tissues.

- All the marine actinomycetes isolated in this study were deposited in the microbial culture collection of NCAAH. These resources could be bioprospected for other bioactivities such as antimalarial, antimicrobial, anti-inflammatory etc. for potential lead compounds.

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APPENDIX

List of Publications

- **Dhaneesha M**, Benjamin NC, Krishnan KP, Rupesh S, Jayesh P, Valsamma J, Singh ISB, William HG, Sajeevan TP (2017) *Streptomyces artemisiae* MCCB 248 isolated from Arctic fjord sediments has unique PKS and NRPS biosynthetic genes and produces potential new anticancer natural products. 3 Biotech 7:32. doi: 10.1007/s13205-017-0610-3
- **Dhaneesha M**, Sajeevan TP, Krishnan KP, Singh ISB (2015) Apoptosis Mediated Anticancer Activity of *Streptomyces sp.* MCCB 248 Isolated from an Arctic Fjord, Kongsfjorden, Svalbard, on NCI-H460 Human Lung Cancer Cell Line. Marine Drugs 13, 7171. doi:10.3390/md131270590 (Special Issue "Selected Papers from the 9th European Conference on Marine Natural Products" (Abstract)).