Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Biomedicine

journal homepage:www.elsevier.com/locate/apjtb

Document heading doi:10.1016/S2221-1691(11)60199-8

Anticancer property of sediment actinomycetes against MCF-7 and MDA-MB-231 cell lines

Ravikumar S[1*], Fredimoses M[2], Gnanadesigan M[1]

[1]*School of Marine Sciences, Department of Oceanography and Coastal Area Studies, Alagappa University, Thondi Campus, Thondi-623 409, Ramanathapuram District, Tamil Nadu, India*
[2]*Department of Biotechnology, K.S. Rangasamy College of Arts & Science, Thiruchengodu-637209, Namakkal District, Tamilnadu, India*

ARTICLE INFO

Article history:
Received 15 July 2011
Received in revised form 4 August 2011
Accepted 20 August 2011
Available online 28 February 2012

Keywords:
Actinomycetes
Breast cancer
MCF-7
MDA-MB-231
Phylogenetic tree
Anticancer property
Multiple sequence analysis
Secondary structure analysis
Sediment
Anticaner agent
Cell line

ABSTRACT

Objective: To investigate the anticancer property of marine sediment actinomycetes against two different breast cancer cell lines. **Methods:** *In vitro* anticancer activity was carried out against breast (MCF-7 and MDA-MB-231) cancer cell lines. Partial sequences of the 16s rRNA gene, phylogenetic tree construction, multiple sequence analysis and secondary structure analysis were also carried out with the actinomycetes isolates. **Results:** Of the selected five actinomycete isolates, ACT01 and ACT02 showed the IC_{50} value with (10.13±0.92) and (22.34±5.82) μg/mL concentrations, respectively for MCF-7 cell line at 48 h, but ACT01 showed the minimum (18.54±2.49 μg/mL) level of IC_{50} value with MDA-MB-231 cell line. Further, the 16s rRNA partial sequences of ACT01, ACT02, ACT03, ACT04 and ACT05 isolates were also deposited in NCBI data bank with the accession numbers of GQ478246, GQ478247, GQ478248, GQ478249 and GQ478250, respectively. The phylogenetic tree analysis showed that, the isolates of ACT02 and ACT03 were represented in group I and III, respectively, but ACT01 and ACT02 were represented in group II. The multiple sequence alignment of the actinomycete isolates showed that, the maximum identical conserved regions were identified with the nucleotide regions of 125 to 221st base pairs, 65 to 119th base pairs and 55, 48 and 31st base pairs. Secondary structure prediction of the 16s rRNA showed that, the maximum free energy was consumed with ACT03 isolate (-45.4 kkal/mol) and the minimum free energy was consumed with ACT04 isolate (-57.6 kkal/mol). **Conclusions:** The actinomycete isolates of ACT01 and ACT02 (GQ478246 and GQ478247) which are isolated from sediment sample can be further used as anticancer agents against breast cancer cell lines.

1. Introduction

Cancer still remains one of the most serious human health problems and breast cancer is the second most universal cause of cancer deaths in women[1]. Therapeutic methods for cancer treatment are surgery, radiotherapy, immunotherapy and chemotherapy[2] and these techniques are individually useful in particular situations and when combined, they offer a more efficient treatment for tumour. Many of the antitumor compounds from marine drugs are derived from algal metabolites[3] and these metabolites play an important role in identification of new pharmaceutical compounds[4,5]. Actinomycetes are one of the most important sources for new bioactive compounds such as antibiotics and enzymes[6-8] which have diverse clinical effects against many pathogenic organisms *viz.*, bacteria, fungi and parasite, *etc*. In fact, more than 60% of approved drugs are derived from natural compounds[9] in that, 50% of the natural antibiotics are produced from actinomycetes, among them marine actinomycetes have been reported to have antifungal, antibacterial and anti-inflammatory activities[10]. But, studies related with the biopotential activities of anticancer metabolites from marine actinomycetes based drug discovery are too limited. In this connection, the present study was made an attempt to find out the anticancer property from marine sediment actinomycetes against two different breast cancer cell lines.

2. Materials and methods

*Corresponding author: Ravikumar S, School of Marine Sciences, Department of Oceanography and Coastal Area Studies, Alagappa University, Thondi Campus, Thondi-623 409, Ramanathapuram District, Tamil Nadu, India.
Tel: +914561-243470, +91-9003306959
E-mail: ravibiotech201321@gmail.com
Foundation Project: This work was financially supported by Indian Council of Medical Research, New Delhi (grant No. 59/6/200/BMS/TRM).

2.1. Isolation and identification of actinomycetes from marine sediments

Mangrove sediment sample was collected from Manakkudi mangrove ecosystem (Lat.8 ° 05′E; Long.77 ° 46′E), Kanyakumari district, Tamilnadu, India. Samples were immediately transferred to the laboratory and 1 g of soil sample was aseptically transferred into 99 mL of presterilized 50% sea water and kept for continuous shaking (100 rpm) for 1 h. About 100 μL of diluted samples were transferred to molten starch casein agar medium (10 g/L soluble starch, 1 g/L of casein and 18 g/L of agar made up with 50% of aged sea water) and incubated at (27±2) ℃ for 7 days. After incubation, colonies appeared on the agar medium were re-streaked in the same agar medium and further subjected for the identification by using conventional[11] and molecular[12] techniques.

2.2. Extraction of bioactive compounds

Mass cultivation of actinomycetes isolates was performed with fermentor. Briefly, 20 mL of overnight culture of actinomycetes (ACT01, ACT02, ACT03, ACT04 and ACT05) isolates were transferred into fermentor containing 1 000 mL of Bennet medium (previously sterilized with steam explosion) individually and incubated for 7 days under continuous shaking. The parameters for our fermentation process were properly set using external controlling device [temperature 24 ℃, dissolved oxygen 3, the agitator at 200 rpm and pH (7.0±0.2)]. Further, the mass cultured broth was adjusted to pH 5.0 (using 1 N HCL) and filtered through cheese cloth to remove mycelia biomass. Further, equal volume (1:1) of ethyl acetate was added and mixed by vigorous shaking and kept without disturbance. The organic phase was collected and evaporated in an incubator at (60-70) ℃ and the residue was stored at -20 ℃ for further use. The biochemical constituents such as sugars, phenol, proteins, amino acids, quinines and alkaloids were analyzed[10].

2.3. Cytotoxicity assay

The breast cancer cell lines such as MCF-7 and MDA-MB-231 were obtained from National Centre for Cell Science, Pune, India. Cells were grown as monolayer culture in RPMI 1640 medium (10% fetal bovine serum, 1 mM sodium pyruvate and 100 U/L of penicillin/streptomycin) and incubated at 37 ℃ in a 5% of CO_2 atmosphere. Cell lines (100 μL) were seeded in 96 well plates at a concentration of 5×10^3 cells/mL for 24 h. After that, culture medium was replaced with 100 μL serum free medium containing various concentrations (25, 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 μg/mL) of sponge associated actinomycete extracts at 24 h and 48 h. Later, the medium was refreshed with 100 μL of serum free medium (RPMI 1640) and 20 μL of MTT (5 μg/mL of (3, 4, 5-dimethylthiazol-2yl)-2, 5-diphenyltetrazo liumbromide). The microtitre plates were incubated for 3 h in dark and the developed color was measured with ELISA reader at 570 nm. Triplicates were maintained for each treatment. Inhibitory concentration (IC_{50}) values were directly determined by linear regression analysis with office XP (SDAS) software.

2.4. 16S rRNA gene amplification and sequencing

Genomic DNA was isolated by using standard method[13] and amplified by PCR with forward primer-F243 (5′-GGATGAGCCCGCGGCCTA-3′) and reverse primer R513GC-(5′-CGGCCGCGGCTGCTGGCACGTA-3′). The reaction mixture contained 25 to 50 ng of DNA, ExTaq PCR buffer, 1.5 mM $MgCl_2$, 10 mM deoxynucleoside triphosphate mixture, 50 pmol of each primer, and 0.5 U of ExTaq polymerase. PCR conditions consisted of an initial denaturation at 94 ℃ for 5 min; 30 cycles at 94 ℃ for 1 min, annealing at 63 ℃ for 1 min and 72 ℃ for 1 min; and final 5 min extension at 72 ℃. The amplification products were examined by agarose gel electrophoresis and purified by using a QIA quick PCR clean up kit with the protocol suggested by Qiagen Inc. The complete 16S rRNA gene was sequenced by using the PCR products directly as sequencing template with above mentioned primers. All sequencing reactions were carried out with an ABI 377 automated DNA sequencer.

2.5. Construction of phylogenetic tree

The retrieved gene sequences were compared with other bacterial sequences by using NCBI BLAST search for their pair wise identities. Multiple sequence alignment and the phylogenetic tree were constructed with MEGA 4.0 software (http://www.megasoftware.net) by using the neighbor-joining (NJ) method with 100 replicates as bootstrap value and NJ belongs to the distance-matrix method[13]. The 16S rRNA sequence was submitted to the GenBank. The RNA secondary structure of the isolates was predicted by using Genebee online software (http://www.genebee.msu.su/services/rna2_reduced.html).

3. Results

Five different sediment actinomycetes were isolated from the Manakkudi mangrove ecosystem. The mycelial characteristics of the isolated actinomycetes were varied as long branched hyphae, curling rod like, branched hypae with pink coloured spores and white coloured spores. Further, the colour of the aerial mycelium was identified as pale pink, white gray, dull white, pale pink and greenish white. Moreover, the riverside colour of the aerial mycelium was differed from white colour to yellowish colour. Size of the colony was differed from 3 cm to 6 cm (Table 1). The anticancer property of the fermented cell free extracts against breast cancer cell lines revealed that, the cytotoxic index of all the five isolates on breast cancer lines (MCF7 and MDA-MB-231) exhibited dose and time dependant growth inhibition (Figure 1 and 2). Among the five isolates, ACT01 showed the minimum concentration of IC_{50} value (10.13 ±0.92 μg/mL) followed by ACT02 (22.34±5.82 μg/mL) at 48 h for MCF-7 cell lines. Similarly, ACT01 showed the minimum concentration of IC_{50} value (18.54±2.49 μg/mL) for MDA-

Table 1
Differential characteristics of *Actinomycetes* sp. isolated from Manakkudi mangrove ecosystem.

Strain numbers	NCBI accession numbers	Characteristics	Colour of the aerial mycelium	Reverse side coloration	Diffusible pigment	Size of the colony (cm)
ACT 01	GQ478246	Spiral spores on substrate mycelium	Pale pink	Yellowish white	–	3
ACT 02	GQ478247	Long branched hypae and spore beared	White grey	Butter white	Brown	5
ACT 03	GQ478248	Curling rod like appearance	Dull white	Butter white	–	3
ACT 04	GQ478249	Branched hypae with pink coloured spores	Pale pink	Dull white	–	6
ACT 05	GQ478250	White coloured spores	Greenish white	Dull yellow	–	5

Table 2
Inhibitory concentration of cell free crude extracts from actinomycetes strains against MCF-7 and MDA-MB-231 breast cancer lines (μg/mL).

Strains	MCF 7		MDA-MB-231	
	24 h	48 h	24 h	48 h
ACT 01	19.49±1.89	10.13±0.92	32.79±6.94	18.54±2.49
ACT 02	29.94±7.71	22.34±5.82	69.84±19.54	60.34±17.34
ACT 03	75.54±19.83	67.94±9.48	84.09±18.93	71.74±13.30
ACT 04	66.04±15.94	58.44±8.94	>100	>100
ACT 05	92.64±12.93	85.99±18.76	>100	>100

MB-231 cell lines at 48 h. But, ACT04 and ACT05 showed IC_{50} values greater than 100 μg/mL for MDA-MB-231 cell lines at 24 and 48 h (Table 2). The preliminary biochemical analysis of the extracts from ACT01 and ACT02 showed the presence of alkaloids and quinines.

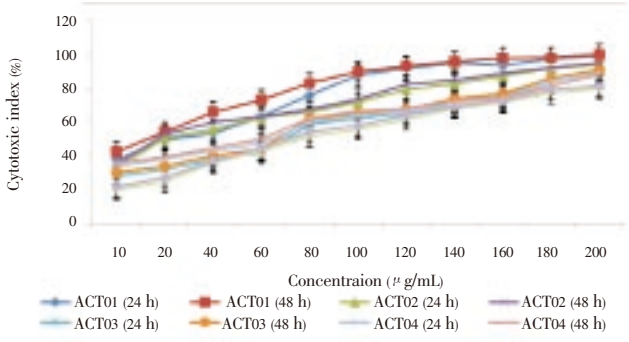

Figure 1. Percentage of cytotoxic index of different concentrations of cell free crude extracts from actinomycetes strains against MCF-7 breast cancer lines.

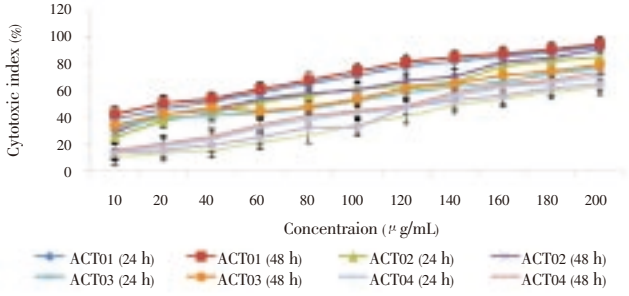

Figure 2. Percentage of cytotoxic index of different concentrations of cell free crude extracts from actinomycetes strains against MDA-MB-231 breast cancer lines.

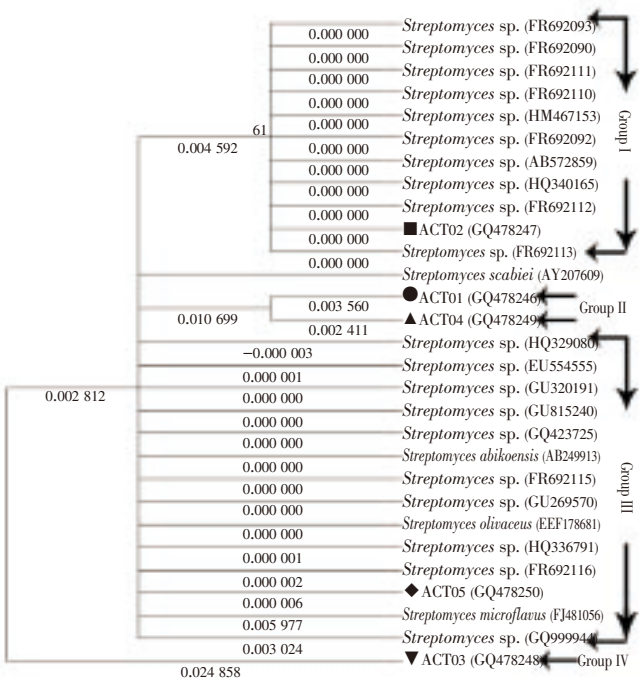

Figure 3. Neighbour joining phylogenetic tree analysis (16s rRNA) of the isolated actinomycete species.

The 16s rRNA sequence analysis of the isolated actinomycetes *viz.*, ACT01, ACT02, ACT03, ACT04 and ACT05 showed 260, 266, 224, 265 and 268 nucleotide base pairs, respectively. The NCBI BLAST analysis showed that, all the isolates showed maximum similarity (>95%) with *Streptomyces* sp. and their accession numbers were mentioned in Table 1. The phylogenetic analysis of the isolated actinomycetes was categorized into four groups on the basis of evolutionary distance with NJ method (Figure 3). It revealed that, the first group was included with the ACT02 isolate. The second (ACT01 and ACT02) and fourth groups (ACT03) were having

distinct relations with the other groups. But, the ACT05 was included with the group 3. Moreover, the multiple sequence alignment was carried out with available sequences of *Streptomyces* sp. from NCBI data bank (first 10 hits in BLAST results) and the results showed that, the maximum identical conserved regions were identified with the nucleotide regions of 125 to 221st base pairs, followed by, 65 to 119th base pairs and 55, 48 and 31st base pairs without the gap alignments (data not shown). Secondary structure prediction of the 16s rRNA showed that, the maximum free energy was consumed with, ACT03 isolate (−45.4 kkal/mol) and minimum free energy was consumed with ACT04 isolate (−57.6 kkal/mol) (Figure 4).

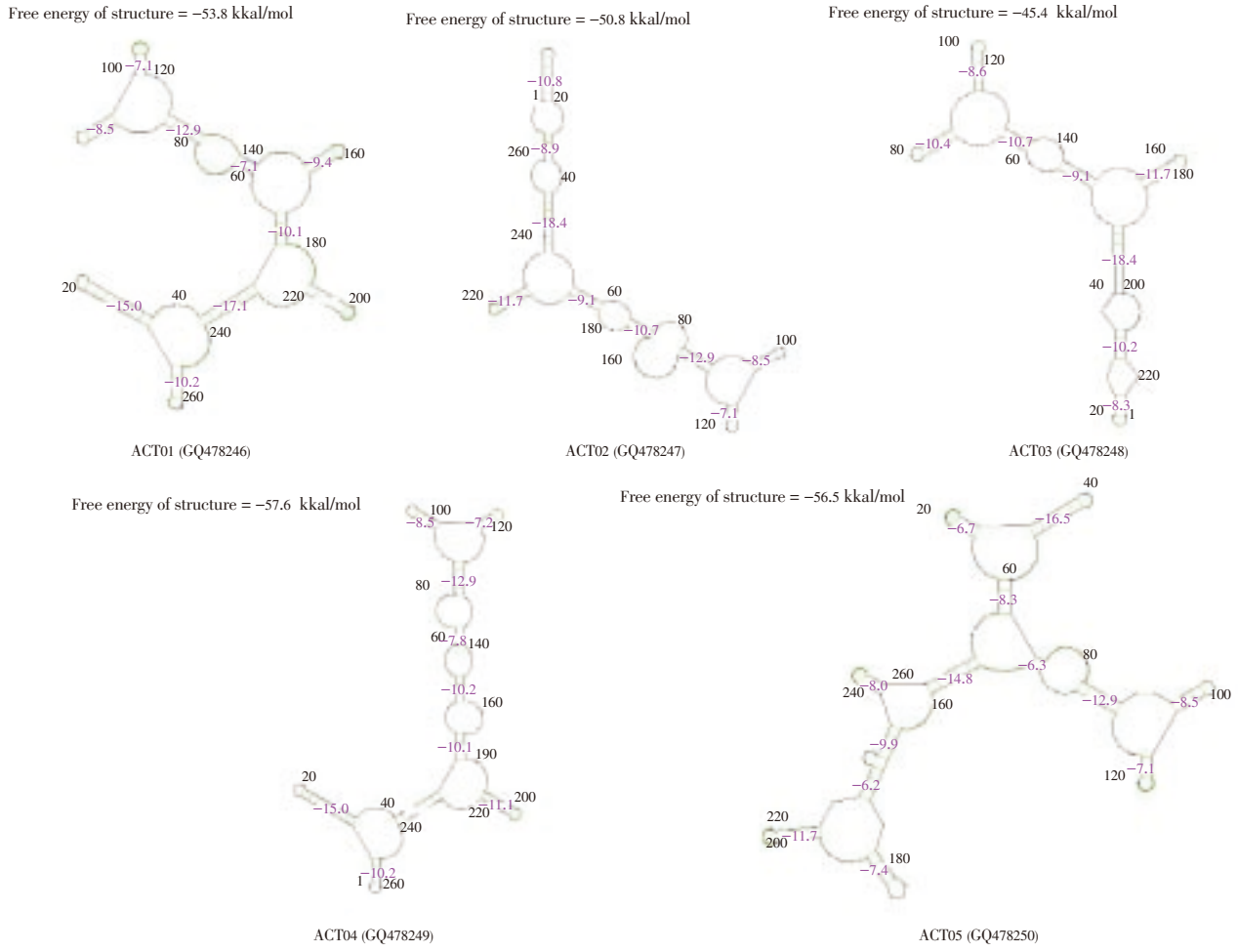

Figure 4. Secondary structure analysis (16s rRNA) of the isolated actinomycete strains.

4. Discussion

Cancer is considered to be the public health problem in developed and developing countries, among which breast cancer is the second most universal cause of cancer deaths in women. Despite the intense efforts to develop treatments, effective agents are still not available. In this regard, natural product extracts continue to be the most promising source of new drugs for cancer. The present study was made an attempt to find out the anticancer compounds from marine actinomycetes. In the present study the actinomycete isolates ACT01 and ACT02 showed IC_{50} values less than 30 μg/mL concentration in both (MCF7 and MDA-MB-231) breast cancer cell lines. Suffness and Pezzuto, reported that, the IC_{50} values less than 30 μg/mL in cancer cell lines can be considered as promising for anticancer drug development[14]. The anticancer property of cell free extracts from actinomycete isolates might be due to the presence of the active secondary metabolites such as alkaloids and quninine[15]. Alkaloids are one of the major physiologically active nitrogenous compounds derived from many biogenetic precursors. Alkaloids are microtubule interfering agents which can bind with beta tublin, thus preventing the cell from making the mitotic spindle fibres necessary to move the chromosome around as the cell divides[16], inhibiting topoisomerase I[17], mitochondrial damage and inducing the release of cytochrome C and apoptosis inducing factor[1]. Moreover, quinine derivatives *viz.*, driamycin, daunorubicin, mitomycin C, streptonigrin and lapachol, can interfere the DNA and RNA replication and mitochondrial oxidative pathways or the formation of super oxide, peroxide and hydroxide radicals as toxic products in the cell line. Several compounds of anthro-quinone families (parimycin, trioxacarcins and gutingimycin) showed antitumor activities[18-20]. The cytotoxic effect of actinomycete extracts seems to be the induction of the cell apoptosis, partial cellular differentiation, and degradation of fusion transcripts, antiproliferation and inhibition of

angiogenesis[1]. The construction of phylogenetic tree was used to find out the relationship between the production of secondary metabolites, nature of product and pathogenic identities. It can be concluded from the phylogenetic tree analysis that, the ACT01 (*Streptomyces* sp. GQ478246) from group I was proved to have good anticancer activity. Similarly, the other species (*Streptomyces* sp. HQ340165) from the group I was also proved to have potential antibacterial properties from the source of marine samples[12]. The ribosome is an important component for the protein synthesis and their structural RNA subunits are highly conserved and these conserved regions can be used as a molecular marker to identify the evolutionary relationships between organisms[21]. In the present study, multiple sequence alignment of the isolated actinomycetes showed to have the conserved regions between 61-115 and 140-260 nucleotide regions. Hence, these regions can be used as a molecular marker for the identification of the *Streptomyces* sp. from other actinobacterium species. Similar multiple sequence identities are already identified with the bacterial isolates of *Acidithiobacillus ferrooxidants*[21]. The rRNA secondary structure of ACT04 showed lower minimal free energy than the other isolated isolates, this may be due to the variations of high G or C contents[21]. Hence, the lowest free energy values of the rRNA secondary structures provides the high relationship with the most primitive organisms, and the highest free energy indicates the less stability during the evolutionary period[22]. Even though, comparison of isolated isolates with the other group organisms is also highly warranted. It is concluded from the present study that, the actinomycete isolates of ACT01 and ACT02 (GQ478246 and GQ478247) from the mangrove sediments of Manakkudi mangrove ecosystem could be used for the development of anticancer drug after completing the successive clinical trials.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors are thankful to the authorities of Alagappa University for providing required facilities and also to Indian Council of Medical Research, New Delhi for financial assistance.

References

[1] Ravikumar S, Gnanadesigan M, Thajuddin N, Chakkaravarthi VSD, Banerjee BM. Anticancer property of sponge associated actinomycetes along Palk strait. *J Pharm Res* 2010; **3**(10): 2415-2417.

[2] Gillet JP, Efferth T, Remacle J. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. *Biochem Biophys Acta* 2007; **1775**: 237-262.

[3] Berdy J. Bioactive microbial metabolites. *J Antibiot* 2005; **58**: 1-26.

[4] Ravikumar S, Suganthi P, Moses FM. Crude bioactive compounds of actinomycetes from Manakkudy mangrove sediment. *J Pharm Res* 2011; **4**(3): 877-879.

[5] Garcia MA, Jamel EM, Ward MM, Center Y, Hao RL, Siegel, et al. *Global cancer facts and figures*. Atlanta: The American Cancer Society; 2007.

[6] Vijayakumar R, Muthukumar C, Thajudin N, Panneerselvam A, Saravanamuthu R. Studies on the diversity of actinomycetes in the Palk Strait region of Bay of Bengal, India. *Actinomycwetologica* 2007; **21**: 59-65.

[7] Singh LS, Indra B, Bora TC. Actinomycetes of loktak habita: isolation and screening for antimicrobial activities. *Biotechnology* 2006; **5**(2): 217-221.

[8] Xu M, Zhu Y, Jin Z, Wu H, Li X, Yang Y, et al. Glycine origin of the methyl substitute on C7′-N of octodiose for the biosynthesis of apramycin. *Sci China C Life Sci* 2006; **49**: 362-369.

[9] Raja M, Ravikumar S, Gnanadesigan M, Vijayaumar V. *In vitro* antibacterial activity of diterpene and benzoxazole derivatives from *Excoecaria agallocha* L. *Int J Biol Chem Sci* 2010; **4**(3): 692-701.

[10] Ravikumar S, Gnanadesigan M, Suganthi P, Ramalakshmi A. Antibacterial potential of chosen mangrove plants against isolated urinary tract infectious bacterial pathogens. *Int J Med Med Sci* 2010; **2**(3): 94-99.

[11] Bergey DH, Holt JG. *Bergey's manual of determinative bacteriology*. 9th ed. Baltimore: Lippincott Williams & Wilkins; 1994.

[12] Singh V, Praveen V, Khan F, Kant C, Tripathi M. Phylogenetics of an antibiotic producing *Streptomyces* strain isolated from soil. *Bioinformation* 2009; **4**(2): 53-58.

[13] Joseph S, Shanmugapriya S, Gandhimathi R, Kiran SG, Ravji RT, Natarajaseenivasan K, et al. Optimization and production of novel antimicrobial agents from sponge associated marine actinomycetes *Nocardiopsis dassonvillei* MA D08. *Appl Microbiol Biotechnol* 2009; **83**: 435-445.

[14] Suffness M, Pezzuto JM. Assay related to cancer drug discovery. In: Hostettman K. (ed.) *Methods in plant biochemistry: assay for bioactivity*. London: Academic Press; 2009, p. 71-133.

[15] Gordaliza M. Cytotoxic terpene quinines from marine sponges. *Mar Drugs* 2010; **8**: 2849-2870.

[16] Solanki R, Khanna M, Lal R. Bioactive compounds from marine actinomycetes. *Indian J Microbiol* 2008; **48**: 410-431.

[17] Facompre M, Tardy C, Bal-Mahieu C, Colson P, Perez C, Manzanares I, et al. A novel potent inhibitor of topoisomerase I. *Cancer Res* 2003; **63**: 7392-7399.

[18] Anibou M, Chait A, Zyad A, Taourirt M, Ouhdouch Y, Benherref A. Actinomycetes from Moroccan habitats: isolation and screening for cytotoxic activities. *World J Microbiol Biotechnol* 2008; **24**: 2019-2025.

[19] Gulecha V, Sivakuma T. Anticancer activity of *Tephrosia purpurea* and *Ficus religiosa* using MCF 7 cell lines. *Asian Pac J Trop Med* 2011; **4**(7): 526-529.

[20] Kumar RS, Rajkapoor B, Perumal P. *In vitro* and *in vivo* anticancer activity of *Indigofera cassioides* Rottl. Ex. DC. *Asian Pac J Trop Med* 2011; **4**(5): 379-385.

[21] Peng H, Yang Y, Li X, Qiu G, Liu X, Huang J, et al. Structure analysis of 16S rDNA sequences from strains of *Acidithiobacillus ferrooxidans* school of minerals. *J Biochem Mol Biol* 2005; **39**(2): 178-182.

[22] Somvanshi P, Seth PK. Predicted RNA secondary structures for the conserved regions in dengue virus. *Bioinformation* 2010; **3**(10): 435-439.