International Journal of Modern Engineering Research (IJMER)
www.ijmer.com Vol.2, Issue.6, Nov-Dec. 2012 pp-4510-4515 ISSN: 2249-6645

# Isolation and Molecular Characterization of Anti-Cancerous Compound Producing Marine Bacteria by Using 16S rRNA Sequencing and GC-MS Techniques

Sai Lakshman Mithun.V[1], C.S.V.Ramachandra Rao[2]
*Department of Biotechnology, DVR & Dr.HS MIC College of Technology, Kanchikacherla, Andhra Pradesh, India.*

***Abstract:*** *Extracts from microorganisms have served as a valuable source of diverse molecules in many drug discoveries. Identification of microbial strains having promising biological activities and purifying the bio-molecules which are responsible for the biological activities, have led to the discovery of many bioactive molecules. Extracts of bacteria in vitro tested on various cancer cell lines. The lyophilized bacterial extract powder was dissolved in various chemical solvents like Methanol, Chloroform and Ethyl acetate. From them cytotoxic assays was performed the extracts were screened on HCT 15 and MES- SA cancer cell lines to study the cytotoxic potential. Where the Ethyl acetate showed the inhibition 87.34% when compared to other solvents. The Ethyl acetate extract of isolate showed promising results by MTT assay and Trypan blue staining. Identification of the microorganism, the selected isolates was characterized by using the 16s rRNA sequencing based on microbial characterization, the compound produced by bacteria was analyzed by GC-MS technique. The organism was identified as Micrococcus luteus and biochemical tests of the extracts were also carried out.*

***Key words:*** *Anticancer, Bacterial extracts, MTT assay, HCT 15 cell line, MES-SA cell line.*

## I. INTRODUCTION

Cancer is a disease in which a cell, or a group of cells represents uncontrolled growth invasion (intrusion on and distortion of adjacent tissues), and metastasis (spread from one part to another part in the body through lymph or blood). These three malignant properties differentiate cancer from benign tumors, which are self-limited and do not invade or metastasize while the malignant tumors are not self limited and metastasize. Most of cancers occur form a tumor; the oncology is deals with the study, diagnosis, treatment, and prevention of cancer and it's a branch of medicine. Cancer is a human tragedy that affects people at all ages with the risk in most types increasing with age. Cancers are primarily an environmental disease with 90-95% of cases due to modification in lifestyle and environmental factors and 5-10% due to genetics Cancer is caused.

By both external factors (tobacco, chemicals, radiation and infectious organisms) and internal factors (inherited mutations, hormones, immune conditions and mutation that occurs from metabolism). Common environmental factors leading to cancer death include: tobacco 25-30%, diet and obesity 30-35%, infections 15-20%, radiation, stress, lack of physical activity and environmental pollutants. [B Dhorajiya et al., 2011].

Early discovery of carcinogens by John Hill, an English physician, 1761. In it he made the first causal link between substances in the environment and cancer when he described a relationship between tobacco snuff and nasal cancer. This brought about the awareness of carcinogens (chemical agents that have been demonstrated to cause cancer). That associated particular occupations with an increased risk of developing specific forms of cancer the forerunner to the field of public health and cancer.[C. A. Almeida and S. A. Barry, 2010].

Natural products have played an important role in treating and preventing human diseases. Natural products with medicinal value have come from various sources *viz.*, terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates the microbial extracts have served as a valuable source of diverse molecules in many drug discovery efforts and led to the isolation of several important drugs. [Newman *et al.*, 2002]. The chemical composition of bioactive compounds of microbial origin is often highly complex. Organic compounds from aqua to terrestrial microorganisms have extensive use in the treatment of many diseases and serve as compounds of interest both in their natural form and as templates for synthetic modification. These compounds provided important contributions to the discovery of antibacterial agents like penicillins, cephalosporins, tetracyclines, and polypeptides, immunosuppressive agents like cyclosporine, ant diabetic agent like acarbose, cholesterol-lowering agents like lovastatin and mevastatin and anti cancer agents like peplomycin, pentostatin, and epirubicin [Butler, 2005; Sneader, 2005]

## II. MATERIALS AND METHODS

**Screening and isolation of bacterial strains:**

**Collection of soil sample:**

Marine soil samples were collected from Bay of Bengal coast of Machilipatnam, Krishna district, AP, India. About 15 grams of soil sediments were collected in a sterile polythene bags and stored at 4$^{o}$C until further use. The soil samples were stored under sterile conditions for preventing the bacterial cross contamination. Then the sample was serially diluted to till $10^{-6}$ dilution by adding 1 gram of soil to 10ml of distilled water.

**Screening for bacterial single isolates:**

From the serially diluted samples, 100µl of sample was poured on Nutrient Agar plates (Himedia). From the $10^{-5}$ and $10^{-6}$ dilutions the single isolated bacterial colonies were obtained on agar plates by the procedure according to [Shirai *et al*. (1989).], The bacterial pure cultures were isolated.

**Sub culturing of bacterial isolates:**

The inoculum was prepared by transferring a single colony of the isolates into 5 ml of the corresponding medium. This inoculum was transferred to 95 ml of the corresponding broth and bacterial isolates were incubated at 37°c for 2 -3 days .this broth was used for the extraction of bioactive metabolites.

**Preparation of bacterial extracts:**

The culture broth was centrifuged at 3000 rpm for 15 min to obtain a clear supernatant. Components were extracted successively dissolved with chemical solvents such as chloroform (C), Methanol (M) and Ethyl acetate (E) and followed by vacuum evaporation of these extracts to obtain the dry extracts by vacuum rotary evaporator. [Angel TreasaThomas etal., 2011]

**Sample preparation for biological studies:**

The bacterial crude extracts were dissolved in 1 ml DMSO and transferred to sterile vials of 2 ml capacity; these samples were stored at room temperature for further use, protected from light.

**Maintenance of cancer cell lines:**

Human colorectal adenocarcinoma Cancer cells (HCT 15) and Human uterine cancer cells (MES-SA) were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 4.5 g/l glucose and 2mm l-glutamine and 5% fetal bovine serum (FBS) (growth medium) at $37^0$c in 5% $Co_2$ incubator.

**Trypan blue dye exclusion technique:**

A cell suspension was made with a fixed volume of cells (e.g. 1ml). Although an aseptic technique is not essential in all stages of this procedure, 50uL of cell suspension was taken and mixed it with an equal volume of trypan blue. Solution was well mixed using a pipette. It transfers to a hemocytometer and then counted live cell as clear form and dead cell as blue cells. After staining with trypan blue solution counting should commence <5minutes as after that time the cells will begin to take up the dye. The hemocytometer was placed on the stage of an inverted microscope.
Focus was adjusted and power until a single counting square fills the field. The number of cells per ml, and the total number of cells were counted using the following formula:
Calculate percent viability by using formula:
% viability = (live cell count/total cell count) ×100

**Anticancer assay:**

**Micro culture tetrazolium (MTT) assay:**

The monolayer cell culture was trypsinized and the cell count was adjusted to 1ml using medium. To each well of 96 well microtitre plates, 0.1ml of diluted cell suspension was added. After 72 hour, the sample solution in wells was flicked off and 50µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37° c in 5% $CO_2$ incubator. The supernatant was removed, 50 µl of Propanol was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a micro plate reader at a wavelength of 490 nm. The percentage growth inhibition was calculated using the Formula below:
The percentage growth inhibition was calculated using following formula:
%cell inhibition= 100-{(At-Ab)/ (Ac- Ab)} x100
Where,
At= Absorbance value of test compound
Ab= Absorbance value of blank
Ac=Absorbance value of control [T. Mosmann, *J. Immunol. Methods]*

**Identification of microorganism:**

Biomass culture for anticancer activity assays was carried out in 500 mL conical flasks at 37°C temperature. Total DNA was extracted and PCR amplification was performed according to the method described by [Fawley and Fawley (2004)] using following primers:
**Forward primer**- $5^1$ GGCGAACGGGTGAGTAA $3^1$
**Reverse primer**- $5^1$ ACTGCTGCCTCCCGTAG $3^1$

The Genomic DNA was isolated from the overnight grown bacterial culture was amplified with universal bacterial primers. A 25µl of reaction mixture contains 15µl of master mix (10 x assay buffer, dntp's, Taq polymerase and $MgCl_2$), 1µl of forward primer, 1µl of reverse primer, 2µl of template DNA and 6µl of distilled water. PCR was carried out by the thermal cycler under the following conditions- An intialization step at 94ºC for 4min followed by 30 cycles of 94ºC for 1min, 52ºC for 1min, 72ºC for 1.15min followed by final extension at 72ºC for 1min and holding temperature at 10ºC for

1min. The amplified DNA fragments were observed by agarose gel electrophoresis in 2% agarose gel and sequenced. The unknown bacterium was identified using
GenBank database. The amplified PCR products were directly sequenced in an ABM Prism 3100-Avant Sequencer. The obtained sequences were analyzed using BLAST tool to get the relative identification of each bacterial species. PCR samples were purified, after that the bacterial extracts were subjected to thin layer chromatography for detecting the activity of extract and their components

**Screening of Bacterial metabolite with GC-MS:**
The active bacterial extract which was showing the maximum inhibition on cancer cell lines growth was analyzed by Gas Chromatography and Mass Spectrophotometer, using this technique the main compound having the anticancerous activity can be detected

## III. RESULTS AND DISCUSSION

Cancer cells were tested with Chloroform, Methanol and Ethyl acetate bacterial extracts cell viability was evaluated by MTT assay. A gradual decrease in the viability of HCT 15 and MES-SA cancer cells were observed in for all the bacterial extracts used in the study. The bacteria was identified as *Micrococcus luteus* by 16s r RNA sequencing.

MTT cell growth inhibition assay was taken as in vitro measure of anticancer activity of bacterial extracts by using different cancer cell lines. Ethyl acetate extracts of bacteria are effective in inhibiting cancer cell growth of the two cancer cell lines. Therefore present invitro studies on ethyl acetate bacterial extracts demonstrate the remarkable anticancer potentials due to the presence of active principles may responsible for this anti cancer activity. Hence Micrococcus luteus Ethyl acetate extracts can be used as a potent natural source of anticancer agent. The GC-MS analyses the bacterial extract and compound identified as pyrrolo (1, 2-alpha) pyrazine1,4 dione hexahydro 3-(2-methylpropyl)

**Isolation of pure cultures:**

The bacterial pure cultures were isolated by serial dilution and streak plating on nutrient agar medium and selected bacterial colonies were sub cultured in to nutrient broth, and then the bacterial cultures were lyophilized and dissolved in chemical solvents. These bacterial extracts were dissolved in DMSO for long period further use.

**FIGURE 1: Isolation of pure culture**

**Trypan blue staining Report:**
The trypan blue staining was performed and the cancer cell line viability cell proliferation as shown below

**Table 1: Trypan blue staining % of Viability of cancer cells**

| Cell line | % viability | Live cell count | Total cell count | $P^H$ |
|---|---|---|---|---|
| MES-SA | 81.1% | $1.72\times10^5$ | $2.12\times10^5$ | 7.5 |
| HCT 15 | 72% | $1.728\times10^5$ | $2.40\times10^5$ | 6.9 |

**GC-MS Report:**

Gas chromatography and mass spectrophotometer analyses the bacterial extract and the metabolite produced by bacteria is identified as Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)

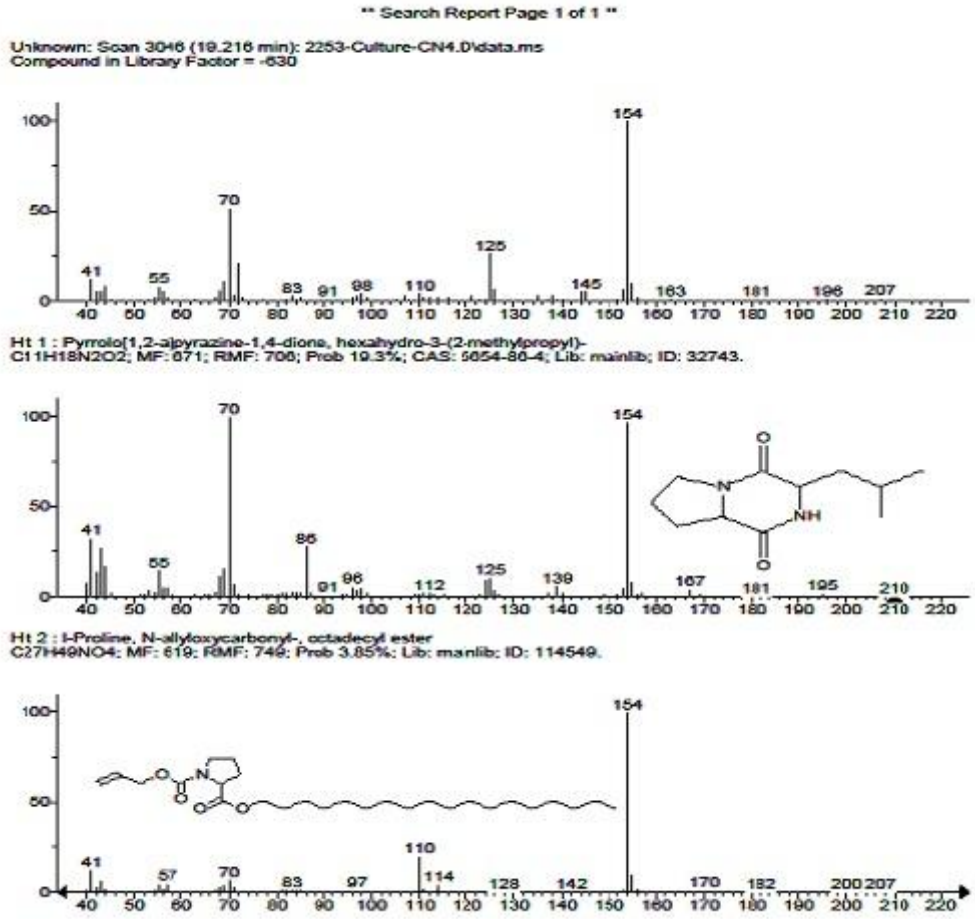



**FIGURE 1: GC-MS analysis report with bacterial extract**

**16s rRNA sequencing report:**

The selected bacterial extract sample was analyzed by using 16s rRNA sequencing, the isolate was identified as *Micrococcus luteus*

>mt_16srRNA

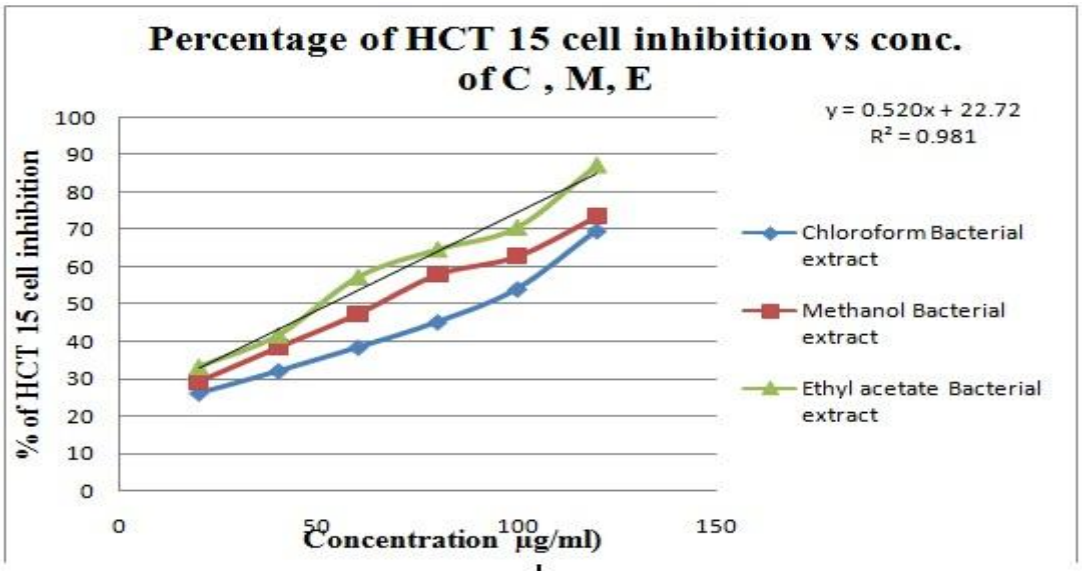
CGCATGGTGGGTGTTGGAAAGATTTATCGGTTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGT
AATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCC
GCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTG
CAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTATCCGGAAT
TATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATC
TGCGGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGC
AGATATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCA
TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGACCA
TTCCACGGTTTCCGCGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAAC
CTTACCAAGGCTTGACATGTTCTCGATCGCCGTAGAGATACGGTTTCCCCTTTGGGGCGGGTTCACAGGT
GGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTT
CCATGTTGCCAGCACGTCGTGGTGGGGACTCATGGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGAGGA
CGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTG
CGATACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCC

**MTT assay Report:**

From the **Table 2 and graph 1:** we can say that as the concentration of Chloroform, Methanol and Ethyl acetate bacterial extracts increased from 20 to 120 µl/ml, the % HCT 15 cancer cell growth inhibition was increased from 26.19 % to 69.76 % in chloroform bacterial extract, 29.45% to 73.56 % in Methanol bacterial extract and 33.24 % to 87.34% in Ethyl acetate bacterial extract, that means they induces a cell arrest to inhibit the growth of the HCT 15cancer cells. The Ethyl acetate bacterial extract showing the promising results

**Table 2: Percentage of HCT 15 cell inhibition at various concentrations**

| Concentration µg/ml | Percentage of HCT 15 cell inhibition | | |
|---|---|---|---|
| | Chloroform Bacterial extract | Methanol Bacterial extract | Ethyl acetate Bacterial extract |
| 20 | 26.19 | 29.45 | 33.24 |
| 40 | 32.12 | 38.62 | 41.77 |
| 60 | 38.47 | 47.56 | 57.33 |
| 80 | 45.28 | 58.08 | 64.73 |
| 100 | 54.11 | 62.9 | 70.65 |
| 120 | 69.76 | 73.56 | 87.34 |




**Graph 1: Effect of Chloroform, Methanol and Ethyl acetate bacterial extracts on HCT 15 cancer cell growth inhibition**

## IV. CONCLUSION

Bacterial extracts of the metabolites were *in vitro* tested for their cytotoxic potential on various cancer cell lines. The extracts were screened on HCT 15 and MES-SA cell lines. The Ethyl acetate extract of bacterial isolate showed promising results by MTT assay and Trypan blue staining. The isolate was identified as *Micrococcus luteus sps* by using 16s rRNA typing, GC-MS analyses the bacterial extract and the metabolite is identified as Pyrrolo [1, 2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) showing promising results were obtained with anti cancerous activity

## V. ACKNOWLEDGEMENTS

SAI LAKSHMAN MITHUN.V would like to thank Department of Biotechnology, DVR& Dr.HS MIC College of Technology, Kanchikacherla, and Andhra Pradesh for their cooperation to complete this work For Masters degree

## REFERENCES

[1] Angel TreasaThomas , Josyula Venkata Rao, Volety Mallikarjuna Subramanian , Hariharapura Raghu Chandrasekhar, Naseer Maliyakkal**1**, Tukaram Kedar Kisan, Alex Joseph, Nayanabhirama Udupa, In vitro anticancer activity of microbial isolates from diverse habitats, Brazilian Journal of Pharmaceutical Sciences, vol. 47, n. 2, apr./jun., 2011

[2] B Dhorajiya, M Malani, B Dholakiya, Extraction and Preservation Protocol of Anti-Cancer Agents from Marine World, Chemical Sciences Journal, CSJ-38, accepted version, Oct 22, 2011

[3] Angel.T.T, Venkata rao.J, Jesilm.A, Subrahmanyam.V.M, Venkatesh.K.B, Udupa.N. Antimicrobial profile of extremophiles from aqua to terrestrial habitats. Pharmacology online, v.1, p.111-126, 2009.

[4] T. Mossman, J. Immunol. Methods; 1983, 65, 55.

[5] Sanjay patel. Nirav gheewala, Ashok suthar, Anand shah, In-vitro cytotoxicity activity of solanum nigrum extract against Hela cell line and vero cell line, International Journal of Pharmacy and Pharmaceutical Sciences, Vol. 1, Suppl 1, Nov.-Dec. 2009
[6] CLARDY. J. Discovery of new compounds in nature. Proc. Amer. Phil. Soc., v.151, p.201-210, 2007.
[7] BUTLER, M.S. Natural products to drugs: natural products derived compounds in clinical trials. Nat. Prod. Rep., v.25, p.475-516, 2008.
[8] HARLEY, J.P.; PRESCOTT, L.M. Laboratory exercises in microbiology. 5. ed. Columbus: The MC Graw Hill Companies, 2002. P.13-16, 125-207.
[9] ATTA–UR-RAHMAN.; IQBAL, C.E.; WILLIAM, J.T. Bioassay techniques for drug development. Amsterdam: Harwood Academic Publishers, 2001. P.1-5
[10] Phillips HJ and Terryberry JE. Counting actively metabolizing tissue cultured cells. Cell Research 1957; 13: 341-347.
[11] Masters RW. Animal cell culture, Trypan Blue Assay sop. 3rd ed. 2000, p. 1 – 3
[12] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D et al. Evaluation of Colorimetric Protein and Biomass Stains for Assaying Drug Effects upon Human Tumor Cell Lines. Proceedings of the American Association for Cancer Research 1989; 30: 612.
[13] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D et al. New Colorimetric Cytotoxicity Assay for Anticancer-Drug Screening. Journal National Cancer Institute 1990; 82(13), 1107-1112.
[14] Masters RW. Animal cell culture, Cytotoxicity and viability assays. 3rd ed. 2000, p. 202-203.
[15] JEFFREY, M.E.; LINDA, S.A.; ANDREW, O.M. A rapid and simple MTT based spectrophotometric assay for determining drug sensitivity in monolayer culture. Tissue Cult. Meth, v.11, p.15-17.
[16] McCloud TG, 2010. High Throughput Extraction of Plant, Marine and Fungal Specimens for preservation of Biologically Active Molecules. Marine Drugs, 15: 4526-4563
[17] MARIANNA, J.; JAMES, P.K.; RONALD, R.R. Macquarimicins, microbial metabolites from Micromonospora I. Discovery, taxonomy, fermentation and biological properties. J. Antiriot., v.48, p.462-466, 1995
[18] Newman DJ, Cragg GM, 2007. Natural products as sources of new drugs over the last 25 years. Natural Products, 70: 461–477.
[19] Rajeev Kumar Jha, and Xu Zi-rong, 2004 Biomedical Compounds from Marine organisms, Marine drugs 2,123-124
[20] Alejandro M.S. MAYER and Kirk R. GUSTAFSON, Marine pharmacology in 2000: Anti tumor and cytotoxic compounds, International journal of Cancer: 105**,** 291–299 (2003)