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Cytotoxicity Activity of Crude Extract Produced by Marine Isolate (MS19) from Sea Water Collected from Bay of Bengal, India

Lalitha P*, Gayathri P† and Paari KA‡

Abstract

Marine bacteria have biological properties like anti-inflammatory, anticancer, antimicrobial, and antioxidant properties. Isolation of 10 strains from sea water was tested to confirm the Actinomycetes character. Among the isolates out of 10, 1 was recorded to be gram positive. Carbon utilisation profile revealed that all the isolates utilised Dextrose and none utilised Ducitol. Production of cytotoxic compound from MS19 strains was carried out by liquid fermentation using ZMB medium and the crude extract was tested with cytotoxicity activity using A549 cell line by MTT assay.

Keywords: Cytotoxicity Activity, AO/EB Staining, Actinomycetes

1. Introduction

Natural resources such as microorganisms, plants, vertebrates and invertebrates are valuable sources of bioactive compounds. A large number of drugs have been developed in medicinal practice from natural products [1]. A few of the potential bioactive compounds

*National Centre for Sustainable Coastal Management, Chennai, India; lalithabiotech2007@gmail.com

†Green Enviro Polestar, Puducherry, India; gaya3.pari@gmail.com

‡CHRIST (Deemed to be University), Karnataka, India; paari.ka@christuniversity.in

obtained from marine and terrestrial sources are Bryostatin, Dolastatin, Auristatin, and Combretastatin. The marine environment covers more than 70% of the world's surface and is a rich source of both biological and chemical diversity [2]. Marine organisms have potential in terms of their ability to produce secondary metabolites which can be utilised as lead compounds in drug discovery. Some of them are anti-inflammatory compounds (e.g. Seudopterosins, Topsentins, Seytonenin and Manoalide), anticancer agents (e.g. Bryostatins, Discodermolide, Eleutherobin and Sarcodictyin), antibiotics (e.g. Marinone), and anti-parasitic compounds (e.g. Valinomycin). So, nature has a rich potential heritage of therapeutic resources that have been exploited for effective and beneficial use against many human cancers such as pancreatic, breast, bladder and lung cancer either as a prevention strategy or as therapeutic armamentaria to kill tumour cells [3].

Cancer is the largest single cause of death in both men and women, claiming over 6 million lives each year in the world. To date, many anti-cancer drugs have been developed and applied in clinical trials. However, resistance to anti-cancer drugs and side-effects has been discovered. The killing of tumours through the induction of apoptosis which would not cause resistance and side-effects has now been recognised as a novel strategy for cancer drugs [4].

Induction of apoptosis is a useful approach in cancer therapies. Apoptosis, a major process of programmed cell death, plays an important role in the regulation of tissue development and homeostasis [5-6]. As compounds which can induce apoptosis are considered to have potential as anti-tumour agents [7], many efforts have been made to discover new drugs through the isolation of apoptosis-inducing agents from natural products.

Cell dying by apoptosis maintain membrane integrity until late in the process but display several morphological and biochemical alterations, including Chromatin condensation, nuclear segmentation, internucleosomal DNA fragmentation, cytoplasmic vacuolisation, cell shrinkage and membrane blebbing with the shedding of apoptotic bodies [8-9]. In this study, the potential of a

cytotoxicity compound from marine bacteria has been isolated and characterised.

2. Materials and Methods

2.1 Reagents

Seawater was collected from Kasimedu, Chennai. Hicarbohydrate™ kits (Himedia Laboratories, Mumbai, India), Zobell Marine Agar (ZMA) and Zobell Marine Broth (ZMB), 3, -(4, 5–dimetthylthiazol-2-yl-)-2, and 5-diphenyltetrazolium bromide (MTT) were obtained from Himedia, India. Hexane, Ethyl Acetate, Acetone, Chloroform, and Methanol were obtained from Solvent, Germany.

2.2 Isolation of Marine Bacteria

Samples were collected from different sampling points and were serially diluted and plated on nutrient agar medium containing 1.5 % NaCl. Zobell Marine Agar medium was incubated at 37^{o}C for 48 hours. The bacterial isolates were selected at random on the basis of their colony morphology. Based on their capacity to produce potential bioactive molecules, these bacterial isolates were chosen for preliminary screening for cytotoxicity activity.

2.3 Morphological, Physiological and Biochemical Characterisation

Taxonomic characterisation of marine bacteria was identified using cell morphology, Gram staining. Growth at 4°C and 42°C were determined following standard methods. Also, routine biochemical tests such as growth kinetics of marine bacteria on Zobell Marine Agar, Urease, Catalase, Oxidase, Nitrate Reduction, Gelatin Hydrolysis production were carried out following standard methods [19]. Utilisation of carbon source such as Lactose, Xylose, Fructose, Maltose, Dextrose, Galactose, Melibiose, L-Arabinose, Mannose, Glycerol, Ribose, A-methyl-D-glucoside, Xylitol, Esculin, D-Arabinose, Malonate, Sorbose, Trehalose, Sorbitol, Mannitol, Adonitol and Glucosamine [17], [18], [20] was tested using

Hicarbohydrate™ kits (Himedia Laboratories, Mumbai, India) according to the manufacturer's instructions. Results of these tests were scored as either positive or negative.

2.4 Extraction of Bioactive Compound

The marine isolate MS19 was grown in Zobell Marine Broth (50 ml) in 250 ml Erlenmeyer flasks on a rotary shaker at 180 rpm for 72 hours at 30 ^{o}C. The cell-free culture supernatant was prepared by centrifuging the culture at 8000 rpm for 20 min at 4 ^{o}C. To the cell-free culture supernatant, equal volume of Ethyl Acetate was added in a separating funnel and vigorously shaken for 5 min. The organic (upper) layer was carefully separated and an equal volume of fresh Ethyl Acetate was added. The extraction process into the organic layer was repeated twice. The pooled organic layer was evaporated to dryness in a Rota evaporator. The viscous dried material was dissolved in DMSO for further use in cytotoxicity studies.

2.5 Cell Culture

Lung cancer (A549) cell line was obtained from the NCCS, Pune. The cells were grown in T25 culture flasks containing DMEM containing 10% FBS and Amphotericin B 3m/l, Streptomycin 75mg/l, Gentamycin 180mg/l, Penicillin 120mg/l, cells were maintained at 37^{o}C in a humidified atmosphere containing 5% CO_2.

2.6 Cytotoxic Activity

Cell survival rate was determined with MTT reduction assay. Exponentially growing A549 cells were seeded at 0.2 $\times 10^6$ cells in 96-well tissue culture plate with a volume of 200µl per well. Cells were incubated with different concentrations (1, 10, 25, 50 and 100 µg/ml) of different crude ethyl acetate extracts of strain MS19 and incubated at 37^{o}C for 72 hours. At the end of the incubation periods, 10µl of MTT stock solution (5mg/ ml) was added to each well and the plates were further incubated for 24 hours at 37 ^{o}C. The Formazan crystals that formed were dissolved by the addition of 100µl of Dimethyl Sulfoxide (DMSO) per well. The soluble

Formazan produced was quantified spectrophotometrically using an ELISA reader at 570nm. The results are based on the cleavage of the Tetrazolium salt by viable cells in the wells. The following formula was used for the calculation.

Cell proliferation inhibited (%) = [1– (A value of the experimental samples/ A value of the 10 control)] ×100%

2.7 Acridine Orange (AO) and Ethidium Bromide (EB) Staining

Cells were trypsinised and washed thrice with PBS. 1×10^6 cells and resuspended in 25 µl of PBS. The cells were treated with AO and EB solution (100 mg ml^{-1} AO and 100 mg ml^{-1} EO in PBS) and examined under a fluorescent microscope using a UV filter (450-490 nm).

3. Results and Discussion

3.1 Isolation of Marine Bacteria

Oceans cover more than 70% of the world's surface. A wide variety of marine bacteria are considered a rich source of natural products [10]. The marine environment is an exceptional reservoir of marine bacteria which are producing novel bioactive metabolites with diverse chemical structures [11]. In recent years, marine bacteria have become important in the study of novel microbial products exhibiting antibacterial, antiviral, anticancer, antifouling, anti-inflammatory, and antimitotic properties. These bioactive compounds may serve as model systems in the discovery of new drugs [12-13]. The first reported bioactive metabolite was isolated from a marine *Pseudomonas* sp. [14]. Blunt et al. (2005) investigated the production of an anti-cancer compound from marine sediment sample *Staphylococcus Aureoverticillatus* [15]. Marine Actinomycetes are responsible for the production of about half of the discovered bioactive metabolites notably antibiotics, antitumor, immunosuppressive, and enzymes [16]. Bioactive metabolites were extracted and purified with various techniques which could be used in the preparation of bioactive peptides, enzymes, oligosaccharides, fatty acids, water-soluble minerals, and

biopolymers for biotechnological and pharmaceutical applications. In the present study, a total of 10 isolates were isolated from seawater samples in the Bay of Bengal. Of 10 isolates, 1 isolate showed potent strain based on screening of cytotoxicity activity. Biochemical characterisation revealed that the strain MS19 was identified as Actinomycetes.

3.2 Biochemical Characterisation of Strain MS19

The strain MS19 was tested for utilisation of an array of carbon sources such as Maltose, Dextrose, Galactose, Glycerol, Sucrose, α −methyl−D−mannoside, Trehalose. The bacterium did not utilise other carbon sources such as Xylose, Fructose, Melibiose, Erythritol, Xylitol, Esculin hydrolysis, L−Arabinose, Mannose, Dulcitol, Inositol, Sorbitol, Mannitol, Adonitol, Arabitol, Lactose, Raffinose, Inulin, Sodium Gluconate, Salicin, Salicin, Rhamnose, Cellobiose, Melezitose, α−methyl−D−mannoside, ONPG, D−arabinose, Citrate utilisation, Malonate utilisation, and Sorbose. The bacterium showed growth at 37 ^{o}C and not at 4^{o}C. The optimum pH was 7 and the optimum salt concentration was 1.5% of growth condition by strain MS19.

3.3 Extraction of Crude by Strain MS19

Strain MS19 extracted bioactive metabolites with Ethyl Acetate and tested for cytotoxicity activity**.**

3.4 Cytotoxicity Activity by Strain MS19

Cytotoxic effect of crude extract of strain MS19 was tested using MTT reduction assay and the results showed that the crude extract of strain MS19 showed a strong cytotoxic effect on A549 cell line and low cytotoxicity effect on PBMC cells viz., less than 20 per cent. The metabolite inhibited proliferation of cell growth of A549 cells in a dose-dependent manner for different time period viz., 24 hours, 48 hours & 72 hours **(**Fig 1**)**. The IC_{50} value is 15.12 ±0.4 which showed at 24 hours.

3.5 AO/ EB Staining

The induction of apoptosis, morphological observation of cell death was investigated by observing the AO/EB stained cells under fluorescence microscopy. After A549 cells were exposed to crude extract IC_{50} concentration of 15.12±0.4 µg/ml for various time intervals, different morphological features of control and treated cells were analysed. Uniformly green live cells with normal morphology were observed in the control group (Fig 2) whereas green early apoptotic cells were noticed in the cells treated with purified metabolite for 6 hrs. The orange coloured apoptotic cells with fragmented chromatin and apoptotic bodies were observed in A549 cells treated with crude extract for 12 hrs. Further complete late apoptotic cells were observed after 24 hrs of treatment. The results observed from our study revealed the ability of the crude extract to induce marked apoptotic morphology in A549 cells. To the best of our knowledge, this is the first report to claim the production of cytotoxicity potential from marine isolates, *Actinomycetes* strain MS19. Further studies are focussing on isolation, purification and structural elucidation of the purified compound with anticancer studies.

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putida, and to psychrophilic pseudomonads associated with food spoilage". J. Gen. Microbiol. Vol 132, pp. 2709-2721.

Appendix

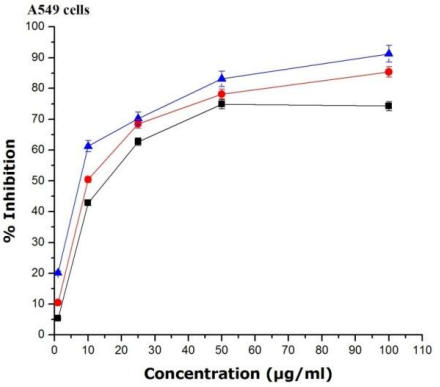

Figure 1: Dose-response analysis of crude extract (15.12±0.4) isolated from strain MS19 on inhibition of anti-proliferation of A549 cell line. 1×10^6 cells/well were seeded in 96 well tissue culture plate followed by treatment with different concentrations (1,10,25, 50, and 100µg/ml) of crude extract for (■) 24hrs,(●) 48hrs & (▲) 72hrs. Inhibition of cell proliferation was determined by MTT reduction assay after the specified incubation periods. Results are mean values ±SD of three independent experiments.

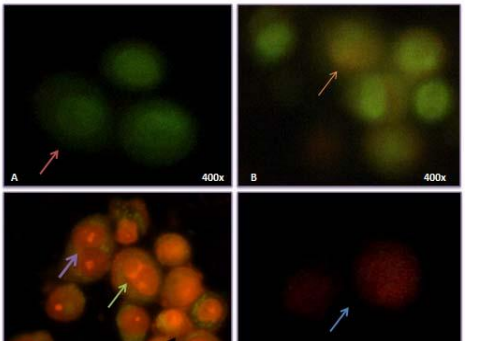

Figure 2: Detection of apoptosis by AO/EB staining. A549 cells were treated with crude extract for different time periods, harvested and stained with AO/EB stain. The green colour represents the control of the viable cell, the greenish yellow colour represents the early apoptotic cells at 6h and reddish orange colour represents the late apoptotic cells at 12h. Blue arrow indicated cells fragmented nuclei. The green arrow indicated cells membrane blebbing 24h. Magnification 400X.