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RESEARCH ARTICLE

INVITRO ANTI-TUMOR ACTIVITY OF THE METABOLITE PRODUCED BY THE BACTERIA *VIRGIBACILLUS SP.* ASSOCIATED WITH THE MARINE SPONGE- CALLYSPONGIA DIFFUSA ON VARIOUS HUMAN CANCER CELL LINES

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Abstract

In the present research, the metabolite produced by the marine bacteria *Virgibacillus sp*. associated with the marine sponge *Callyspongia diffusa* was evaluated for its potential anti tumor activity against various human cancer cell lines viz., MCF-7 (Breast carcinoma), A-549 (Lung carcinoma), HeLa (Cervix carcinoma) and Caco-2 (Human colorectal adenocarcinoma) using MTT assay. The metabolite produced by the bacterium *Virgibacillus sp*. displayed potential cytotoxic activity against the cancer cell line A-549 (Lung carcinoma) and HeLa (Cervix carcinoma). At concentration 1000 µg/ml the cytoxic activity was found to be 57.37% against A-549(Lung carcinoma) cell line and in the mean time it was 45.67 against HeLa (Cervix carcinoma) cell line. The CTC_{50} value in inhibiting A-549 Lung cancer cell line was 350 µg/ml and it was <1000 µg/ml for HeLa (Cervix carcinoma) cell line. Moderate activity was found against MCF-7(Breast carcinoma) and Caco-2(Human colorectal adenocarcinoma) cell lines. At concentration 1000 µg/ml the metabolite of *Virgibacillus sp.* displayed 33.12 % of cytotoxic activity against MCF-7(Breast carcinoma) cell line and in the meanwhile displayed 31.27 percentage of cytotoxic activity against Caco-2 (Human colorectal adenocarcinoma) cell line. The CTC_{50} value was found <1000 µg/ml concentration for both of the cell lines. The antitumor activity was recorded in a dose dependent manner.

Introduction

Marine environment play a major role in the development of novel drugs with potential biomedical activity. One of the important contributors in drug development from the marine environment is marine microorganisms. Biologically active molecules obtained from marine flora and fauna have been found with potential applications in pharmaceuticals, nutritional supplements, cosmetics, agrochemicals, molecular probes and fine chemicals (Faulkner, 2002). Especially marine bacteria have significant consequence in the development of medical science. They produce biologically active substances that can cure infections (Jensen and Fenical 2000). But only limited bacterial strains have still been utilized for the production bioactive compounds. In the marine environment the bacterial colonies inhabit either in the open ocean or in association with marine macro organism. Marine sponges are one of the kinds of macro organism in which the bacterial colonies live in the mesophyl regions. They contribute in the epibiotic defense of the sponge specimen (Thakur et al., 2003), they have potential biomedical applications. Secondary metabolites produced by sponge- associated bacteria far exceed those produced by planktonic bacteria (Lu et al., 2009). Bacteria associated with marine invertebrates are known to display significant antifouling, antibacterial and cytotoxic activities (Li Zheng et al., 2006). However, there are only limited reports on the cytotoxic potential of sponge- associated marine bacteria. And that, anti-tumor activity of metabolites produced by the marine

bacteria *Virgibacillus sp.* associated with the marine sponge *Callyspongia diffusa* was evaluated for its biopotential against various human cancer cell lines.

2 MATERIALS AND METHODS

2.1 Sponge collection and identification

The sponge specimen was collected from Gulf of Mannar region, Hares Island, Tuticorin coast from the net of fisherman and was brought about to the laboratory in a sterile polyethylene bag along with the habitat water during the month of February 2012. The sponge specimen was then identified by Dr. P.A. Thomas, Retired Scientist, CMFRI, Kerala, India

2.2 Isolation of sponge-associated bacteria

In order to remove surface associated microbes, the sponge specimen was subjected to 70% alcohol treatment. Then the mesophyll region (1-cm2) of the collected marine sponge specimen was sliced into small pieces and ground well with the application of a mortar and pestle. The homogenate was subjected to serial dilution technique and each dilution was plated on Zobell marine agar medium. Incubation of the plates were carried out at 37^{0} C for 24 hrs. After the due incubation time was over, the plates were observed for the presence of bacterial colonies. One of the isolated strains was identified as *Virgibacillus sp.* based on 16 S rRNA sequencing.

2.3 *Fermentation* of *Virgibacillus sp.* for secondary metabolites production

Mass culturing of the bacterial strain *Virgibacillus sp*. was carried out by using 20 L (10X2L) of Zobell marine broth. The inoculums of the strain *Virgibacillus sp*. was allowed into the medium and kept for incubation for 72 hrs at room temperature on a shaker. After the appropriate incubation time, the bacterial suspension was centrifuged at 12000 rpm for 20 mins as to separate the culture supernatant without the presence of any bacterial cells. The cell free culture supernatant was extracted three times with ethyl acetate using a separating funnel and was condensed with the application of a rotary vacuum evaporator under reduced pressure at 40^{0} C . The crude metabolite of about 20 g was obtained

2.4 Invitro Anti - tumor activity

2.4.1 Chemicals

3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

2.4.2 Cell lines and Culture medium

MCF-7 (Breast carcinoma), A-549 (Lung carcinoma), HeLa (Cervix carcinoma) and Caco-2 (Human colorectal adenocarcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

2.4.3 Preparation of Test Solutions

The metabolite of *Virgibacillus sp.* was dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

2.4. 4 Determination of cell viability by MTT Assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^{o} C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 hrs interval. After

72 hrs, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 hrs at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 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 540 nm (Francis and Rita 1986). The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \text{ X } 100 \right)$$

3 RESULTS AND DISCUSSION

In the present study the metabolite produced by the marine bacteria *Virgibacillus sp*. associated with the marine sponge *Callyspongia diffusa* was evaluated for its invitro anti-tumor activity using MTT assay against various human cancer cell lines viz., MCF-7 (Breast carcinoma), A-549 (Lung carcinoma), HeLa (Cervix carcinoma) and Caco-2 (Human colorectal adenocarcinoma). The results of the study revealed that the metabolite produced by the bacterium *Virgibacillus sp*. displayed potential cytotoxic activity against the cancer cell lines viz., A-549 (Lung carcinoma) and HeLa (Cervix carcinoma). At concentration 1000 µg/ml the cytoxic activity was found to be 57.37% towards A-549(Lung carcinoma) cell line and in the mean time it was 45.67 towards HeLa (Cervix carcinoma) cell line. The CTC_{50} value in inhibiting A-549 Lung cancer cell line was 350 µg/ml and it was <1000 µg/ml for HeLa (Cervix carcinoma) cell line. Moderate activity was found against MCF-7(Breast carcinoma) and Caco-2(Human colorectal adenocarcinoma) cell lines. At concentration 1000 µg/ml the metabolite of *Virgibacillus sp* displayed 33.12 percentage of cytotoxic activity against MCF-7(Breast carcinoma) cell line and in the meanwhile displayed 31.27 percentage of cytotoxic activity against Caco-2 (Human colorectal adenocarcinoma) cell line. The CTC_{50} value was found <1000 µg/ml concentration for both of the cell lines. The antitumor activity was recorded in a dose dependent manner.

Table 1: Cytotoxic properties of metabolite produced by *Virgibacillus sp*. against MCF-7 cell line

Sl. No	Metabolite Concentration (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	1000 500 250 125 62.5	33.12±1.0 27.22±1.2 23.53±1.2 16.24±0.7 12.19±1.5	**>1000±0.00**

Table 2: Cytotoxic properties of metabolite produced by *Virgibacillus sp* .against A-549 cell line

Sl. No	Metabolite Concentration. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	1000 500 250 125 62.5	57.87±2.6 55.13±0.3 47.36±0.7 44.36±0.7 38.32±4.2	**350.00±1.7**

Table 3: Cytotoxic properties of Metabolite produced by *Virgibacillus sp.* against HeLa cell line

Sl. No	Metabolite Concentration. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	1000 500 250 125 62.5	45.67±2.2 44.11±2.5 39.75±3.4 27.93±2.3 21.04±0.8	**>1000±0.00**

Table 4: Cytotoxic properties of metabolite produced by *Virgibacillus sp*. against Caco-2 cell line

Sl. No	Metabolite Concentration. (µg/ml)	% Cytotoxicity	CTC_{50} (µg/ml)
1	1000 500 250 125 62.5	31.27±3.2 23.69±2.8 22.30±3.6 19.94±3.4 15.33±4.0	**>1000.00**

The alkaloids and anthroquinones present in the crude metabolite may be the reason for its potential anticancer activity. Alkaloids are microtubule interfering agents which can bind with beta tubulin, thus preventing the cell from making mitotic spindle fiber necessary to move the chromosome around as the cell divides, inhibiting toposiomerase (Facompre et al.,2003) , mitochondrial damage and inducing the release of cytochrome C and apoptosis inducing factor (Ravikumar et al.,2010). 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 anthroquinone families (parimycin, trioxacarcins and gutingimycin) showed antitumor activities (Gulecha and Shiva Kumar, 2011; Kumar et al., 2011). Li Zheng et al., (2006) reported that the bacterial strain *Pseudoalteromonas piscicida* associated with the marine sponge *Hymeniacidon perleve* had significant cytotoxic activity on HeLa and BGC-823 cell lines with IC50 values of 150 ± 4.6 μg/ml and 192 ± 3.5 μg/ml respectively. The other strain, *Bacillus megaterium* only inhibited the growth of BGC-823 cell line, and the measured IC50 value was 185±2.8 μg/ml. Marine natural products have been prominently featured in the area of cancer research (Da Frota et al., 2008). The bioactive compound Alteramide A produced by the marine sponge *Halichondria okadai* associated bacteria *Alteromonas sp* showed potential cytotoxic activity (Kelecom, 2002 and Shigemori et al., 1992). The bioactive compound Bromo-alterochromide A produced by the marine sponge *Fascaplysinopsis reticulate* associated bacteria *Pseudoalteromonas maricaloris* KMM 636T (γ-Proteobacteria showed potential cytotoxic activity (Blunt et al., 2009). The secondary metabolites present in the crude extract of *Virgibacillus sp.* would have been induced the apoptosis of cancer cells and in turn inhibited the proliferation of cancer cells

4 CONCLUSIONS

Considering the deleterious effect of various cancer outbreaks, an attempt was made to trial the metabolite produced by the marine bacteria *Virgibacillus sp*. associated with the marine sponge *Callyspongia diffusa* on various human cancer cell lines. Our compound showed potential cytotoxic activity against all the above cancer lines even in the tested low concentrations. Further research is going to separate the individual compounds present in

the crude metabolite produced by the marine bacteria *Virgibacillus sp*. associated with the marine sponge *Callyspogia diffusa*

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