

Evaluation of anticancer and antibacterial activity of crude protein extract from saltpan actinomycetes

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Abstract

Naturally occurring antibiotics and other bioactive compounds have been shown to be mostly derived from Streptomyces. Finding and identifying the actinomycetes exhibiting antagonistic activity was the primary the current investigation's objective. Out of the 17 actinomycetes that were isolated and evaluated against four bacterial pathogens, a strain was identified from marine sand samples that were obtained off the Indian coast of the Kodiyakkarai region. It generated compounds that were effective against pathogens that are Gram positive and Gram negative. Shake flask conditions have been shown to maximize the nutritional needs and cultural conditions for the highest possible growth and yield of bioactive chemicals. Furthermore, research on the crude extract's anticancer efficacy against the human breast cancer cell line MDA-MB-231 was conducted. Nonetheless, in the future, the actinomycetes found in the marine ecosystem will be helpful in the search for novel medications.

Key words : Marine actinomycetes, Antibacterial, Anticancer.

The novel class of marine microorganisms and potential source of biologically active substances, are currently gaining popularity¹⁰. They create a wide range of metabolites, some of which can be exploited in medication development²⁶. These microbes provide almost limitless sources of new substances with numerous therapeutic applications. Actinomycetes

are renowned because of their great diversity and demonstrated ability to create novel chemicals. Furthermore, Using microbial secondary metabolite screening to identify new antibiotic and non-antibiotic lead compounds is becoming more and more important³¹. Most actinomycetes are found in both terrestrial and aquatic environments^{5,16}. In an effort to identify

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the bioactive compounds of actinomycetes, contemporary researchers extensively examined marine plants, medicinal plants, sediments, and soil habitats^{1,17,25,27}. The prokaryotes with the greatest commercial and biotechnological value are actinomycetes¹⁹.

Approximately It has been shown that 23,000 bioactive secondary metabolites are produced by microorganisms, with actinomycetes producing over 10,000 of these chemicals. This amounts to 45% of all bioactive microbial metabolites that have been found³⁰. Known to synthesize are species of *Streptomyces* around 7600 chemicals that belong to actinomycetes¹². The Marinlit database contains information on about 289 secondary metabolites from the marine derived species *Streptomyces*. These metabolites have a wide range of chemical structures, including peptides, lactones, macrolides, indoles, terpenes, and quinones¹⁵. Many industrially useful activities like cytotoxic, immunosuppressive, antibacterial, antifungal, antimalarial, anticancer, anti-inflammatory, anthelmintic, herbicide, and enzyme properties, are demonstrated by these compounds^{18,21,32}. The second most common cause of cancer-related fatalities in women is breast cancer and cancer continues to rank among the most significant health issues facing people²⁸. Chemotherapy, immunotherapy, radiotherapy, and surgery are therapeutic approaches used to treat cancer¹³. These methods work well separately in specific contexts, and when combined, they provide a more effective tumor treatment. Algal metabolites are the source of many antitumor compounds found in marine drugs⁴, and these metabolites are crucial for the discovery of novel pharmaceutical compounds^{14,29}.

Isolation and identification of Actinomycetes From Saltpan sediments :

A sample of marine sediment was taken from the Kodiyakkarai ecosystem in the Nagapattinam district of Tamilnadu, India, at a depth of 5 to 15 cm (Lat. 10.336727; Long. 79.842984). Samples were immediately transferred to the laboratory. The sample was first pretreated at ambient temperature until it dried. Grinding the sample using mortar and pestle and make 1g of sample was transferred into 9 mL of distilled water and perform serial dilution method for isolation. After incubation colonies appeared on the agar medium and make it sub culture in the same agar medium for pure isolates. Store the pure cultured plate in refrigerator for further studies^{20,23}. Place a colony of actinomycetes in a clean glass slide from a pure isolation culture plate. Place a cultivated slide under a microscope to observe its morphology.

Selection and maintenance of Actinomycetes colonies :

Actinomycetes isolates were chosen with consideration for the cottony, gummy growth that developed over the media, based on the various morphological features of the colonies. Sub-culturing was used to protect the viability of the actinomycetes isolates pure cultures until after they were used afterwards.

Preparation of cell free culture extracts of Actinomycetes isolates :

Actinomycetes isolation broth was used to cultivate the isolated microorganisms in rotary shaker for a period of six days at a temperature of 30°C. After cultivation the broth was carried out in centrifuge tube and

the culture were centrifuged at 10,000 rpm for 10 minutes at 4°C. The culture pellet was taken out in a sterile container and stored in a suitable atmosphere for further study.

Extraction of Bioactive compounds :

A 250 mL conical flask was filled with 100 mL of actinomycetes isolation broth, which was used to inoculate an isolated actinomycetes. For seven days, the inoculated culture broth was maintained in a rotating shaking incubator at 27°C and 120 rpm. A separating funnel filled with the appropriate solvents in a 1:1 ratio was used to separate the culture after incubation. Place a separated compound in a sanitized petriplate and dry it in a hot air oven. A powder should be taken in a sterile container when the chemical has reached the appropriate expected format in powder form. The powder used for anticancer activity.

Production and purification of protein fraction from Actinomycetes isolates :

The actinomycetes isolates were cultured in 100 milliliters of actinomycetes isolation broth medium at 30°C for six days using a rotary shaker set at 120 rpm. After growing, the mycelium of actinomycetes was removed from the broth using centrifugation, which was done for 10 minutes at 4°C and 10,000 rpm. Isolates of actinomycetes that exhibited inhibitory action were chosen to be potential isolates for further study.

Antibacterial activity of crude Protein extracts :

The well diffusion method experiment was used to evaluate the crude protein extracts

antibacterial activity (Reeves, 1989). 100 ml of distilled water was used to dissolve 3g of Muller Hinton agar, which was then autoclaved for 15 minutes at 121°C. This medium was allowed to cool before being put into sterilized petri dishes and let to solidify. The bacterial pathogens were cross-streaked using sterile cotton swabs on Muller Hinton Agar medium. A sterile well borer was used to create the wells, which were then filled with 100 µL of crude protein extracts and incubated for 24 hours at 37°C. Each sample was split into three replicates. Clear zones were created and microbial growth was inhibited by the extracts with antibacterial activity. Millimeters were used to measure the zone of inhibition.

Isolation of Actinomycetes :

From the sampling locations 50 soil samples in total were collected. The isolated soil samples were pretreated at room temperature in the lab. Afterwards, the sample performed a serial dilution technique to separate actinomycetes from the soil. Then the isolated actinomycetes were subcultured on Actinomycetes Isolation Agar medium. It is possible to separate actinomycetes from a variety of habitats, including soil, the rhizosphere, marine environments, insects, and plant materials. Appropriate techniques are applied to soil samples, and colonies containing actinomycetes are chosen and cultivated in suitable environments².

Extraction of bioactive compounds from Actinomycetes :

Bioactive secondary metabolites found in actinomycetes have a wide range of uses in industry, agriculture, and medicine.



Fig. 1. Pure culture of Actinomycete

Numerous substances, including enzymes, enzyme inhibitors, antiviral drugs, anticancer agents, and antibiotics, are produced by these microbes. Researchers use a variety of methods to extract and identify these bioactive chemicals. To obtain the required secondary metabolites, the actinomycete isolates are fermented. Ethyl acetate solvent are subsequently used to extract the chemicals^{3,11,27}. The bioactive compound was taken in clean petri dishes and put into a hot air oven for drying. The sample changed into powder after drying.



Fig. 2. Extraction of bioactive Compound

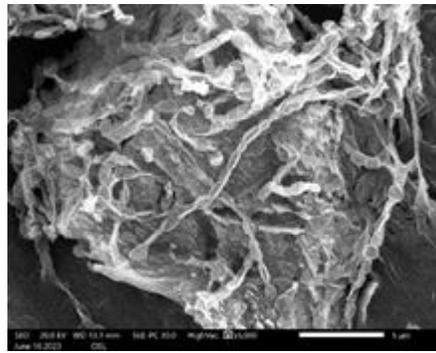
Scanning electron microscope (Sem) :

Using a scanning electron microscope for examination. Coverslips with coating and metal stubs that went with them were piled inside the specimen chamber of the microscope. The field was scanned at low magnification in order to locate the growth line. Next, areas with unique, unaltered sporing structures were selected for additional scrutiny. Ilford HP-3 film was utilized to obtain relevant subjects. This device scans the specimen with an electron beam that creates secondary electrons as it ionizes the material's atoms. A portion of these are gathered along with some reflected primary electrons that are energetic enough to escape into the vacuum to form the screen image. The specimen was positioned with the secondary electron collector at an angle to the original beam, giving it a "shadowed" appearance that highlights surface details⁹⁻²².

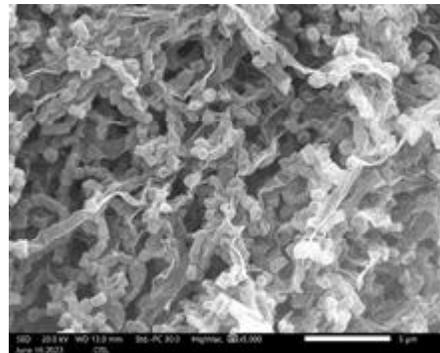
Purification of protein :

Use a centrifugation speed 10,000 rpm to separate large amounts of cell debris and intact cells from the crude extract⁶. After making a crude extract, the big material is usually removed by a standard centrifugation process. Centrifugation is a technique where mixtures are sedimented using a centrifuge by applying centrifugal force. Two immiscible liquids are separated by this process; the denser components of the mixture migrate in the direction of the centrifuge's axis, while the less dense components migrate in the opposite direction. Centrifugation speeds up and completes the precipitate (or "pellet") accumulation at the tube's bottom by altering the effective gravitational force acting on the tube or bottle. The leftover solution is

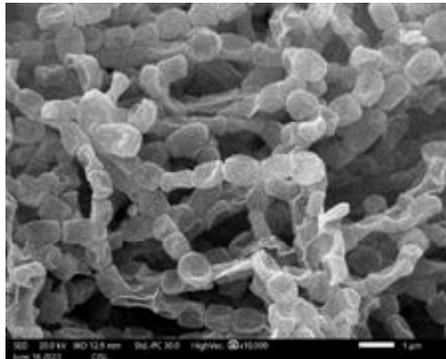
(462)



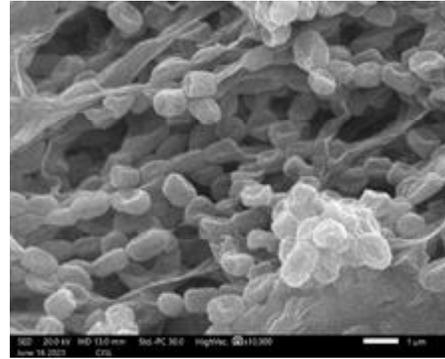
x 5000



x 5000



x 10000



x 10000

Fig. 3. Morphology of sem analysis



Fig. 4. Purification of protein by centrifugation method.

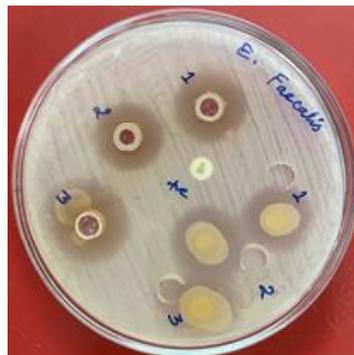
appropriately referred to as a “supernatant”. The liquid supernatant is drained out of the tube or container quickly and without disturbing the precipitate.

Antibacterial activity of crude protein extract :

The test pathogens were swabbed across the surface of Muller-Hinton Agar plates. Swabbed plates were positioned with extract of protein in well and also the sterile disc over the test organisms surface. The temperature of the agar plates was kept at 27°C. Recorded was the zone of inhibition.



Staphylococcus aureus



Enterococcus faecalis



Escherichia coli



Pseudomonas sp

Fig. 5. Antibacterial activity of crude protein extract

Table-1. Assesment of zone of inhibition

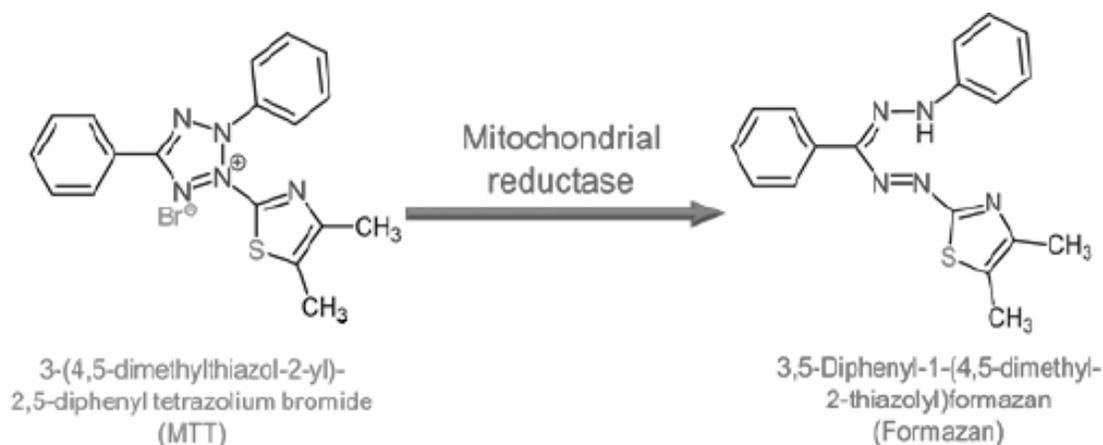
PATHOGENS	Well 1	Well 2	Well 3	Disc 1	Disc 2	Disc 3
<i>Staphylococcus aureus</i>	20 mm	19 mm	20 mm	20 mm	14 mm	20 mm
<i>Enterococcus faecalis</i>	20 mm	20 mm	22 mm	20 mm	20 mm	20 mm
<i>Escherichia coli</i>	20 mm	20 mm	23 mm	23 mm	20 mm	23 mm
<i>Pseudomonas sp</i>	18 mm	19 mm	20 mm	18 mm	20 mm	23 mm

+ve – Chloramphenicol

Anticancer activity of crude extract of Actinomycetes :

Traditionally, the method used to assess unknown substances' detrimental effects in vitro has been counting live cells after coloring them with a vital dye. Some methods that are employed are automated counter counting, the measurement of DNA synthesis by radioisotope incorporation, and other methods that depend on dyes and cellular activity. Mitochondrial dehydrogenases are used in the MTT procedure to gauge the activity of living cells. The MTT method is simple, accurate and yields reproducible results. The main ingredient is 3-[4, 5-dimethylthiazol-

2-yl]-2, 5-diphenyl tetrazolium bromide, or MTT. It is a tetrazolium salt that dissolves in water and, when mixed with salt solutions or other media without phenol red, yields a yellowish solution. The tetrazolium ring in dissolved MTT is broken down by the enzyme mitochondrial dehydrogenase in living cells, producing an insoluble purple formazan. This water-insoluble formazan can be dissolved using DMSO, acidified isopropanol, or other solvents (pure propanol or ethanol). Using spectrophotometry, the purple solution that results is measured. The amount of formazan generated changes in tandem with changes in the number of cells, providing insight into the test material's degree of cytotoxicity.



Required materials :

1. MTT Powder (the solution is filtered through a 0.2 μ m filter and stored at 2–8 °C for frequent use or frozen for extended periods)
2. DMSO
3. CO₂ incubator
4. Tecan Plate reader

Preparation of test solution :

Serial two-fold dilutions were generated for cytotoxicity experiments, ranging from 100 μ M to 0 μ M, and then used for treatment.

Cell lines and culture medium :

Each and every cell line was purchased from ATCC. The stock cells were cultured at

37°C in a humidified atmosphere with 5% CO₂ in DMEM / F12 supplemented with 10% inactivated Fetal Bovine Serum (FBS), 100 µg/ml streptomycin, and 100 IU/ml penicillin. Cell dissociating solution (0.2% trypsin, 0.2% EDTA, and 0.05% glucose in PBS) was used to separate the cell. After being centrifuged, the cells' vitality is examined. Additionally, a 96-well plate containing 50,000 Jurkat cells per well was seeded, and the plate was incubated for 24 hours at 37 °C with 5% CO₂.

Source of reagents : F12, Dmem, Fbs, Penstrep, Trypsin Procured From Invitrogen.

Procedure :

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/ml using the proper medium containing 10% FBS. A volume of 100 µl of the diluted cell suspension, comprising 50,000 cells per well, was added to each well of a 96-well microtiter plate. After 24 hours, when a partial monolayer had developed, 100 µl of different test drug doses were applied to the partial monolayer in microtiter plates, and the supernatant was removed. After that, the plates were incubated for 24 hours at 37°C in an atmosphere with 5% CO₂. Following the incubation period, each well received 100 µl of MTT (5 mg/10 ml of MTT in PBS), and the test solutions within were disposed of. The plates were incubated in an environment containing 5% CO₂ at 37°C for 4 hours. Once the supernatant was taken out, 100 µl of DMSO was added, and the plates were shaken gently to dissolve the formazan that had formed. A microplate reader was used to measure the absorbance at 590 nm in wavelength.

The percentage growth inhibition was computed using the following formula, and the concentration of test medicines needed to inhibit cell growth by 50% (IC₅₀) values was found using the dose-response curves for each cell line^{8,24}.

Calculating inhibition :

$$\% \text{ Inhibition} = 100 - (\text{OD of Sample} / \text{OD of Control}) \times 100$$

Statistical Evaluation :

IC₅₀ Value :

The half maximal inhibitory concentration (IC₅₀) of a medication is used to determine how effective it is at inhibiting biological or biochemical processes. This quantifiable metric shows the amount of a specific medicine or other substance (inhibitor) required to halve the inhibition of a given biological process (or process component, such as an enzyme, cell, cell receptor, or microbe). By creating a dose-response curve and analyzing the impact of various antagonist concentrations in reversing agonist activity, one can ascertain a drug's half-life (IC₅₀). The concentration required to inhibit half of an antagonist's maximal biological reaction can be used to compute the antagonist's IC₅₀ values. Graph Pad Prism 6 (Graph Pad, San Diego, CA, USA) was used to compute the IC₅₀ values for cytotoxicity tests, which were obtained by a nonlinear regression analysis (curve fit) based on the sigmoid dosage response curve (variable).

Nonlinear Regression :

In nonlinear regression, observational data is represented by a function that depends

on one or more independent variables and is a nonlinear combination of the model parameters. Nonlinear regression is a sort of regression analysis used in statistics. Fitting the data involves a series of consecutive approximations.

MTT Absorption value

CELLS	MDA-MB-231				SAMPLES NAME				ACT			
BLANK	0	10	20	30	40	50	60	70	80	90	100	
0.019	0.923	0.801	0.655	0.532	0.413	0.322	0.211	0.122	0.056	0.033	0.008	
0.016	0.924	0.804	0.648	0.529	0.418	0.337	0.213	0.113	0.062	0.043	0.01	
0.015	0.918	0.807	0.649	0.532	0.417	0.336	0.212	0.114	0.063	0.045	0.012	
	0.922	0.804	0.651	0.531	0.416	0.332	0.212	0.116	0.060	0.040	0.010	
MEAN	0	13	29	42	55	64	77	87	93	96	99	
VIABILITY	100	85	71	58	45	36	23	13	7	4	1	
	$IC_{50} = 37.87 \pm 0.09 \mu g$											

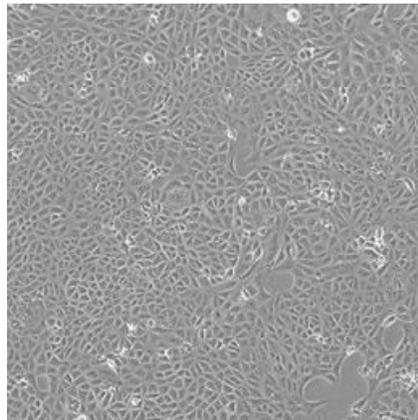
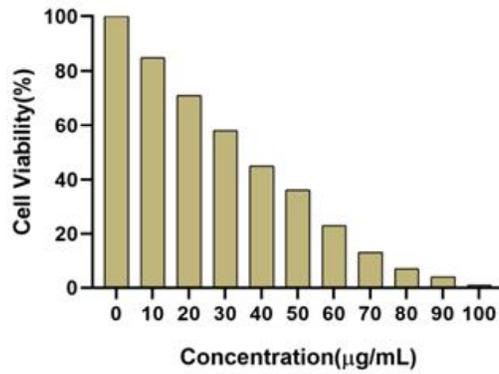
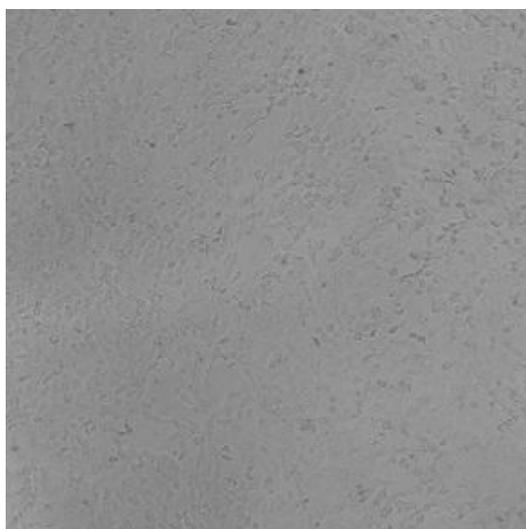
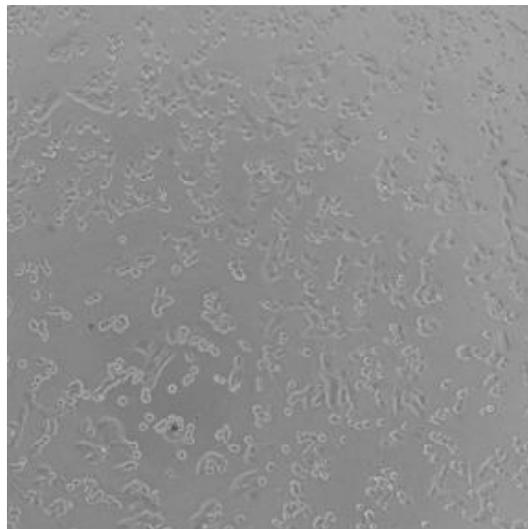


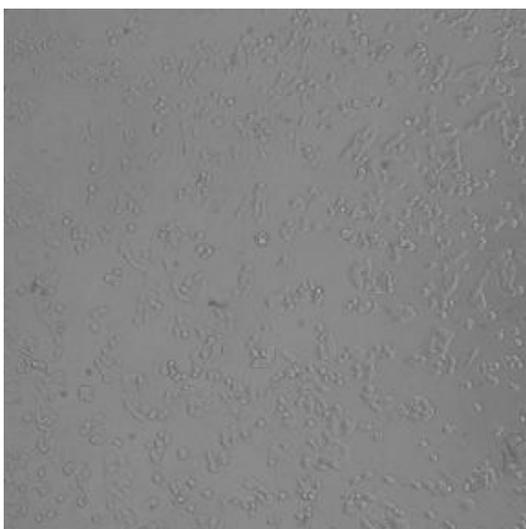
Fig. 6. Mda-Mb-231 Control Cells



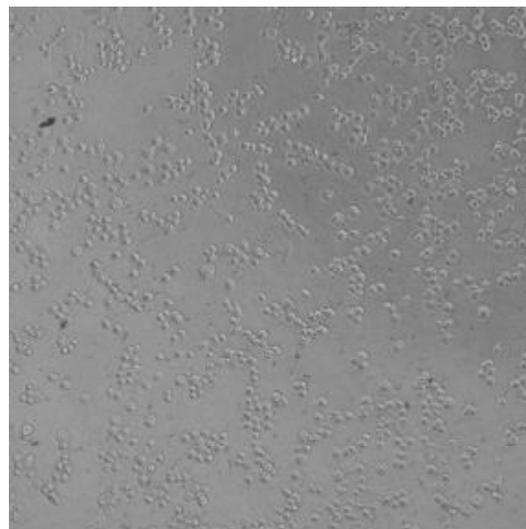
Mda-Mb-231 cells – Treated 1



Mda-Mb-231 cells – Treated 2



Mda-Mb-231 cells – Treated 3



Mda-Mb-231 cells – Treated 4

Fig. 7. Treated Cell Lines

A fantastic place to find important organisms that require further research is the marine environment. The current study set out to investigate our natural surroundings in quest of actinomycetes that produce bioactive

substances. The results of this investigation revealed that the antimicrobial compounds produced by the antagonistic marine actinomycetes isolates may find usage as antibiotics in the future, as well as in aquaculture systems.

Furthermore, the chemicals have potential applications as anticancer agents. Additional research is needed to identify the product.

Conflict of interest

Statement We Declare that we Have No conflict of interest.

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