Antagonistic, Antibacterial and Anticancer Activity of Marine Macroalgae-Associated Bacteria Collected from Kanyakumari coast

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Abstract: Marine macroalgae or seaweed associated bacteria gains much importance because of its crucial role in the production of certain compounds that are found to be active against various pathogenic bacteria and cancer cells. In this study the antagonistic, antibacterial and anticancer potential of marine macroalgae associated bacteria were analysed. 14 epiphytic and 6 endophytic bacterial strains were isolated. Among the isolated bacteria 3 strains showed antagonistic activity against Staphylococcus aureus and 1 strain showed activity against Escherichia coli. Antibacterial potential of seaweed associated bacteria revealed that the bacterial extracts have the potential to inhibit the growth of Escherichia coli, Bacillus subtilis and Staphylococcus aureus. Further the strains also inhibited the proliferation of SK-MEL-28 cell line in a dose dependent manner.

Keywords: Seaweed, Antagonistic, Antibacterial, Anticancer, Bacteria.

Introduction

Marine organisms are a profound source of novel and active metabolites that differs structurally and biologically. Several chemical compounds of marine origin that have different biological activity have been isolated and studied extensively for being developed as new products or pharmaceuticals (Faulkner *et al*.,, 2000). Various bioactive compounds have been isolated from marine microorganisms. Since 1980, more than 50,000 bioactive natural products have been discovered of which 8000 compounds exhibited bactericidal activity (Berdy, 1989).

Seaweeds acts as a host for several types of marine bacteria and many of them play a vital role in maintaining the overall health of the associated host organism by producing unique biologically active secondary metabolites. Hence, seaweed-associated bacteria are useful in the development and production of compounds with various biological activities (Bolinches *et al*., 1988).

Certain species of marine algae need specific nutrients for their growth that could be partly obtained from associated bacteria. In addition they also produce a range of active compounds with antiviral, antibacterial, antifungal and cytotoxic activity in view of maintaining a mutual relationship with epiphytic micro-environments (Jasti *et al*., 2005).

Seaweed associated microorganisms have been identified as a potent source in promoting the morphological development of seaweeds as well as in the protection of seaweeds from pathogens and other competitive organisms. This property of bacteria is because of the bactericidal compounds produced by them (Avendaño *et al*., 2005). Other Symbiotic functions characterised to seaweed associated bacteria includes stabilization of seaweeds, processing of metabolic waste and secondary metabolite production (Hentschel *et al*., 2002).

Microorganisms show certain highlighting competitive mechanisms against other organisms of which antagonism is highly commendable. Microbial antagonism is a natural phenomenon where certain microorganisms present in normal microbiota prevents the proliferation of other harmful microorganisms by creating an environment that is deprived of nutrients or by the secretion of growth inhibitory substances. Few microorganisms can inhibit or reduce the growth of other microorganisms because of the metabolites or toxic components produced by them. Microorganisms are not only the causative agents of infection but also they provide relief from the infections caused by several harmful pathogenic organisms (Kirkup and Riley, 2004). Bacteria in aquatic environments are also found to produce substances with antimicrobial activity thereby inhibiting the growth of other microorganisms (Fabregas *et al*., 1991).

Cancer is one of the dreadful diseases that affect the human population globally. The various causes underlying this disease are lifestyle, radiation and exposure to several carcinogens (Choucry, 2017). Marine floras synthesize different compounds that are found to exhibit an array of medicinal properties including anticancer activity (Sithranga and Kathiresan, 2010).

Material and Methods

1. Collection and Identification of Marine Macroalgae

Marine seaweeds were collected from Kadiapattinam coast, Kanyakumari district, Tamilnadu, India by hand picking during low tide. Specimens were brought to the laboratory in sterile polythene bags and stored in refrigerator for further studies. Collected Seaweeds were identified at CMFRI, Vizhinjam.

2. Isolation and Purification of Marine Macroalgae-Associated Bacteria

2.1. Isolation of Epiphytic Bacteria

Collected seaweeds were rinsed with sterile ocean water and by using sterile cotton swab its surface was swabbed and spread on Zobell Marine Agar plates, followed by incubation for 48 hours at room temperature. Colonies formed after incubation were again sub-cultured to get pure colonies and stored at 4°C for further analysis.

2.2. Isolation of Endophytic Bacteria

From the collected marine algae epiphytes were removed by washing with ocean water followed by washing in 70% ethanol and 2% sodium hypochlorite. Then the samples were cut into pieces of size 2-3cm, placed in Zobell Marine Agar plates and incubated for 48 hours at room temperature. Colonies formed after incubation were again sub-cultured to get pure colonies and stored at 4°C for further analysis.

3. Antagonistic Activity of Marine Macroalgae-Associated Bacteria

Potential antagonists were identified by streaking the isolated bacterial strains in dual culture media of nutrient agar and Mueller Hinton Agar swabbed with either *Escherichia coli* or *Staphylococcus* *aureus*. After 48 hrs of incubation at 25ºC, the plates were observed for clear zones around the bacterial isolates (Lindow, 1988).

3.1Antagonistic Activity of cell free lysate of Marine Macroalgae-Associated Bacteria

3.1(a) Mass Production and TCA/Acetone Protein Precipitation

The organisms showing antagonistic properties were mass cultured and centrifuged to remove the cell lysate. The supernatant containing the secondary metabolites were then used for protein precipitation.

Procedure

1. The cell lysate was mixed with 100% ice-cold acetone and 100% trichloro acetic acid in the ratio 1:4:0.5 and Precipitated at -20 °C for 1 hr.

2. After incubation supernatant was collected by centrifuging the cell lysate at 11,500 rpm for 15 minutes at 4ºC (Lindow, 1988).

3. Antagonistic activity of the test organisms were detected based on well diffusion method. Standardized inoculum of *E.coli/ S. aureus* was spread uniformly over the surface of agar plates and four wells of 8mm diameter were made aseptically using a cork borer. To the two wells Supernatant obtained after centrifugation (40 µL and 80 µL) was added. Gentamycin was used as positive control and a well without sample was maintained as negative control. Plates were incubated at 37ºC for 24 hrs, after which the antagonistic activity of the test organism was measured in mm.

4. Antibacterial Activity of Marine Macroalgae-Associated Bacteria

4.1 Antibacterial Assay

Agar well diffusion method was used to screen the antibacterial activity of marine macroalgae-associated bacteria. Mueller – Hinton agar plates having wells of diameter 8 mm were used. Test organisms such as *Staphylococcus aureus* (MTCC 87), *Escherichia coli* (MTCC443), *Bacillus subtilis* (MTCC 2413), *Pseudomonas aeruginosa* (MTCC 424) *Klebsiella pneumoniae* (MTCC 109) procured from MTCC, Chandigarh were spread on the surface of these plates. 50 µL of the extract solution at desired concentration was added to the wells. Gentamycin and DMSO were used as positive and negative control respectively. Zone of inhibition was measured in mm after incubating the plates under optimal conditions.

5. Invitro Anticancer Activity of Marine Macroalgae-Associated Bacteria

For anticancer activity screening SK-MEL-28 cell line was selected and it was cultured in DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: penicilin (100U/ml), streptomycin (100U/ml) and Amphotericin B (2.5µg/ml). Cultured cells were kept in a humidified CO_2 incubator at 37ºC and the anticancer activity was analysed by microscopic observation followed by MTT assay.

5.1. Anticancer Screening by Microscopic Observation

Prepared bacterial sample (1 ml) was mixed with 1 ml of DMEM and dissolved completely by cyclomixer. After that extract solution was sterilized by filtering it through 0.22µm Millipore syringe.

The culture medium was removed after 24 hours and samples were freshly prepared in 5% DMEM, five times serially diluted by two fold dilution (6.25µg, 12.5 µg, 25 µg, 50 µg, 100 µg in 100 µl of 5% MEM). Each concentration of 100µl sample was added in triplicates to the well plates and incubated in a humidified CO_2 chamber (5%) at 37ºC.

After incubation the plate was observed through an inverted microscope at different time interval (24, 48 and 72 hours respectively) and the morphological changes in the cells were documented. The cytotoxicity was determined based on the changes in cell morphology.

Anticancer Anticancer screening by MTT Assay

15 mg MTT was mixed with 3ml of phosphate buffered saline and filter sterilized for anticancer screening.

Samples present in the wells were decanted after 24 hours incubation. 30 µl of reconstituted MTT solution was added to the wells marked as test and control. Gently shake the plates and incubate for four hours in a humidified CO_2 chamber (5%) at 37ºC. After incubation remove the supernatant and add 100 µl of DMSO (MTT soubilization solution) to the wells and mixed well. The cytotoxicity was assessed by measuring the absorbance at 570nm using a micro plate reader (Talarico *et al*., 2004).

The growth of inhibition (%) was calculated using the following formula

$$\text{\% of viability} = \frac{\text{Mean OD Samples}}{\text{Mean OD of control}} * 100$$

Results

1. Isolation of Marine Macroalgae-Associated Bacteria

Marine macroalgae were collected from Kadiapattinam, Kanyakumari district, Tamilnadu, India and were identified as *Hypnea musciformis* and *Sargassum wightii*. From the two collected marine macroalgae, based on the colony morphology a total of 20 associated bacteria were isolated. Among the isolated strains 14 were epiphytes and 6 were endophytes.

2. Antagonistic Activity of Marine Macroalgae-Associated Bacteria

Antagonistic activity of the 20 isolated bacterial strains were tested against *Escherichia coli* and *Staphylococcus aureus* by streaking them in a dual culture media of NA and MHA followed by incubation at 25ºC for 48 hrs. Among the tested bacteria, strains A_1S_1-3, B_1S_1-5 and B_1S_2-2 showed antagonistic activity against *Staphylococcus aureus* whereas the strain B_1S_1-2 alone showed antagonistic activity against *Escherichia coli*. All other strains never showed any activity against neither *Escherichia coli* nor *Staphylococcus aureus*.

Further the antagonistic strains were mass cultured and their cell free lysate was obtained and again tested for antagonistic property. All the cell free lysates exhibited antagonism against the tested organisms with the maximum zone of inhibition (24mm) by strain B_1S_1-2 against *E.coli* (Table - 1). Hence further experiments were carried out with the strains that showed positive antagonistic activity.

Table - 1: Antagonistic Activity of Marine Macroalgae-Associated Bacteria

Sl. No.	**Sample Name**	**Antagonistic property**	**Diameter of zone (mm)**		
			+	**T1** (40 µL)	**T2** (80 µL)

1.	A_1S_1-3	*S. aureus*	23	18	22
2.	B_1S_1-2	*E.coli*	32	21	24
3.	B_1S_1-5	*S. aureus*	24	17	22
4.	B_1S_2-2	*S. aureus*	23	16	22

3. Antibacterial Activity of Marine Macroalgae-Associated Bacteria

Antibacterial activity of marine macroalgae-associated bacteria was analysed against five different pathogenic bacteria by agar well diffusion method. The associated bacterial strain B_1S_1-2 showed activity only against *Bacillus subtilis* whereas the B_1S_1-5 strain showed antibacterial activity against *Escherichia coli* and *Bacillus subtilis*. The strain B_1S_2-2 was also found to supress the growth of *Staphylococcus aureus and Escherichia coli.* Meanwhile no activity was exhibited by the strain A_1S_1-3 against any of the tested organisms. Further none of the tested organisms showed activity against *Pseudomonas aeruginosa* and *Klebsiella pneumonia* ((Table - 2).

Table - 2: Antibacterial Activity of Marine Macroalgae-Associated Bacteria

Test Organism		***Staphylococcus aureus***	***Escherichia coli***	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Klebsiella pneumoniae***
Sample	**Sample Concentration (µg)**	**Zone of Inhibition (mm)**				
A_1S_1-3	S- 80	20	20	20	23	27
	C	-	-	-	-	-
	T1- 400	-	-	-	-	-
	T2- 800	-	-	-	-	-
B_1S_1-2	S- 80	19	20	20	20	25
	C	-	-	-	-	-
	T1- 400	-	-	11	-	-
	T2- 800	-	-	13	-	-
B_1S_1-5	S- 80	19	20	25	22	25
	C	-	-	-	-	-
	T1- 400	-	-	13	-	-
	T2- 800	-	11	15	-	-
B_1S_2-2	S- 80	20	24	20	20	21
	C	-	-	-	-	-
	T1- 400	15	12	-	-	-
	T2- 800	17	18	-	-	-

4. Invitro Anticancer Activity of Marine Macroalgae-Associated Bacteria

The most two potent bacterial extracts (B1S1-5 and B1S2-2) that showed high antibacterial activity was tested for anticancer activity.

4.1 Anticancer Effect by Direct Microscopic observation

SK-MEL-28 cells that were treated with B1S1-5 and B1S2-2 bacterial extracts were observed using an inverted microscope and the microscopic images were documented. A decrease in the number of cells with increasing concentration of extract, a remarkable variation in cell morphology was observed (Plate 1, 2), which indicates the cytotoxicity.

4.2 Anticancer Effect by MTT Assay

Various concentrations of B1S1-5 and B1S2-2 bacterial extracts were added to SK-MEL-28 cells and tested for anticancer activity. The IC_{50} value of the bacterial extracts, B1S1-5 and B1S2-2 against the tested cancer cell lines were found as 31.537µg/ml and 28.064µg/ml respectively. Hence both the extracts were found to inhibit the growth of tumor cells, which could be inferred from the decrease in percentage viability of the cells with increasing concentration of extracts (Table-3).

Table-3: Anticancer Activity of Marine Macroalgae-Associated Bacteria by MTT assay

Sample	Concentration (µg/ml)	Percentage viability	IC_{50} (µg/ml)
B1S1-5	6.25	91.76	31.537
	12.5	84.7	
	25	63.52	
	50	42.35	
	100	25.88	
B1S2-2	6.25	90.66	28.064
	12.5	85.33	
	25	58.66	
	50	34.66	
	100	20	

Plate 1: Anticancer Effect of B1S1-5 extract by Direct Microscopic observation

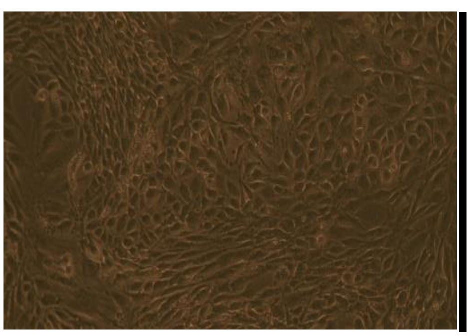

Plate 1.2: Control

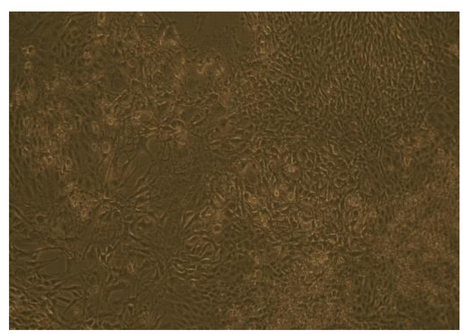

Plate 1.2:6.25µg/ml

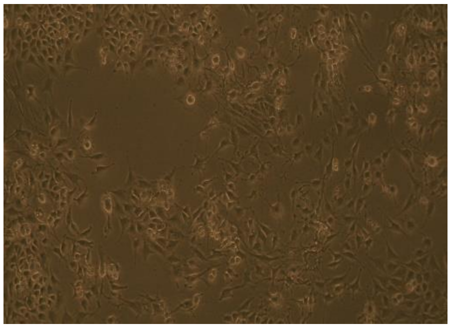

Plate 1.3: 12.5µg/ml

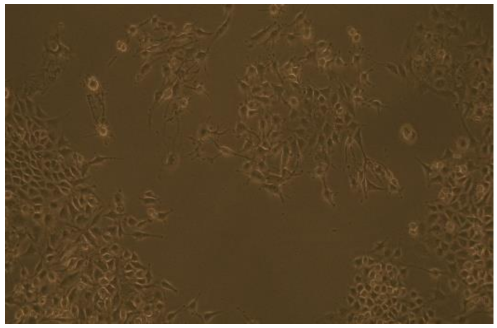

Plate 1.4:25 µg/ml

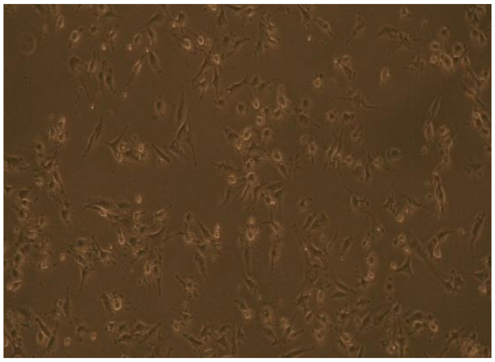

Plate 1.5: 50 µg/ml

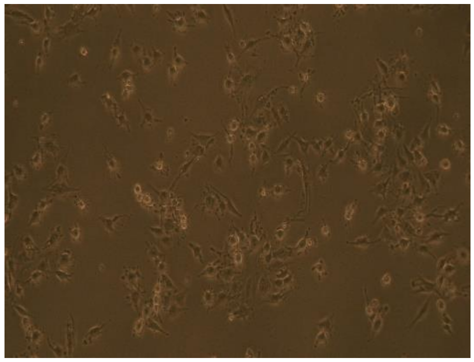

Plate 1.6:100µg/ml

Plate 2.2: Anticancer Effect of B1S2-2 extract by Direct Microscopic observation

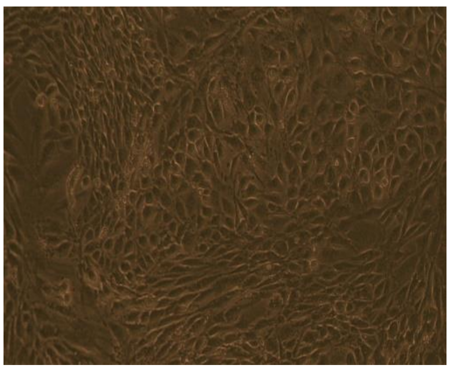

Plate 2.1: Control

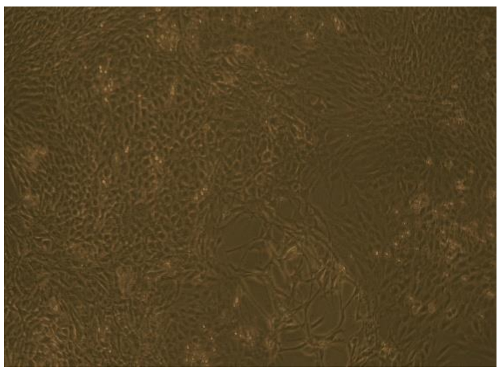

Plate 2.2: 6.25µg/ml

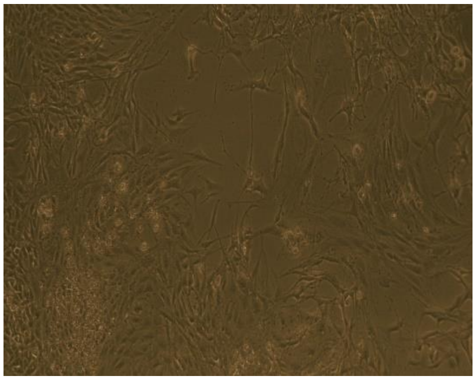

Plate 2.3: 12.5 µg/ml

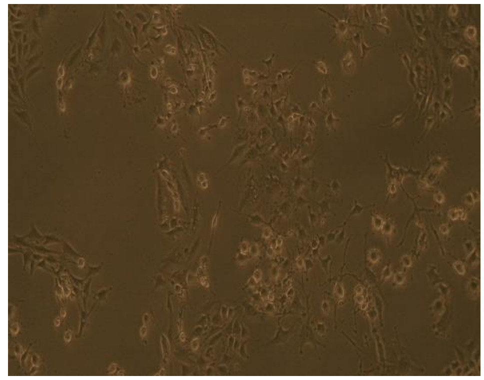

Plate 2.2: 25 µg/ml

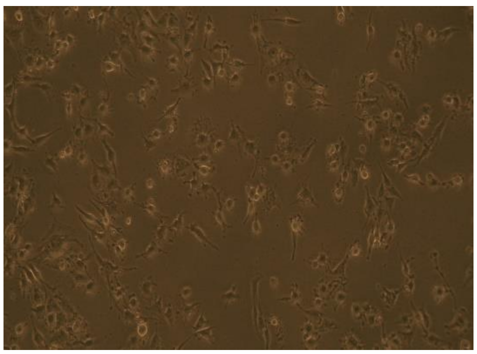

Plate 2.5: 50 µg/ml

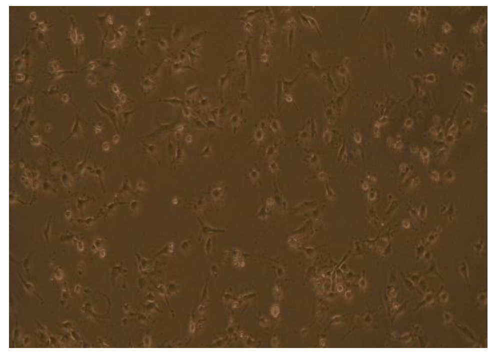

Plate 2.6: 100µg/ml

Discussion

Marine macroalgae- associated bacteria gains much significance among the researchers because of its highly varying and rich source of biologically active compounds. They produce such bioactive compounds not only to protect them from other microorganisms but also for ensuring their association with the host organism. These compounds are found to exhibit an array of different bioactivities including antimicrobial, antiviral, antiprotozoan, antiparasitic, and antiproliferative activities. Because of such highlighting properties Seaweed- associated bacteria are being explored for biotechnology and pharmaceutical applications (Ravindra *et al*., 2015).

Marine water comprises a vast diversity of microbial life including bacteria, fungi, actinomycetes, viruses and spores (Harder, 2009) and they settle on marine flora and fauna thereby forming unique associations with the host organisms (Singh and Reddy, 2014). In this study seaweed associated bacteria were isolated from the two seaweeds collected from Kadiapattinam coast, Kanyakumari district, Tamilnadu, India and their bioactive properties were analysed. A total of 20 different associated bacterial strains were obtained of which 14 were epiphytes and 6 were found to be endophytes. Clayton *et al.*, (1990) also isolated different bacteria from marine red, green and brown algae. Bioactive compounds as well as other secondary metabolites produced by marine algae associated bacteria inhibits the attachment of other bacteria to the host (Holmstrom and Kjelleberg, 1994).

In the present study antagonistic activity of the 20 isolated bacterial strains was evaluated against *Escherichia coli* and *Staphylococcus aureus* and it was found that only 25% of the tested bacteria showed antagonism. The strains A_1S_1-3, B_1S_1-5 and B_1S_2-2 showed antagonism against *Staphylococcus aureus* by interrupting their growth whereas the strain B_1S_1-2 alone showed antagonistic activity against *Escherichia coli*. Cell free lysate of antagonistic organisms also showed positive antagonism against the tested organisms with the maximum zone of inhibition (24mm) by strain B_1S_1-2 against *E.coli*. Studies made by Janaki *et al.*, (2013) also showed that the bacterial isolates associated with five different seaweeds collected from Gulf of Mannar, South east coast of India exhibited good antagonistic activity activity against human pathogens.

The association between marine microorganisms and host organisms are based on the bioactive compounds produced by associated marine microorganisms. Antibacterial activity of marine macroalgae-associated bacteria was analysed against five different human pathogenic bacteria and most of the strains showed activity against *Bacillus subtilis* and *Escherichia coli*. Few strains inhibited the growth of *Staphylococcus aureus*. Several studies also highlights that, marine algae acquire epiphytic and endophytic microbes that produces antimicrobial substances (Harborne *et al.*, 1998). Experiments done by Balakrishnan *et al.*, (2009) also revaled that microbes associated with different marine macroalgae were found to be bactericidal against *Staphylococcus aureus* and *Escherichia coli*.

A vast number of marine natural products have been found to exhibit antitumor activity and those compounds have been isolated from algae and other associated organisms (Chapman and Gellenbeck, 1983). In this study an effort was taken to evaluate the anticancer activity of most potent bacterial extracts against human melanoma cell line (SK-MEL-28). The bacterial extract B1S2-2 showed higher anticancer activity with an IC_{50} value of 28.064 µg/ml when compared with B1S1-5 extract whose IC_{50} value is 31.537µg/ml. The ability of the extracts to induce apoptosis can be inferred from the changes in morphology of cells, a decrease in percentage viability of the cells with increase in extract concentration. Findings of Luis *et al*., (2010) also showed that seaweed associated bacteria have gained the capability to synthesize compounds that supresses the proliferation of human colon cancer cells (HCT-116).

Conclusion

Findings of the present study reveals that marine macroalgae - associated bacteria isolated from Kadiapattinam coast, Kanyakumari district, Tamilnadu, India are promising sources of producing various bioactive compounds that have the ability to inhibit the growth of pathogenic microbes as well as to induce the apoptotis of cancer cells. Hence these organisms can be further explored for the innovation of novel compounds with therapeutic efficacy.

References

1. Avendaño-Herrera R, Lody M and Riquelme C.E (2005). Producción de substancias inhibitorias entre bacterias de biopelículas en substratos marinos. Revista de Biología Marina Oceanografía. 40(2): 117-125.
2. Balakrishnan C.P, Venkataraman K, Mohan V.R, Louis J.L, Athiperumal S.T, (2009). A general survey of the common agarophytes in the Gulf of Mannar in relation to agar ecology. Seaweed Research and Utilisation; 31(1&2):33–46.
3. Berdy J., 1989. The discovery of new bioactive microbial metabolites: screening and identification. In: Bushell ME & U Grafe (eds). Bioactive metabolites from microorganisms. Prog. Industrial Microbiol. 27: 3-25.
4. Bolinches J, Lemos M.L and Barja J.L (1988). Population dynamics of heterotrophic bacterial communities associated with *Fucus vesiculosus* and *Ulva rigida* in an estuary. Microb. Ecol 15: 345–357.
5. Chapman David J and Gellenbeck Kevin (1983), Seaweed uses: the outlook for mariculture, *Endeavour*, vol. 7(1), pp.31–37.
6. Choucry M. A. (2017). Chemical composition and anticancer activity of *Achillea fragrantissima* (Forssk.) Sch. Bip. (Asteraceae) essential oil from Egypt. Journal of Pharmacognosy and Phytotherapy, 9(1), 1-5.

7. Clayton M. N. (1990). The adaptive significance of life history characters in selected orders of marine brown macroalgae. Aust. J. Ecol., 15: 439-452.
8. Fabregas J, Munoz A, Otero A, Barja J.L. and Roruaris M. 1991. A preliminary study on antimicrobial activities of some bacteria isolated from the marine environment, Nippon Suisan Gakkaishi, vol.57, pp.1377– 1380.
9. Faulkner D.J, Harper M.K, Haygood M.G, Salomon C.E and Schmidt E.W (2000). Symbiotic bacteria in sponges: sources of bioactive substances. In: Fusetani N, editor. Drugs from the Sea. Karger; Basel. pp. 107–119.
10. Harborne J. B. (1998). *Phytochemical Methods*. London: Chapman & Hall; 60–66.
11. Harder T (2009), Marine Epibiosis: Concepts, Ecological consequences and host defence. In Costerton JW (ed). Marine and Industerial Biofouling Springer, Berlin, pp219-231.
12. Hentschel U, Hopke J, Horn M, Friedrich A. B, Wagner M, Hacker J, and MOORE S.. 2002. Molecular evidence for a uniform microbial community in sponges from different oceans. Applied and Environmental Microbiology 68:4431–4440.
13. Holmstrom C and Kjelleberg S (1994). The effect of external biological factors on settlement of marine invertebrate and new antifouling technology, Biofouling, vol. 8(2), pp.147-160.
14. Janaki Devi V, Yokesh Babu M, Umarani R and Kumaraguru A.K (2013). Antagonistic activity of seaweed associated bacteria against human pathogens. International Journal of Current Microbiology and Applied Sciences. Volume 2 (12) pp. 140-147.
15. Jasti S, M Sieracki, N Poulton, MW Giewat & JN Rooney- Varga. 2005. Phylogenetic diversity and specificity of bacteria closely associated with *Alexandrium spp*. and other phytoplankton. Applied and Environmental Microbiology 71(7): 3483-3494.
16. Kirkup B.C. and Riley M.A (2004). Antibiotic-mediated antagonism leads to a bacterial game of rock-paper – scissors in vivo, Nature, vol.428, pp.412–414.
17. Lindow S.E., 1988. Lack of correlation of *in vitro* antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. Phytopathology 78: 444-450.
18. Luis Jesús Villarreal-Gómez, Irma E.Soria, Graciela Guerra Rivas and Nahara E.Ayala-Sanchez (2010). Antibacterial and anticancer activity of seaweeds and bacteria associated with their surface. Revista de Biologia Marina Y Oceanografia 45(2):267-275.
19. Ravindra Pal Singh, Puja Kumari, and Reddy (2015). Antimicrobial compounds from seaweeds-associated bacteria and fungi. Appl. Microbiol Biotechnol. 99(4):1571-86.
20. Singh R.P and Reddy CRK (2014) seaweed-microbial interactions: key functions of seaweed – associated bacteria. FEMS Microbial Ecol 88:213-230.
21. Sithranga, N. and Kathiresan K. (2010). Anticancer drugs from marine flora: An overview. Journal of Oncology. vol.21, 41-86.
22. Talarico LB, Zibetti RG, Faria PC, Scolaro LA, Duarte ME, Noseda MD, Pujol CA and Damonte EB (2004). Anti-herpes simplex virus activity of sulphated galactans from the red seaweed *Gymnogongrus grifithsiae* and *Cryptonemia crenulata*. International Journal of Biological Macromolecules. 34 (1-2): 63-71.