Bioactive potential of exopolysaccharide from marine bacteria *Halomonas titanicae*

Pandiaraj Maheswari[1], Shunmugiah Mahendran[1]*, Subbiah Sankaralingam[2] and Natesan Sivakumar[3]
1. Department of Microbiology, Ayya Nadar Janaki Ammal College (Autonomous), Sivakasi, INDIA
2. Department of Botany, Saraswathi Narayanan College, Madurai, INDIA
3. School of biotechnology, Department of Molecular Microbiology, Madurai Kamaraj University, Madurai, INDIA
*mahi.ran682@gmail.com

Abstract

The present investigation was to study the purification and cell lines activities of the exopolysaccharide(EPS) from marine bacteria Halomonas titanicae. The exopolysaccharide was isolated and purified from bacteria by ethanol precipitation and chromatography using Sephadex G-50 respectively. Further, to confirm the biological potential, the antibacterial and MIC properties of exopolysaccharide, the exopolysaccharide was evaluated for in vitro antioxidant activity in various scavenging models including total antioxidant activity (80.27± 0.45%), DPPH (74.12 ± 0.25%) and ABTS (70.24 ± 0.34%) respectively.

The effect of exopolysaccharide had cytotoxic effects on MCF7 cell lines. FTIR analysis indicated that the EPS under study contained functional group in EPS.

Keywords: Sephadex G-50, MIC, DPPH, ABTS and MCF7.

Introduction

Exopolysaccharides (EPS) are often found in the surrounding as the outer most structures of both prokaryotic and eukaryotic microbial cells. They may be closely associated with the cell in the form of discrete capsules or else excreted as slime unattached to the cell surface. EPS exists in a wide variety of unique and often complex chemical structures and believed to provide self protection against anti microbial substances[7]. The water-soluble polysaccharide (SPS-CF) isolation and purification from Korean Caps siphon fulvescens by dilute acid extraction, ethanol precipitation and DEAE-cellulose ion exchange chromatography[22].

Cancer causes the highest mortality around worldwide, particularly in undeveloped countries. Chemotherapy drugs are still standard cancer treatments in spite of its toxicity against normal cells and tissues. In contrast, natural products for cancer treatments have relatively few side effects on normal cells and tissues[5] and have become an essential goal in many studies of immunopharmacology[21]. In the current decade, cancer has still remained the top listed death-causing disease despite of developments in tools of diagnosis, treatment and prevention and it is the most common-death causing disease[14]. There are recent reports for anti-cancerous activities of various polysaccharides from different sources[10]. Recently, there is a shift from microbial polysaccharides to plant polysaccharides because latter are mainly non-toxic and may do not cause side effects[17]. Plant polysaccharides from different sources have long been studied and are widely used for a variety of purposes including food, animal feed, medicine and papermaking[8].

Generally, polysaccharides work via two mechanisms against tumor cells: direct action (inhibition of tumor cell growth and apoptosis induction) and indirect action (immunostimulation). Besides the indirect action, several polysaccharides have shown direct effects on cancer cells. Many *in vitro* and *in vivo* studies suggested that inhibition of tumor cell proliferation induces their death by apoptosis after treatment with polysaccharides[4,23]. The present study was for isolation, purification and cell lines activities of marine bacterial strain that produced EPS. In addition, to analyze the antibacterial activity, *in vitro* antioxidant activities and cytotoxic effects on MCF7 cell lines assay were studied.

Material and Methods

Isolation and identification of the bacteria: Marine sediment soil samples were collected from Rameswaram, South east coast of India. Isolates were obtained by serial dilution plating on Zobell marine agar medium. Exopolysaccharide producing bacteria were screened based on their morphological characters and mucous appearances. A total of 100 colonies were isolated and the exopolysaccharides producing bacteria were screened for their ability to produce exopolysaccharide based on colony morphology (mucous and ropy).

Culture process and EPS production from bacteria: *Halomonas titanicae* culture was maintained on nutrient agar plates. It was sub cultured and slants were inoculated and maintained at 28ºC for 24 hrs. Experiments were done using 250 ml flask each containing 100 ml of Zobell marine agar medium inoculated with the bacterial culture. The flask was incubated at 28°C on an orbital shaker incubator at 110 rpm for 72 hrs.

Bacterial EPS quantification: After 72 hrs of incubation, Zobell marine agar medium samples were centrifuged at 5000 rpm for 20 min. The EPS was then precipitated from the supernatant by addition of equal volume of methanol. The mixture was agitated with addition of methanol to prevent local high concentration of the precipitate and left over night at 4ºC and centrifuged at 7000 rpm for 20 mins. After centrifugation the precipitate was collected in a Petri plate and dried at 60ºC [9].

Fractionation of EPS: The crude EPS was further purified by column chromatography. The crude EPS was dissolved in distilled water (10 mg/ 10ml) centrifuged at 6000 rpm for 10 min and the supernatant was applied to a Sephadex G-25 column (3×45 cm) equilibrated with distilled water. The column was eluted stepwise with distilled water with a linear gradient of 0-3 mol/ NaCl at a flow rate of 0.55 ml/min. The collected fractions were estimated for the carbohydrate content by phenol-sulphuric acid method[3]. The fractions showing higher yield were pooled together dialyzed for 24 hours against distilled water and then lyophilized.

Antibacterial activity

Target microorganisms: *In vitro* antibacterial susceptibility tests were performed using a panel of seven human pathogenic strains which include *Streptococcus pyogenes, Staphylococcus aureus, Klebsiella oxytoca, Proteus mirabilis, Escherichia coli, Pseudomonas aeruginosa* and *Bacillus subtilis* obtained from the laboratory in Department of Microbiology, Ayya Nadar Janaki Ammal College, Sivakasi, Tamil Nadu, India. All the strains were maintained in nutrient agar slants. Muller Hinton agar plates were prepared in a sterile condition and inoculated with 0.1ml of 24hrs old culture of the bacterial strains and spread properly throughout the solid media in a Petri dish with the help of a spreader.

The wells were cut in a single Petri dish using sterile cork borker one well for one extract. The EPS 1000µg was dissolved in 100µl of sterile distilled water separately and from each 20µl of the sample was injected into the wells. One of the control well was filled with 10µl of ampicillin (100µg/100µl). The plates were then incubated at 37 ^{0}C for 24hrs. After incubation, in the case of positive antibacterial activity, the zone of inhibition was measured and expressed in millimeters[6].

Determination of MIC: Minimum inhibitory concentration (MIC) of EPS was performed by macro-dilution method. The crude EPS extract was dissolved in distilled water. Bacterial suspension of the test organisms was prepared in sterilized Mueller-Hinton broth. Then 1 ml of the dilution was added to each sterilized screw cap tube containing 1 ml of compound suitably diluted in the sterilized broth medium to make final volume of 2 ml.

Culture media without sample and other without micro-organism were used in test as control. Tubes were incubated at 370C for 24 hours. Later they were analyzed by spectrophotometer at 600 nm[6].

***In vitro* antioxidant activity**

Determination of total antioxidant capacity: Total antioxidant activity of EPS was determined according to the method of Prieto et al.[13] Briefly, 0.3 ml of sample was mixed with 3.0 ml reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95 ºC for 90 minutes under water bath. Absorbance of all the sample mixtures was measured at 695 nm after 15 min. Ascorbic acid was used as standard.

DPPH radical scavenging assay: The free radical scavenging activity of EPS was measured by the 1-1-Diphenyl-2-picryl-hydrazyl (DPPH) following the method of Blois[2]. DPPH was used as a reagent which evidently offers a convenient and accurate method for titrating the oxidizable groups of natural (or) synthetic antioxidants. 0.1 mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 3ml of seaweed extracts of different concentration (100, 250, 500, 750 and 1000µg). After 10 minutes, absorbance was measured at 517 nm.

ABTS inhibition assay: The ability of the extract to scavenge ABTS (2,2 azino bis (3-etheylbenzothiazoline-6-sulphonicacid) diammonium salt) radical scavenging was determined by the method of Re et al.[15] ABTS was generated by mixing 5 ml of 7 mM ABTS with 88 µl of 140 mM potassium persulfate under darkness at room temperature for 16 hours. The solution was diluted with 50% ethanol and the absorbance at 734 nm was measured. The ABTS radical cation scavenging activity was assessed by mixing 5 ml ABTS solution (absorbance of 0.7± 0.05) with 0.1ml polysaccharide (100, 250, 500, 750 and 1000 µg). The final absorbance was measured at 743 nm with spectrophotometer.

Cell lines and culture condition: MCF-7 Breast cancer cell line was obtained from NCCS Pune, India. Cells were cultured in DMEM medium and supplemented with 10% of fetal bovine serum (FBS) then the culture flasks were maintained at 37˚C in 5% CO_2 incubator.

Cell viability (MTT) assay: The cytotoxicity test for the EPS was evaluated using MTT method. MCF-7 cells were seeded in 24 well plates at the concentration of $1x10^5$ cells/ml using DMEM medium and incubated for 24 hrs. When cells reached >50% confluence, the medium was replaced and the cells were treated with the jellyfish venom at 10, 30, 40, 50 and 60 µg/ml dissolved in DMSO and culture was incubated for 24 hrs. After incubation the medium was discarded. Then MTT solution was prepared by adding 0.5 mg/ml of MTT in 15 ml of DMEM medium. 100 µl of the MTT stock solution were added in each well and incubated at 37˚C for 4 hrs.

The MTT stock solutions were replaced by DMSO 200µl to each well and the colour in each well was observed. The amount of MTT- formazan that is directly proportional to the number of living cells was determined by measuring the optical density (OD) at 540 nm[11]. Cell images were captured under microscope to analyze its morphological structure.

FT-IR spectrophotometer analysis: IR spectroscopy's of sulfated polysaccharides was tested using Perkin-Elmer FT-IR instrument[19].

Statistical analysis: Each data point was obtained by making at least 3 independent measurements. All data are expressed as means ± S.D.

Results and Discussion

Yield of EPS: The crude EPS was partially purified in Sephadex G-25 using 1M sodium chloride and yield was 1g. The active fraction was identified by carbohydrate estimation using phenol sulphuric acid method and it was used for the further study. Exopolysaccharides play important roles in many biological processes and they can function as the virulence determinants in the pathogens[12]. Wenxia et al[20] elucidated the main structure of polysaccharides purified with DEAE and Sephadex G-25.

Antibacterial activity and MIC of EPS: The EPS showed maximum of 12 mm of inhibition zone against *Proteus mirabilis* and maximum of 6 mm of inhibiting zone against *Klebsiella oxytoca* shown in table 1. In addition, determination of the MIC values suggested that EPS was effective at minimum concentrations to inhibit the growth of different test organisms shown in table 2. Similarly, Patel et al[6] reported that the evaluation of antibacterial activity of EPS suggested that all 4 test microorganisms (*E. coli, B. cereus, B. subtilis* and *V. cholerae*) and sensitivity against EPS but the sensitivity is different from one bacteria to another bacteria. The MIC values of EPSs suggested that they can be used at lower concentration to inhibit test organisms. Abid et al[1] reported that the EPS of lactic acid bacteria showed an antibacterial activity against *E. coli, E. faecalis* and *L. monocytogenes*.

Free radical scavenging activity of EPS: The total antioxidant activity of EPS was calculated based on inhibition percentage as 80.27 ± 0.45% for *H. titanicae*. The DPPH radical scavenging assay for the *H. titanicae* is 74.12 ± 0.25%. The ABTS inhibition assay for the EPS from *H. titanicae* is 70.24 ± 0.34% shown in table 3. Similarly, Mahendran et al[9] reported that bacterial exopolysaccharides were evaluated for their antioxidant properties. The total antioxidant capacity of EPS extracts was found to be maximum in 80.13 ± 0.26%. The DPPH radical scavenging activity was found to be maximum in 48.39 ± 2.15%. ABTS inhibition assay was found to be maximum in 50.75 ± 3.85%.

***In vitro* cytotoxic activity of EPS:** *In vitro* cytotoxic activity of EPS at various concentrations against MCF-7 cancer cell line was studied using MTT assay.

Table 1
Antibacterial activity of EPS against pathogens

S.N.	Pathogens	Ampicillin (mm)	EPS (mm)
1	*Streptococcus pyogenes*	19	7
2	*Staphylococcus aureus*	20	10
3	*Klebsiella oxytoca*	17	6
4	*Proteus mirabilis*	18	12
5	*Escherichia coli*	21	9
6	*Pseudomonas aeruginosa*	22	11
7	*Bacillus subtilis*	16	8

Table 2
MIC of EPS for test microorganisms

S.N.	Pathogens	MIC (mg/ml)
1	*Streptococcus pyogenes*	5.5
2	*Staphylococcus aureus*	6.3
3	*Klebsiella oxytoca*	4.2
4	*Proteus mirabilis*	9.1
5	*Escherichia coli*	7.8
6	*Pseudomonas aeruginosa*	8.4
7	*Bacillus subtilis*	4.9

Table 3
Free radical scavenging activity of EPS

S.N.	Antioxidant activity	Standard Gallic acid	EPS (%)
1	Total antioxidant capacity	89.12 ± 0.23	80.27 ± 0.45
2	DPPH	81.09 ± 0.31	74.12 ± 0.25
3	ABTS	80.17 ± 0.63	70.24 ± 0.34

Antiproliferative activity of EPS at various concentrations against MCF-7 cancer cell line was 10 µg/ml (55.16%), 20 µg/ml (50.01%), 30 µg/ml (39.42%), 40 µg/ml (37.14%), 50 µg/ml (33.55%) and 60 µg/ml (31.32%) represented in figure 1. The cell viability was decreasing by increasing concentration of EPS depicting the cytotoxic effect of EPS shown in figure 2. The cells were aggregated in treatment group and its morphological disturbance revealed the cell death. Then the percentage of cell density has decreased indicating evidently the cell death.

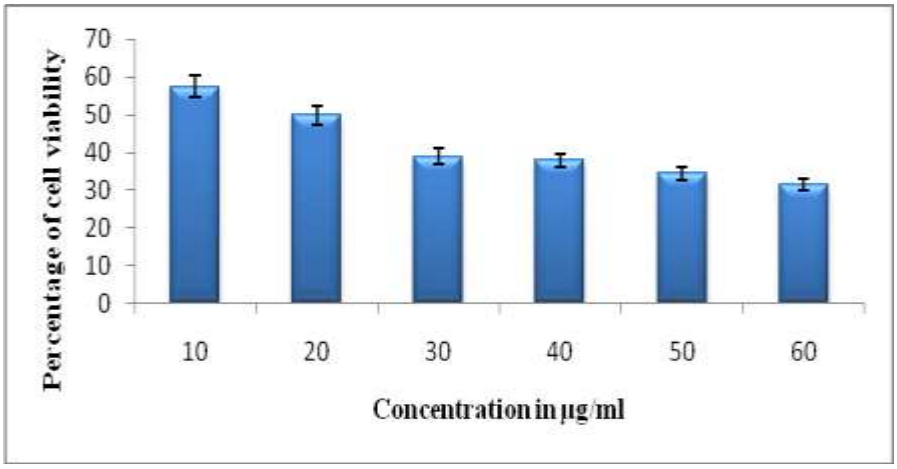

Fig. 1: Antiproliferative effect of EPS against MCF-7 cell line

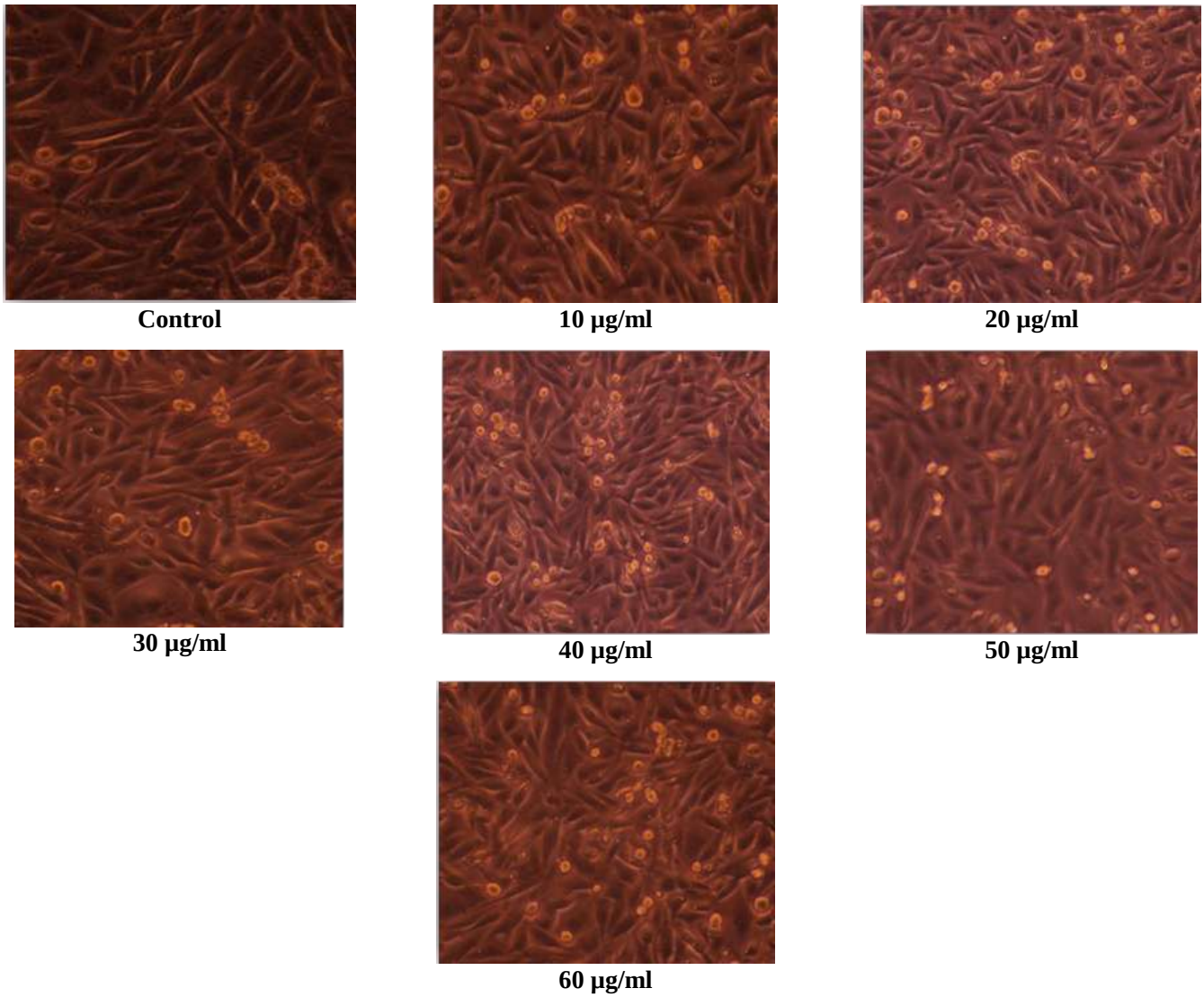

Fig. 2. MTT assay of EPS against breast cancer cell line MCF-7

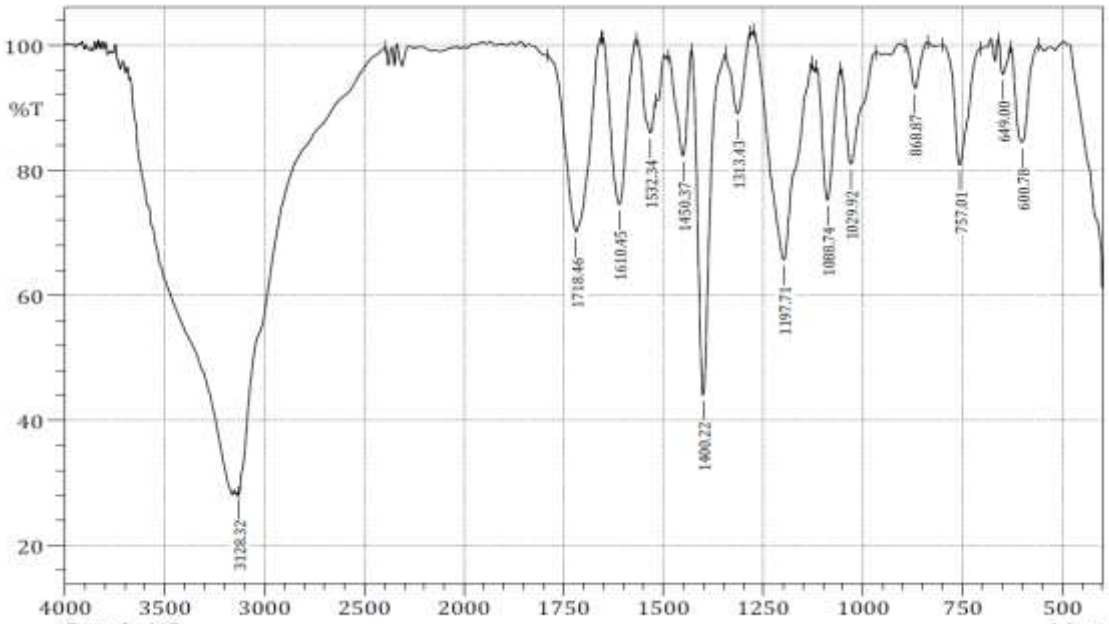

Fig. 3: FT- IR spectrum of EPS

Saly et al[16] reported that the effect of *J. rubens* polysaccharide had cytotoxic effects on MCF7 and CoCa2 cell lines. Annexin v concentrations and LDH activity were increased in the polysaccharide treated MCF7 and CoCa2 cells as compared with the untreated ones.

FT- IR analysis: The FT-IR spectrum of purified EPS of *H. titanicae* is shown in figure 3. The strong peak at 3128.32 cm^{-1} explained O-H stretching vibration. The signal at 1718.46 cm^{-1} explained C=O stretch vibration. 1610.45 cm^{-1} contributed N–H bend vibration. The band 1532.34 cm^{-1} explained N–O asymmetric stretch. The signal at 1450.37 and 1400.22 cm^{-1} corresponding to C–C stretch bending is plane. The signal at 1313.43, 1197.71 and 1088.74 cm^{-1} concluded C–O stretch bending. The signals at 868.87, 757.01, 649.00 and 600.78cm^{-1} concerned CH-deformation. The signals 586.36 and 584.43 cm^{-1} designed ring formation for C-C stretching.

Sutherland[18] reported the presence of carboxyl, hydroxyl and alkyl groups in exopolysaccharides. The bacterial EPS extracts gave characteristics bands for EPS. Here, carbonyl (C= O) stretching peak and OH stretching peak were at broad and the maximum peak and the band at 1000-1500 showed the presence of polysaccharide. The cultivation temperature by 10˚C below optimal level inhibits the EPS biosynthesis by microbial cells. However, under low temperature of the growth, environment profiles of the high productivity of extracellular poly-saccharide occur by bacterial cells.

Conclusion

It can be concluded from this study that marine bacteria *Halomonas titanicae* exopolysaccharide showed considerable content of bioactive compounds explaining the antibacterial, antioxidant capacity and cell line (MCF-7) with a potential for future pharmacological and medical applications.

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(Received 11th May 2020, accepted 18th July 2020)