Characterization and Application Study of Exopolysaccharide Isolated from Cronobacter muytjensii ATCC 51329 (T)

Gitanjali G. Mane, Govind S. Bhosle and Venkat S. Hamde

Research Journal of Agricultural Sciences
An International Journal

P- ISSN: 0976-1675
E- ISSN: 2249-4538

Volume: 12
Issue: 03

Res Jr of Agril Sci (2021) 12: 781–788

CARAS

Res. Jr. of Agril. Sci. (May-Jun 2021) 12(3): 781–788
www.rjas.org

Full Length Research Article

Characterization and Application Study of Exopolysaccharide Isolated from *Cronobacter muytjensii* ATCC 51329 (T)

Gitanjali G. Mane*[1], Govind S. Bhosle[2] and Venkat S. Hamde[3]

Received: 10 Mar 2021 | Revised accepted: 05 May 2021 | Published online: 17 May 2021

A B S T R A C T

In present study exopolysaccharide was produced from *Cronobacter muytjensii* ATCC 51329 (T) and purified by extraction followed by dialysis. The present exopolysaccharide was characterized as heteropolysaccharide in nature by RP-HPLC. HRMS analysis revealed that the peak at 9.8, 10.1, 13.8, 15.0, 15.9 were corresponds to Glucose, Galactose, Arabinose, Xylose, Rhamnose respectively. The partially purified exopolysaccharide obtained from *cronobacter spp.* showed 28.40% and 244.905±2.69% of water solubility index and water holding capacity respectively. The emulsifying activity for xylene was higher (63.955±0.45) followed by coconut oil (61.225±0.19). Exopolysaccharide has maximum antioxidant activity (100%) at 5 mg/ml with an EC_{50} value was 0.1 mg/ml. Exopolysaccharide showed significant antibacterial activity against *E. coli, P. auroginosa, S. aureus* and *B. subtillus* with MIC values 11.41 µg/mL, 16.98 µg/mL, 18.11 µg/mL, 13.16 µg/mL respectively. Anticancer activity of exopolysaccharide was studied by MTT assayat the highest tested concentration (1000 µg/mL). The inhibitory effects of exopolysaccharide increased to 91.6±6.5% and 91.5±5.8% against HepG-2 and HeLa cell lines respectively. Hemolytic activity showed that the EPS has negligible toxicity on human RBCs. The present properties specify the potential use of EPS as water holding, emulsifying, antibacterial, antioxidant, and anticancer agent.

***Key words*:** RP-HPLC, Anticancer, MTT assay, Antioxidant, Emulsifying

EPSs are high molecular weight polymers composed of saccharide subunits and are synthesized and secreted by microorganisms into the surrounding environment. Microorganisms produce large spectrum multifunctional polysaccharides including intracellular polysaccharides, structural polysaccharides and extracellular polysaccharides or exopolysaccharides (EPSs). Exopolysaccharides generally consist of monosaccharides subunits linked with α- or β-glycosidic linkage and some non-carbohydrate substituents (such as protein, nucleic acids, lipids, acetate, pyruvate, succinate, and phosphate) [1]. EPSs can be classified into homo- or hetero- as well as neutral, acidic, or basic polysaccharides based on monosaccharide content, organic and inorganic substituents present [2-3]. These EPSs are metabolic products accumulated in the bacterial cell wall, provide protection to microbial cells and serve as carbon and energy source [4]. EPS plays an important task in interaction between bacteria and their surrounding environment [1] (Orsod *et al.* 2012). EPS helps bacteria in the cell adherence to surfaces, in biofilm formation, pathogenesis, protecting against osmotic shock, toxic stress, and antibacterial compounds microbial aggregation, cell protection, also involved in symbiosis [5-7].

Natural polysaccharides have the advantages of being, non-toxic, ecofriendly and biodegradable are less harmful than synthetic polymers [8-9]. Over the past few decades, the number of known exopolysaccharides (EPSs) produced by microbial fermentation has gradually increased [10]. Microbial EPSs showed diversity in physico-chemical properties due to differences in monosaccharide composition and sequence, branching degree, and type of linkage and polymerization. This diversity of characteristics has drawn attention to the industrial applicability of EPSs in different fields [11-12]. Interest in the exploitation of microorganisms for production of valuable polysaccharides has greatly increased in recent years, since these biopolymers produced by a variety of microorganisms are chemically well defined and have attracted worldwide attention due to their novel and unique physical properties such as bioadhesives, bioflocculants, biosorbents, gelling agents, probiotics, stabilizing, emulsifying, gelling, thicking agent in different fields. EPS used in various industries, such in the bionanotechnology, food, pharmaceutical, cosmetic, concrete additives, in enhanced oil recovery, metal recovery and waste water treatment. In medical field EPS used as immune-

* Gitanjali G. Mane
✉ gitanjalimane@gmail.com

[1-2] Department of Microbiology, Yogeshwari Mahavidyalaya, Ambajogai, District Beed - 431 517, Maharashtra, India

[3] Organic Chemistry Division, CSIR-National Chemical Laboratory, Pune, Maharashtra, India

modulator, antitumor, antiviral, and antiulcer and antioxidant, and in drug delivery as coating agent [13].

It was considered from long time that root nodule occupation was restricted to rhizobia, but later wide range of α-, β-, and γ- Proteobacteria have been isolated from root nodules of different leguminous plants as root nodulating bacteria. Non-symbiotic nodule endophytes have been poorly studied as compared to symbiotic root nodulating bacteria [14-15]. Different genera of bacteria have been presented as endophyticinclude *Bacilus, Staphylococcus, Mycobacterium, Rhodopseudomonas, Enterobacteria* etc. [16]. It was previously reported that the *Cronobacter* species might be of plant origin due to its physiological features such as gum like exopolysaccharide production, and its desiccation resistance [17]. The isolation of *Cronobacter* has been reported from wide spectrum of environmental sources including soil, water, vegetables, and herbs, and also from rice, and soybean plants [18]. Patent for food thickener was reported for polysaccharide material extracted from *Enterobacter sakazakii* isolated from Chinese tea [19-20].

Based on characterization data, suitability of EPS for product and industrial application can be determined easily. If properly characterized, these biopolymers could serve as stabilizers, restorative agents, thickeners, excipients, emulsifiers, bio based encapsulants, coating agents and texture modifiers [21-22]. In the present research EPS obtained from *Cronobacter muytjensii* was characterized by RP-HPLC; furthermore, its various applications were studied such as anticancer, antibacterial, antioxidant and hemolytic activities.

MATERIALS AND METHODS

Isolation and identification of Cronobacter muytjensii

EPS producing bacterial culture was isolated from healthy, pink colored, unbroken root nodules of leguminous plant soybean (*Glycin max*). Isolated bacterial culture further identified by morphological, biochemical characterization and by 16s rRNA technology.

EPS production and extraction

Inoculum preparation

For inoculum preparation one loopful of isolated bacterial culture was transferred in Erlenmeyer flask (100 mL) containing 25 mL YEM broth supplemented with 1% mannitol as carbon source. The flasks were incubated at 30±20C for 24 hours on a rotary shaker. After 24 hours of incubation 1.5 mL inoculum with constant growth O.D. (1.054 at 600nm) was used and transferred in fermentation media.

Fermentation

YEM broth (YEMB) used as fermentation broth was inoculated with isolated bacterial culture and incubated at 30±20C on a rotary shaker for 72 hours [23].

Extraction and partial purification of EPS

After 66 hours of incubation, fermentation broth was treated with 10% TCA (Trichloro acetic acid) to remove protein and after half an hour fermentation broth centrifuged (at 10,000 rpm for 20 minutes) after centrifugation three volume of ice-cold ethanol was added to one volume of supernatant and kept at 40C for 24 hours. After incubation EPS precipitate were collected by centrifugation (2500 rpm for 15 minutes). Obtained EPS precipitate was suspended in distilled water again precipitated with equal volume of ice-cold ethanol. The solution was again centrifuged at 2500 rpm for 15 minutes. The final pellet obtained was dried at 60°C in oven [24]. Obtained EPS was dissolved in water and dialyzed again double distilled water for two to three days with changing water. Dialyzed EPS was again purified by lyophylization and used for application.

EPS characterization

Hydrolysis of Exopolysacchride

The obtained EPS was characterized by the method given by [25]. For hydrolysis 2 mg of partially purified EPS was dissolved in 2 mL of 4M trifluoroacetic acid in a screw capped autoclavable tube and heated to 110°C in an oil bath for 8 h. The reaction mixture after cooling was centrifuged at 1000 rpm and later neutralized by the addition of 0.3 M NaOH. This mixture was then used for derivatization with Anthranilic acid (AA).

Derivatization of oligosaccharide with Anthranilic acid (AA)

As optimized by [26] AA labeling of monosacchrides for capillary electrophoresis. They suggested 0.2M AA and 1 M $NaBH_3CN$ (Sodium cyanoborohydried) as the optimum condition for the reaction. We found this condition worked well on our polyssachride. Although AA is difficult to dissolve in water, it does dissolve well in water when heated in presence of $NaBH_3CN$. Therefore, $NaBH_3CN$ is prepared first and then AA reagent is dissolved in this solution by heating at the reaction temperature (65°C).

Recovery of AA tagged saccharide

Precipitation with acetonitrile made it convenient to collect AA tagged monosacchrides. Most of which were attached to the wall of container after addition of acetonitrile and vortexing. This precipitate after careful removal of acetonitrile can be used directly for RP-HPLC.

RP-HPLC purification and HRMS analysis of AA labeled saccharide

The resulting precipitate was dissolved in 50% acetonitrile in water. The solution was then filtered through 0.22 µm filter (Millipore Inc, USA) diluted with water and analyzed by RP-HPLC instrument (waters) at 303 nm, in a C18 column 250 x 4.16 mm, stainless steel column packed with Octadecylsilane (C18) bonded to porous silica. The flow rate was maintained at 1.5 mL/min and the injected volume is 50µl.

Application study

Water solubility index (WSI) and Water holding capacity (WHC)

The WSI and WHC of EPS were determined by [27]. Two hundred milligrams of EPS sample was dissolved in 5mL distilled water and stirred for 30 minutes in a water bath at 40 to 60°C to get uniform suspension of EPS sample. Then the suspension was centrifuged at 10,000 rpm for 15 min.

WSI

To calculate the WSI, the supernatant was positioned in a Petrie dish and dehydrated out at 105ºC in oven for 4 hours to attain the dry solid weight. Water solubility index was calculated as per the following formula [28-29].

$$WSI = \frac{\text{Weight of dry solid in supernatant}}{\text{Weight of dry sample}} \times 100$$

WHC

The pellet was dropped on pre-weighed filter paper for removal of excess water. Then the filter paper was weighed and recorded. The percentage of Water-Holding capacity was calculated using following formula [28-29].

$$\text{WHC \%} = \frac{\text{Total sample weight after absorption}}{\text{Total dry sample weight}} \times 100$$

Emulsifying activity

The emulsification activity of EPS was measured as per [30-31] method. Different hydrocarbons (petrol, xylene, and toluene) and oils commercially available in India were used to study the emulsifying activities of EPS. Hydrocarbon or oil (1.5 mL) was added to 1.5mL of various concentrations of EPS (0.5, 1.0, 2 and 3 mg/mL) and vortexed vigorously for 1 to 2 min. The emulsifying activity (% EA) was determined after 1 hour whereas the emulsion index (stability) was determined as (% EI) after 24, 48, and 72 hours. The % EA and % EI were calculated by dividing the height of the emulsion layer (in cm) by the total height of the mixture (in cm) multiplied by 100.

Antioxidant activity tests using DPPH radical scavenging assay

The antioxidant activity of partially purified EPS was measured, based on the scavenging of stable 1-1-Dipheny1-2picryl-hydrazyl (DPPH) following the previously determined method [32]. Used as a reagent, DPPH 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 1 mL of this solution was added to 3 mL of exopolysaccharides extract at different concentrations (50, 100, 250, 500 and 5000 µg/ml) and reference compound ascorbic acid (10, 20, 50, 100, 200, 400 and 500µg/ml). After 10 min, absorbance was read at 517 nm. The percentage scavenging activity values were calculated as below:

$$\text{Percentage of scavenging} = (A^{\circ} - A^{1}) / A^{\circ} \times 100$$

A°- Absorbance of control

A^{1}- Absorbance of sample

Anti-bacterial activity

All bacterial cultures were first grown in LB (Luria Bertani) media at 37°C at 180 rpm. Once the culture reaches 1 O.D, it is used for anti-bacterial assay. Bacterial strains *E. coli* (NCIM 2688), *P. aeruginosa* (NCIM 2036) as gram-negative and *B. subtilus* (NCIM 2079), *S. aureus* (NCIM 2010) as gram-positive were obtained from NCIM (NCL, Pune) and were grown in LB medium from Himedia, India. The assay was performed in 96 well plates after 8 hours and 12 hours for gram negative and gram-positive bacteria respectively. 0.1% of 1 OD culture at 620 nm was used for screening. 0.1% inoculated culture was added in to each well of 96 well plates containing the compounds to be tested. Optical density for each plate was measured at 620 nm after 8 hours for gram negative bacteria and after 12 hours for gram positive bacteria [33].

Hemolysis assay

Freshly drawn human red blood cells (hRBCs) with additive K_2 EDTA (spray-dried) were washed with PBS buffer several times and centrifuged at 1000 g for 10 min until a clear supernatant was observed. The hRBCs were re-suspended in PBS to get a 0.5% v/v suspension. EPS dissolved in PBS were added to a sterile 96-well plate to a volume of 75 µL in each well. Then 75 µL of 0.5% v/v hRBC solution was added to make up a total volume of 150 µL in each well. TritonX-100 and PBS were used as positive and negative controls respectively. The plate was then incubated at 37°C for 1 hour, followed by centrifugation at 3500 rpm for 10 min. The supernatant (120 µL) was transferred to fresh wells and absorbance was measured at 414 nm on a VarioskanFlash (4.00.53) Microplate Reader. Results were with respect to the positive (Triton X-100) and negative (PBS) controls. % hemolysis was determined by the equation:

$$\% \text{ hemolysis} = (\text{Abs.}_{\text{sample}} - \text{Abs.}_{\text{PBS}}) / (\text{Abs.}_{\text{Triton-X 100}} - \text{Abs.}_{\text{PBS}}) \times 100$$

Cytotoxicity studies by MTT assay

The cytotoxicity of EPS to HeLa cells and HEP G2 cells was determined by the MTT cell viability assay. All cells were plated overnight in 96-well plates at a density of 10,000 cells per well in 0.2 mL of appropriate growth medium with 10% FBS (Fetal Bovine serum) at 37°C. Different concentrations of EPS up to a maximum of 1000µg/mL were incubated with the cells for 12 hours, following which; the EPS were removed by replacing the media by fresh media. The cell culture medium alone and with cells, and both without EPS were included in each experiment as controls. After 12 hours incubation, 20 µL of a 5 mg/mL solution of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added and further incubated for 4 hours. Conversion of MTT into purple formazan product by metabolically active cells is used as an indication of cell viability. The crystals of formazan were dissolved with DMSO (Dulbecco's Eagle Medium) and the optical density was measured at 570 nm using a microplate reader VarioskanFlash (4.00.53), for quantification of cell viability. All assays were run in triplicates.

RESULTS AND DISCUSSION

Extraction and purification of EPS

After fermentation, obtained EPS was dissolved in water and dialyzed again double distilled water for two to three days with changing water. Dialyzed EPS was again purified by lyophylization and used for application. The purified EPS was further used for characterization and application study.

Table 1 HRMS analysis of the collected peaks of hydrolyzed EPS of the study

Sugar (Retention Time)	Calculated Mass	Observed Mass
Glucose (9.8)	299	298
Galactose (10.1)	299+Na	322
Arabinose (13.8)	269+Na	293
Xylose (15.0)	269	270
Rhamnose (15.9)	283	282

Characterization of EPS by RP-HPLC

Analytical chromatogram of hydrolyzed EPS is shown in (Fig 1), six peaks were observed at 9.8, 10.1, 13.8, 15.0, 15.9, 16.8 minutes. All the peaks were collected and subjected to HRMS (High Resolution Mass Spectroscopy) analysis. HRMS analysis revealed that the peak at 9.8, 10.1, 13.8, 15.0, 15.9 were corresponds to Glucose (Fig 2), Galactose (Fig 3), Arabinose (Fig 4), Xylose, (Fig 5) Rhamnose (Fig 6) respectively (Table 1). The peak at 16.8 remains unidentified.

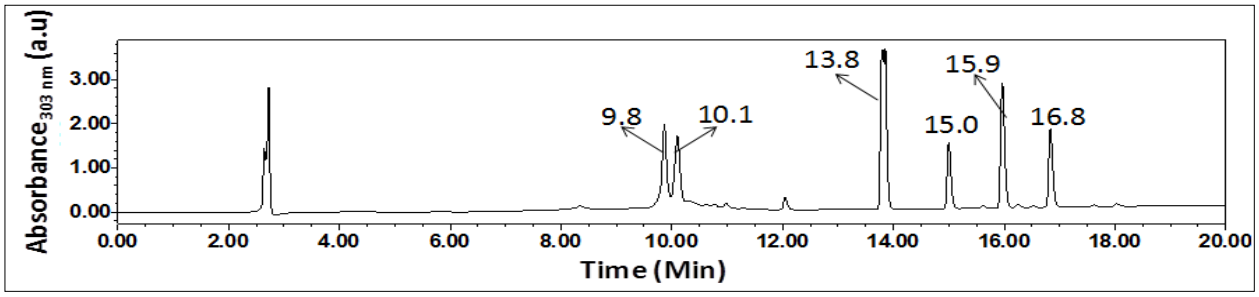

Fig 1 RP-HPLC chromatogram of hydrolyzed EPS of the study

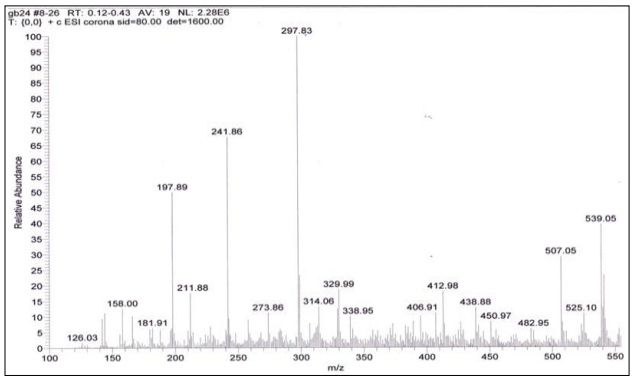

Fig 2 Mass spectra of Glucose + Anthranilic acid

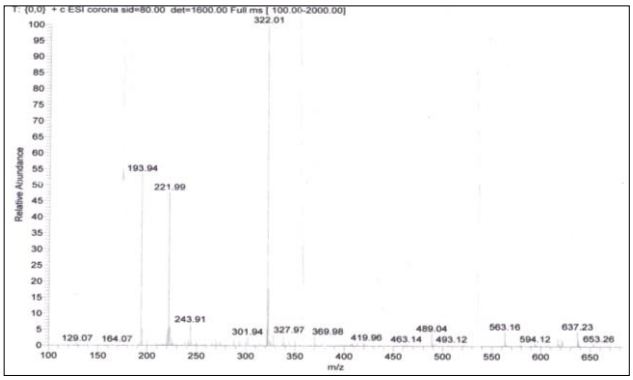

Fig 3 Mass spectra of Galactose + Amino benzoic acid

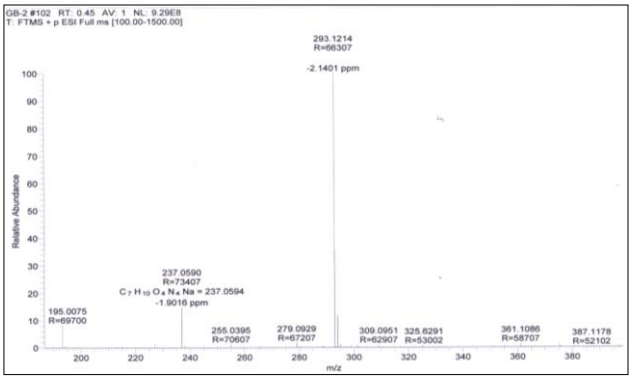

Fig 4 Mass spectra of Arabinose + Amino benzoic acid

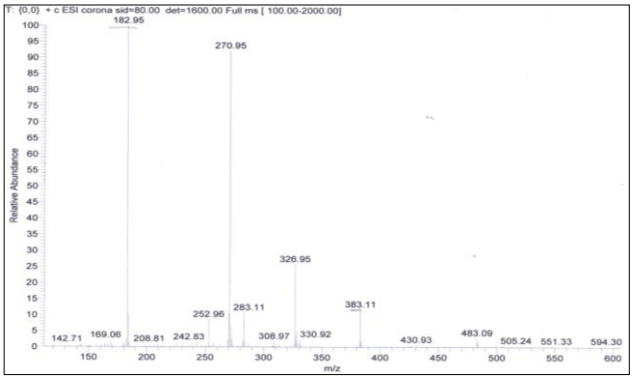

Fig 5 Mass spectra of Xylose + Amino benzoic acid

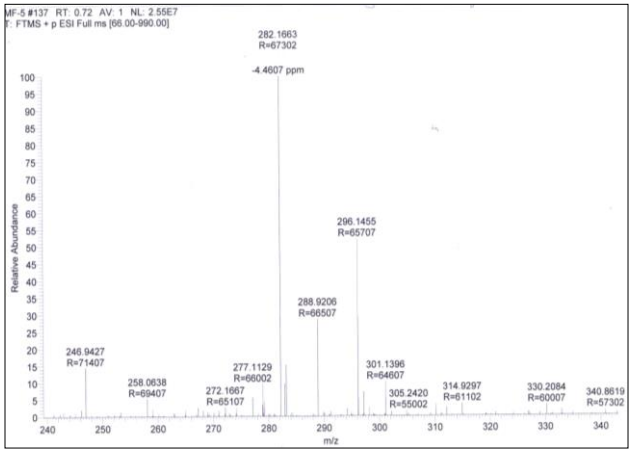

Fig 6 Mass spectra of Rhamnose + Amino benzoic acid

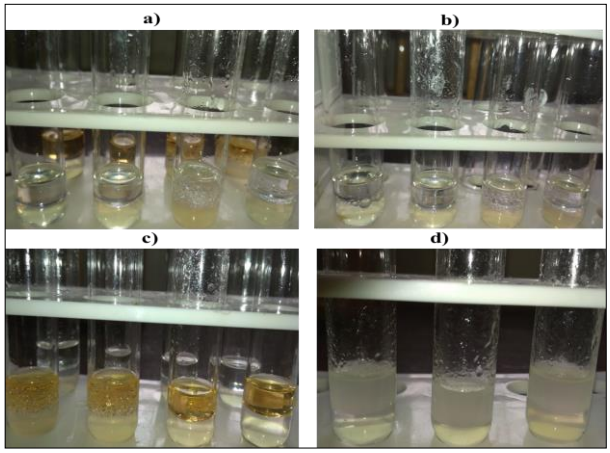

Fig 7 Emulsifying activity of xylene (a), toluene (b), petrol (c), and coconut oil (d)

Carbohydrates neither have chromophores nor fluorophores, ruling out direct photometric or fluorometric detection. Refractive index detection is the method of choice, but this technique is not very sensitive and unsuited for gradient elutions. Pulsed amperometric detection (PAD) is a common method for the quantification of saccharides, amino sugars, and uronic acids applying HPAEC with gradient elution. Recently, capillary electrophoresis (CE) in combination with PAD has shown promise as a new tool for this analytical task because of rapid and high resolution of complex matrixes. Since the PAD sensitivity does not meet the analytical demands, it does have detection limits. Higher

detection sensitivities are attainable by labeling of the analytes with fluorometric or chromophoric tags.

A number of photometric and fluorometric tags have been developed for pre- and postcolumn analysis by HPLC and CE. The advantages of precolumn derivatization are the modification or removal of the sample matrix and the selective enrichment of the derivatives. Several reagents have been developed for precolumn tagging of carbohydrates, amino sugars, and uronic acids. A popular reagent for this purpose is 1-phenyl-3-methyl-5-pyrazolone (PMP). The derivatization procedure comprises several steps including phase-transfer reactions and phase separation and seems difficult for automation.

Labeling by reductive amination using a combination of aromatic amines and reducing reagents is conducted most frequently. Many reagents classified as monosubstituted aminobenzene derivatives (2-aminobenzamide, 4-aminobenzoic acid esters, 4-aminobenzonitrile, 2-aminobenzoic acid, 3-aminobenzoic acid, 4-aminobenzoic acid) were successfully applied for the formation of derivatives of mono- and oligosaccharides, suited for CE or HPLC analysis. Primarily these derivatization reagents are used in the bioanalytical research for determination of carbohydrates after hydrolysis of oligo- and polysaccharides and glycoproteins.

In this experiment, we have used fluorescent derivatization of sugar units obtained by acid hydrolysis of exopolyssachrides using anthranilic acid in aqueous solution. In HPLC analysis different independent peaks were observed and molecular mass was determined with retention time, and it was revealed that the present EPS is heteropolysaccharide in nature. HRMS analysis revealed that the peak at 9.8, 10.1, 13.8, 15.0, 15.9 were corresponds to Glucose, Galactose, Arabinose, Xylose, Rhamnose respectively (Table 4). The peak at 16.8 remains unidentified. [34] Characterized isolated EPS by RP-HPLC analysis after PMP derivatization as heteropolysaccharide in nature contains glucose, galactose, rhamnose, manose, glucuronic acid and galacturonic acid. [35] the monosaccharide composition of EPS was determined by a PMP-derivatization method using a HPLC analysis and found that the isolated EPS consists of only glucose as monosaccharide unites.

Water solubility index (WSI) and water holding capacity (WHC)

The partially purified EPS obtained from *cronobacter spp.* showed 28.40% and 244.905±2.69% of WSI and WHC respectively. [36] reported the water solubility index and water holding capacity of the EPS *Leuconostoclactis KC117496* isolated from idli batter were 14.2 ± 0.208%, 117 ± 7.5% respectively. [37] reported that the water solubility index and water holding capacity of EPS from *Lactobacillus kefiranofaciens ZW3* was 14.2% and 496% respectively. EPS is water soluble compound with good water holding capacity due to absorptive structure of the EPS polymer which can contain huge quantities of water through hydrogen bonds [38]. The presence of a large number of hydroxyl groups and residual proteins are responsible for absorption of moisture in these polysaccharide structures. Due to its Physico-chemical properties such as viscosity, water holding capacity and solubility; Carbohydrates can also improve the textural and rheological properties of food products.

Table 2 Emulsifying activity (EA) of EPS with different concentrations under different hydrocarbons and oil

Hydrocarbon/oil	EPS mg/mL	% EA	Emulsifying index		
			E_{24}	E_{48}	E_{72}
Petrol	0.5	43.745±2.94	34.165±1.18	35.355±0.50	45±0
	1	49.995±6.42	37.17±0.46	45.115±0.16	42.3±0.28
	2	52.17±0	41.425±2.01	47.61±0	47.135±0.12
	3	57.14±0	44.585±0.20	53.585±2.00	47.555±0.07
Xylene	0.5	38.09±0	39.495±1.98	37.495±1.60	41.1±0.28
	1	46.53±1.52	51.135±1.60	44.04±1.68	50.58±0.82
	2	58.33±1.68	51.19±1.68	54.88±0.16	56.475±0.94
	3	63.955±0.45	59.635±0.77	57.14±0	57.32±0.25
Toluene	0.5	37.495±1.60	41.425±2.01	37.88±1.41	43.925±1.52
	1	45.115±0.16	45.115±0.16	43.925±1.52	52.53±0.04
	2	51.135±1.60	56±1.41	58.33±1.68	57.35±0.29
	3	60.495 ±1.98	58.57±2.02	58.8±2.34	60.71±1.68
Coconut oil	0.5	46.735±1.53	48.805±1.68	52.595±0.04	54.54±0
	1	54.325±0.95	46.305±1.84	56.2±1.32	54.88±0.16
	2	55.43±1.54	56.665±0.20	59.205±1.85	61.18±1.01
	3	61.225±0.19	60.495±1.98	60.71±1.68	59.305±0.30

Emulsifying activity

Emulsifying activity of EPS with different solvents is shown in (Table 2, Fig 7). The EPS produced by *Cronobacter muytjensii* ATCC 51329(T) was capable of stabilizing emulsions with different hydrocarbons and oil. The emulsifying activity for xylene was higher (63.955±0.45) followed by coconut oil (61.225±0.19). Both EA and EI were higher for toluene followed by coconut oil when compared with other solvents (oils and hydrocarbons).

The emulsification activity of the EPS is determined by its strength in retaining the emulsion for a certain period of time. The ability of the EPS to form stable emulsions through emulsification activity test revealed that the emulsifying activity of EPS was found to be higher for the Xylene followed by coconut oil and less for toluene and petrol. But emulsifying index of coconut oil was more than xylene. Similar with our results the EPS produced by *Bacillus megaterium* showed the highest emulsion index with coconut oil (76%) [39]. [40] Studied emulsifying activity of EPS produced by Soil-degrading *Ochrobactrum anthropi MP3* isolated from refinery waste and found maximum emulsifying activity with diesel (60±1%). Whereas EPS produced by *Streptococcus thermophilus CC30* show Both Emulsifying activity and index higher for sunflower oil (58%). The presence of acetyl group imparts somewhat hydrophobicity to the EPS that might contribute to its emulsifying capacity and

surface-active properties [41]. From this experiment it might be concluded that, the purified EPS would be a better bio emulsifier with significant emulsifying activity in different industrial applications.

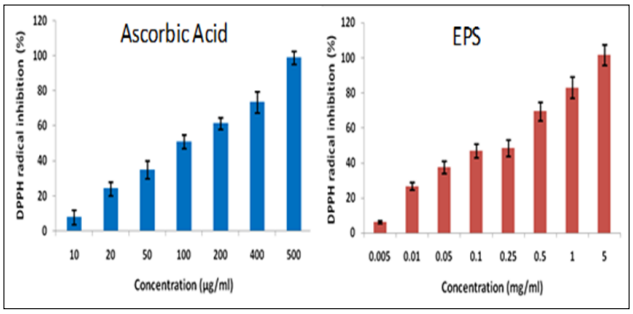

Fig 8 Scavenging effects of EPS and ascorbic acid during DPPH test by changes in
Each value expressed as mean ± standard deviation (n = 3)

Antioxidant activity

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds [42]. In the DPPH test, the antioxidants were able to reduce the stable DPPH radical to the yellow colored diphenylpicryl hydrazine. The effect of antioxidants on DPPH radical scavenging was conceived to be due to their hydrogen-donating ability. The DPPH radical scavenging activities of EPS and Ascorbic acid were evaluated. Ascorbic acid was used as a positive control [43] and the results are shown in (Fig 8). As shown in (Fig 8), EPS exhibited the scavenging activity toward DPPH radicals in a concentration-dependent manner, with an EC_{50} value of 0.25 mg/mL. Scavenging activity of EPS was compared with known antioxidant ascorbic acid (EC_{50}= 0.1 mg/mL) under the same conditions, ascorbic acid, a free radical scavenger, showed a slightly higher effect on the hydroxyl radicals, with an EC_{50} value of 0.1 mg/mL. Both of them presented approximately identical change trend of antioxidant activity. These results indicated that the EPS had a noticeable effect on scavenging free radical, especially at high concentration. It is well known that reactive oxygen species (ROS), such as hydroxyl radicals, super oxide anion and hydrogen peroxide, are related to the pathogenesis of various diseases [44]. Hydroxyl radical is the most reactive among the oxygen radicals and induces severe damage to the adjacent biomolecules [45-46]. However, the scavenging activity of EPS against the DPPH-radical was slightly less than that of ascorbic acid.

Table 3 MIC values of EPS against the bacteria

Compound ID	MIC (µg/mL)			
	E. coli	*S. aerogenosa*	*S. aureus*	*B. subtilis*
EPS	11.14	16.96	18.11	13.16
Ampicillin	1.46	4.36	1	10.32
Kanamycin	1.62	0.49	30	1.35

Antibacterial activity

EPS were found to show excellent activity against all the tested bacteria as shown in the killing curves (Fig 3). The results of antibacterial activity are summarized in (Table 3). The EPS showed MIC values 11.41 µg/mL, 16.98 µg/mL, 18.11 µg/mL, 13.16 µg/mL, against *E. coli, P. auroginosa, S. aureus and B. subtilis* respectively. [47] reported antibacterial activity of Exopolysaccharides of *Lentinussubnudus*by injecting Swiss Albino Rats intraperitoneally in aqueous solution (20mg/mL) into infected with pathogenic strains of *Escherichia coli* and *Pseudomonas aeruginosa*. The results showed that exopolysaccharides of *L. subnudus* possesses antibacterial properties and is a, non-toxic substance of medicinal value.

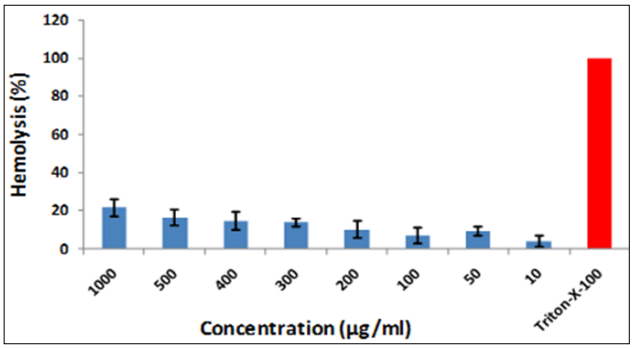

Fig 9 Hemolysis of EPS on human RBCs
Each value expressed as mean ± standard deviation (n = 3)

Hemolysis assay

The hemolytic activity of a representative EPS, in comparison to the known haemolytic agent, Triton X-100, was measured as an indication of its effect on the mammalian cell membrane (Fig 9). This *in vitro* assay is an indicator of red blood cell lysis and evaluates the hemoglobin released in the plasma spectrophotometrically at 540 nm, after exposure to the test EPS. An increase in absorbance at this wavelength is therefore, indicative of increased hemolysis and toxicity. In this assay, the EPS showed negligible hemolytic activity compared to Triton X-100. As the EPS has negligible toxicity on human RBCs, it can be further explored for many applications such as food processing, antioxidant, antibacterial and anticancer agents. Treatment with Triton X-100 and phosphate buffered saline were used as positive and negative controls respectively.

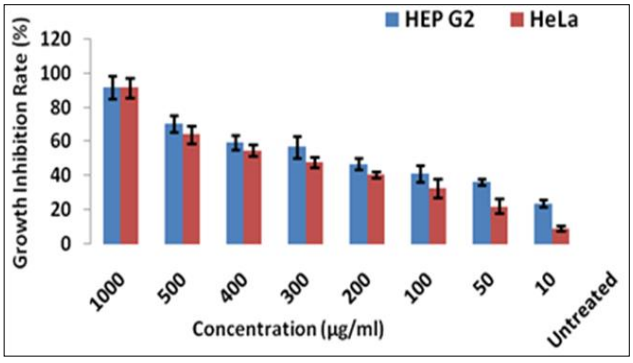

Fig 10 Cytotoxicity profiles of EPS against $HEPG_2$ and HeLa cells
Each value expressed as mean ± standard deviation (n = 3)

Cytotoxicity studies by MTT assay

As shown in (Fig 10), the inhibitory effects of partially purified EPS on HepG-2 and HeLa cells significantly increased with increasing concentration. At the highest tested concentration (1000 µg/mL), the inhibitory effects of EPS increased to 91.6 ± 6.5% and 91.5 ± 5.8% for HepG-2 and HeLa, respectively. These results indicated that EPS from *Cronobacter muytjensii* ATCC 51329(T) has significant anticancer activities against HepG-2 and HeLa cells.

Antitumor activity of EPS produced by endophytic bacteria *Bacillus amyloliquefaciens* was studied against gastric carcinoma cell lines (MC-4 and SGC-7901) by 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT assay. Tumor cells were treated with different concentrations of EPS (14, 22 and 30 µg/µl), and the results revealed that the exopolysaccharides displayed concentration-dependent inhibitory effects against the MC-4 and SGC-7901 tumour cells, with an IC50 of 19.7 and 26.8 µg/µl, respectively [48]. [49] studied the antitumor activity of EPS (BMEPS) from *Bacillus marinus*against human breast adenocarcinoma cell line (MCF-7) and human alveolar basal epithelial cell line (A-549) by MTT assay. MCF-7 cells showed maximum viability 82% at 12.5 µg/mL of EPS concentration and minimum 55% viability at 100 µg/mL of EPS concentration; and the calculated IC50 for cell line MCF-7 was 118.0µg/mL for BMEPS. The cell line A-549 cells showed 88% viability with 12.5µg/mL and minimum 63% viability at 100µg/mL of EPS. The calculated IC50 values–indicating low antitumor affinity to be 220µg/mL for EPS showed a weak cytotoxic activity [50] reported that the apoptosis induced by EPSAH from *Aphanothece halaphytica*. Exposure of EPS is associated with an up-regulation of Bax and simultaneous down regulation of Bcl-2 proteins, eventually leading to an increase in the ratio of Bax/Bcl-2 proteins which plays very important role in apoptosis.

CONCLUSION

EPS produced from present organism *Cronobacter muytjensii* found to be potent agent in different industrial applications such as in medical as anticancer, antioxidant, antimicrobial, and hemolytic agent. FTIR spectrum showed that the obtained EPS is heteropolymeric in nature.

Acknowledgements

The author is grateful to the INSPIRE DST (Department of Science and Technology) fellowship, New Delhi for providing financial support as INSPIRE fellowship. The author is also thankful to Yogeshwari Mahavidyalaya, Ambajogai, Maharashtra.

LITERATURE CITED

1. Orsod M, Joseph M, Huyop F. 2012. Characterization of exopolysaccharides produced by *Bacillus cereus* and *Brachybacterium Sp.* isolated from Asian sea bass (LatesCalcarifer). *Malaysian Journal of Microbiology* 8(3): 170-174.
2. Sutherland IW. 1997. Microbial biofilm exopolysaccharides - superglues or Velcro? *In*: (Eds) Wimpenny JWT, Handley P, Gilbert P, Lappin-Scott HM, Jones MV. *Biofilms: Community Interactions and Control*. Bioline Publications, Cardiff. pp 33-39.
3. El-Awady ME, Eldin MAN, Heba M, Ibrahim HM, Bahnasy ME, Aziz SHA. 2019. In vitro evaluation of antioxidant, anticancer, and antiviral activities of exopolysaccharide from *Streptomyces hirsutus NRC2018*. *Journal of Applied Pharmaceutical Science* 9(11): 010-018.
4. Ledezma OEV, M´endez, HIP, Manjarrez, LAM, Cano ETQ, Alvarez NM, Lopez-Luna A. 2016. Characterization of extracellular polymeric substances (EPS) produced by marine *Micromonospora sp*. *Jr. Chem. Pharmaceut. Res*. 8(6): 442-451.
5. Razack SA, Velayutham V, Thangavelu V. 2013. Medium optimization for the production of exopolysaccharide by *Bacillus subtilis* using synthetic sources and agro wastes. *Turkish Journal of Biology* 37: 280-288.
6. Naseem H, Bano A. 2014. Role of plant growth-promoting *rhizobacteria* and their exopolysaccharide in drought tolerance of maize. *Journal of Plant Interactions* 9(1): 689-701.
7. Mane GG, Hamde VS. 2015. Optimization of *Cronobacterdublinensis subsp. dublinensis*DES187(T) isolated from root nodules of Soybean for exopolysaccharide production. *International Journal of Global Science Research* 2(4): 265-270.
8. Freitas F, Alves VD, Reis MAM. 2011. Advances in bacterial exopolysaccharides: from production to biotechnological applications. *Trends in Biotechnology* 29: 388-398.
9. Castellane TCL, Otoboni AMMB, Lemos EDM. 2015. Characterization of Exopolysaccharides Produced by *Rhizobia Species*. *R. Bras. Ci*. 39: 1566-1575.
10. Liang TW, Tseng SC, Wang SL. 2016. Production and characterization of antioxidant properties of exopolysaccharide(s) from *Peanibacillusmucilaginosus*TKU032. *Marine Drugs*14(40): doi: 10.3390/md14020040.
11. Patel S, Goyal A. 2010. Isolation, characterization and mutagenesis of exopolysaccharide synthesizing new strains of lactic acid bacteria. *The Internet Journal of Microbiology* 8(1): 1-10.
12. Ye S, Liu F, Wang J, Wang H, Zhang M. 2012. Antioxidant activities of an exopolysaccharide isolated and purified from marine PseudomonasPF-6. *Carbohydr. Polym*. 87: 764-770.
13. Sivakumar T, Narayani SS, Shankar T, Vijayabaskar P. 2012. Optimization of cultural conditions for exopolysaccharides production by *Frateuriaaurentia*. *International Journal of Applied Biology and Pharmaceutical Technology* 3(3): 133-143.
14. Saidi S, Chebil S, Gtari M, Mhamdi R. 2013. Characterization of root nodule bacteria isolated from *Viciafaba* and selection of plant growth promoting isolates. *World Jr. Microbiol. Biotechnology*. DOI: 10, 1007/S1 1274-1278-4.
15. Mane GG, Hamde VS. 2018. Isolation and identification of Exopolysaccharide producing Cronobacter species from root nodules of leguminous plants. *Journal of Biochemical Technology* 9(2): 1-6.
16. Surjit SD, Rupa G. 2014. Beneficial properties, colonization, establishment and molecular diversity of endophytic bacteria in legumes and non-legumes. *African Journal of Microbiology Research* 8: 1562-1572.
17. Kucerova E, Joseph S, Forsythe S. 2011. The *Cronobacter* genus: ubiquity and diversity. *Quality Assurance and Safety of Crops and Foods* 3: 104-122.
18. Schmid M, Iversen C, Gontia I, Stephan R, Hofmann A, Hartmann A, Jha B, Eberl L, Riedel K, Lehner A. 2009. Evidence for a plant-associated natural habitat for *Cronobacter spp*. *Research in Microbiology* 160: 608-614.
19. Larry R Beuchat LR, Kim H, Gurtler JB, Lin LC, Ryu JH, Richards GM. 2009. Cronobacter sakazakii in foods and factors affecting its survival, growth, and inactivation. *Int. Jr. Food Microbiology* 136(2): 204-213.
20. Scheepe-Leberkuhne M, Wagner F. 1986. Optimization and preliminary characterization of an exopolysaccharide synthesized by *Enterobactersakazakii*. *Biotechnology Letter* 8: 695-700.

21. Duta FP, Franca FP de, Lopes LM de A. 2006. Optimization of culture conditions for exopolysaccharides production in *Rhizobium sp*. using the response surface method. *Electronic Journal of Biotechnology* 9(4): 391-399.
22. Timilsena YP, Adhikari R, Kasapis S, Adhikari B. 2015. Molecular and functional characteristics of purified gum from Australian chia seeds *Carbohydrate Polymers* http://dx.doi.org/10.1016/j.carbpol.2015.09.035.
23. Nirmala P, Aysha OS, Valli S, Reena A, Kumar PV. 2011. Production of extracellular polysaccharides by a *Rhizobium species* from root nodules of *Vigna mungo* (Hepper). *International journal of pharmaceutical and Biological Archives* 2(4): 1209-1214.
24. Gharzauli R, Benahmed A, Benhizia Y, Benguedouar A. 2012. Influence of carbon sources on the production of exopolysaccharide by *Rhizobium sullae* and on the nodulation of *Hedysarumcoronarium L*. legume. *African Journal of Microbiology Research* 6(30): 5940-5946.
25. Qiang L, Yumei L, Sheng H, Yingzi L, Dongxue S, Dake H, Jiajia W, Yanhong Q, Yuxia Z. 2013. Optimization of fermentation conditions and properties of an exopolysaccharide from *Klebsiella sp*. H-207 and Application in Adsorption of *Hexavalent Chromium*. *Plos One* 8: e53542.
26. Sato K, Okubo A, Yamazaki S. 1998. Optimization of derivatization with 2-aminobenzoic acid for determination of monosaccharide composition by capillary electrophoresis. *Analytical Biochemistry* 262: 195-198.
27. Saravanan C, Shetty PK. 2015. Isolation and characterization of exopolysaccharide from *Leuconostoclactis* KC117496 isolated from idli batter. *International Journal of Biological Macromolecules* 90: 100-106.
28. Rashid F, Hussain S, Ahmed Z. 2017. Extraction purification and characterization of galactomannan from fenugreek for industrial utilization. *Carbohydrate polymer* DOI: https://doi.org/10.1016/j.carbpol.2017.10.025.
29. Reddy CK, Suriya M, Haripriya S. 2013. Physico-chemical and functional properties of resistant starch prepared from red kidney beans (*Phaseolus vulgaris* L) starch by enzymatic method. *Carbohydrate Polymers* 95: 220-226.
30. Cameron DR, Cooper DG, Neufeld RJ. 1988. The mannoprotein of *Saccharomyces cerevisiae* is an effective bioemulsifier. *Applied and Environmental Microbiology* 54(6): 1420-1425.
31. Kanamarlapudi SLRK, Muddada S. 2017. Characterization of exopolysaccharide produced by *Streptococcus thermophilus CC30*. *BioMed Research International* Article ID 4201809. pp 11.
32. Blois MS. 1958. Antioxidant determinations by the use of a stable free radical. *Nature* 181: 1199-1200.
33. Singh R, Shushni MAM, Belkheir A. 2015. Antibacterial and antioxidant activities of *Menthapiperita* L. *Arabian Journal of Chemistry* 8: 322-328.
34. Chouly C, Colquhoun IJ, Jodelet A, York G, Walker GC. 1995. NMR studies of succinoglycan repeating-unit octasaccharides from Rhizobium meliloti and Agrobacterium radiobacter. *Inter. Jr. Biol. Macromology* 17: 357-363.
35. Jeong SM, Kang BW, Park JU, Kim MJ, Lee HH, Choi YH, Jeong YK. 2011. Biochemical characterization of the exopolysaccharide purified from *Laetiporussulphureus. Mycelia Jr. of Microb. and Biotechnology* 21(12): 1287-1293.
36. Navarini L, Abatangelo A, Bertocchi C, Conti E, Bosco M, Picotti F. 2001. Isolation and characterization of the exopolysaccharide produced by Streptococcus thermophilus SFi20. *International Journal of Biological Macromolecules* 28(3): 219-226.
37. Ahmed Z, Wang Y, Anjum N, Ahmad A, Khan ST. 2013. Characterization of exopolysaccharide produced by *Lactobacillus kefiranofaciens ZW3* isolated from Tibet kefir e Part II. *Food Hydrocolloids* 30(1): 343-350.
38. Maina NH, Tenkanen M, Maaheimo M, Juvonen MR, Virkki L. 2008. NMR spectroscopic analysis of exopolysaccharides produced by *Leuconostoccitreum* and *Weissella confuse*. *Carbohydrate Research* 343: 1446-1455.
39. Chowdhury RS, Basak R, Sen R, Adhikaria B. 2011. Optimization, dynamics, and enhanced production of a free radical scavenging extracellular polysaccharide (EPS) from hydrodynamic sediment attached *Bacillus megaterium RB-05*. *Carbohydrate Polymers* 86: 1327-1335.
40. Ramasamy S, Prabhakaran P, Chandran P. 2014. Characterization and optimization of EPS-producing and diesel oil-degrading *Ochrobactrumanthropi* MP3 isolated from refinery wastewater. *Petroleum Science* 11: 439-445.
41. Ashtaputre AA, Shah AK. 1995. Emulsifying property of a viscous exopolysaccharide from *Sphingomonaspaucimobilis*. *World Journal of Microbiology and Biotechnology* 11: 212-219.
42. Lee SE, Hwang HJ, Ha JS, Jeong HS, Kim JH. 2003. Screening of medicinal plant extracts for antioxidant activity. *Life Sciences* 73: 167-179.
43. Shimada K, Fujikawa K, Yahara K, Nakamura T. 1992. Antioxidative properties of xanthan on the autoxidation of soybean oil in cyclodextrin emulsion. *Journal of Agricultural and Food Chemistry* 40: 945-948.
44. Abe J, Berk BC. 1998. Reactive oxygen species as mediators of signal transduction in cardiovascular disease. *Trends in cardiovascular medicine* 8: 59-64.
45. Chance B, Sies H, Boveris A. 1979. Hydroperoxide metabolism in mammalian organs. *Physiological Reviews* 59: 527-605.
46. Jahanbina K, Goharib AR, Moinic S, Emam-Djomehc Z, Masid P. 2011. Isolation, structural characterization and antioxidant activity of a new water-soluble polysaccharide from *Acanthophyllumbracteatum* roots. *International Journal of Biological Macromolecules* 49: 567-572.
47. Majolagbe ON, Oloke JK, Adebayo EA, Adewoyin AG, Ayandele A, Bamigboye CO. 2013. Study on the antibacterial activity of exopolysaccharides of *lentinussubnudus* using swiss albino rats as animal model. *American-Eurasian Journal of Scientific Research* 8(1): 47-52.
48. Chen J, Qin G, Wang J, Yu J, Shen B, Li S, Ren Y, Zuo L, Shen W, Das B. 2013. One-step fabrication of sub-10-nm plasmonic nanogaps for reliable SERS sensing of microorganisms. *Biosensors and Bioelectronics* 44: 191-197.
49. Sayed OHE, Kader AE, El-Sayed M, Salem HM, Mahmoud MG, Asker MS, Mohamed SS. 2015. Isolation, characterization and biological activities of exopolysaccharide produced by *Bacillus marinus*. *Der Pharma Chemica* 7(2): 200-208.
50. Ou Y, Xu S, Zhu D, Yang X. 2014. Molecular mechanisms of exopolysaccharide from *Aphanothecehalaphytica* (EPSAH) induced apoptosis in HeLa cells. *PLoS One* 9: e872232014.