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Production of exopolysaccharides from novel marine bacteria and anticancer activity against hepatocellular carcinoma cells (HepG2)

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Abstract

Background: The aim of the current study based on the production and characterization of exopolysaccharides (EPSs) isolated from marine sediment of the Mediterranean and Red Seas is to study its cytotoxic activity against HepG2 cells.

Results: Eleven isolates have the ability to produce EPSs and also decreased the viability of HepG2 cell line in different manners. The five most promising isolates that produce high yield of EPSs and high cytotoxicity were identified by 16S RNA as *Brevundimonas subvibrioides* MSA1, *Bacillus thuringiensis* E4, *Bacillus amyloliquefaciens* MGA2, *Pseudomonas fluorescens* SGA3, and *Advenella Kashmirensis* NRC-7. The chemical composition of the following EPSs (M1, M3, M6, M15, M19, E2, E4, E10, S5, S7, and S11) demonstrates that they are acidic sulfated heteropolysaccharides with different relative ratios of monosugars of glucose, mannose, galactose, glucuronic acid, and mannouronic acid. The average molecular weights from 1.94×10^4 to 7.95×10^5 g/mol and the number average molecular weight from 1.51×10^4 to 7.53×10^5 g/mol. FTIR spectrum of the five EPSs indicated the presence of sulfate and carboxylic groups in different percentages.

Conclusions: The EPSs produced from marine bacteria are very promising for treating the HepG2 cells.

Keywords: Exopolysaccharides, Marine bacteria, Cytotoxicity, Hepatocellular carcinoma cells

Background

Essential liver tumor is the fifth most normal threat on the planet, with a worldwide yearly occurrence of around one million new patients (Shah and Bhowmick 2006). The malady wins in parts of Asia and Africa, yet seems overflowing in numerous European nations lately. As need be, it is encouraged to investigate another approach for improvement of a viable treatment against this illness. In spite of the fact that chemotherapy is one of the compelling medication strategies against liver cancer, chemotherapeutic operator prompts serious unfavorable impacts while murdering tumor cells (Drake and

Antonarakis 2010). At that point, it is extremely essential to discover novel bacteria hostile to tumor specialists with high natural exercises and low danger to host. Natural products have been the pillar of tumor chemotherapy for as far back as 30 years and are probably going to give a considerable lot of the lead structures, and these will be utilized as layouts for the development of novel compounds with upgraded organic properties (Wani et al. 1971). The marine condition is a rich wellspring of remarkable bioactive compounds, for example, exopolysaccharides (EPSs) from microorganisms with boundless chemical and functional varieties. Pretty much 30,000 regular items have been segregated from marine organisms, and a few of the medication competitors are right now in clinical trials. Marine microorganisms frequently deliver bioactive substances

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with novel capacities and structures in view of their uncommon living conditions (Fenical 1993). EPSs from marine microorganisms are important to new medication disclosure (Miranda et al. 2008; Xu et al. 2009; Casillo et al. 2018). EPSs have different biological activities with low poisonous quality and have pulled in broad considerations of researchers (Huang et al. 2012; Wang et al. 2014; Cai et al. 2017). As of late, various investigations on the mechanism of action of EPSs have exhibited that EPSs could repress the tumor development in vivo for their immunomodulatory activities. They apply hostility to tumor movement by boosting the host's common insusceptible resistance. Along these lines in this investigation, different EPSs segregated from various marine bacteria were tried for their capacity on liver malignancy cell line (Yang et al. 2013; Peng et al. 2014; Yang et al. 2014). The present study aimed to isolate and identify new marine bacterial strains that produced EPSs. The cytotoxic effects of these crude EPSs were tested against hepatocellular carcinoma (HepG2) cells.

Methods

Sampling

Three samples were collected from marine sediment at different locations from (Mediterranean Sea) Sidi Bishr beach at Alexandria, (Red Sea) Marsa-Alam (rhizosphere around the mangrove tree) areas, and El-Ain El-Sokhna beach in September 2014.

Isolation of bacteria

The collected samples (10 g) were placed in 100 mL of sterile seawater and homogenized by shaking at 200 rpm for 15 min, and a serial dilution was performed (Hayakawa and Nonomura 1987). Finally, 50 μL of the supernatant of each dilution was inoculated on marine nutrient agar, and the plates were incubated at 37 °C for 24 h. The colonies that appeared per plate of each sample were subjected to purification. Isolated microorganisms showing mucous growth on these media were further screened for EPS production by inoculation into shake flasks.

Screening for production of EPSs

Isolates were screened for production of EPSs in a different liquid media which were composed of the following ingredients (g/L): peptone 4.0, yeast extract 2.0, and sucrose 20 (Jiang et al. 1999). The ingredients were dissolved in 750 mL seawater. After adjusting the pH, the final volume was completed up to 1 L with distilled water. After incubation at 37 °C for 3 days, the culture medium was centrifuged at 5000 rpm for 20 min (Sigma Laborzentrifugen, 2 K 15) to remove bacterial cells. Trichloroacetic acid (5%) was added and left overnight

at 4 °C and centrifuged at 5000 rpm again. The pH of the clear solution was adjusted to 7.0 with NaOH solution and dialyzed three times against distilled water. The supernatant was completed to four volumes with absolute ethanol and left overnight at 4 °C. The precipitated polysaccharides were separated by centrifugation at 5000 rpm, washed twice with acetone, dehydrated by ether, and finally dried under vacuum at 40 °C (Shene et al. 2008).

Cytotoxic effects of isolated EPSs on HepG2 cells

Cell propagation and maintenance

Hepatocellular carcinoma HepG2 cells were purchased from ATCC (American Type Culture Collection) and maintained in the proper conditions. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Belgium) supplemented by 10% fetal bovine serum, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate at 37 °C in a humidified incubator with 5% CO_2. The cells were harvested after trypsinization (0.025% trypsin and 0.02% EDTA) and washed twice with Dulbecco's phosphate-buffered saline (DPBS). When the cell density reached approximately 80%, cells were split for further culture. The experiments were made up when the cells were in the logarithmic growth phase.

Cytotoxicity assay

Cell viability was measured by neutral red uptake assay (Repetto et al. 2008). The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes. The cells were incubated with various concentrations of the test compounds (125, 250, 500, and 1000 μg/mL) for 48 h at a cell density of 10^4 cells/well of a 96-well plate. Using the relation between used concentrations and neutral red intensity value, IC_{50} of tested compounds was calculated (Repetto et al. 2008). Four parameter equation logistic curve was used (log concentration vs. percentage cell growth as compared to control cells). For the untreated cells (negative control), medium was added instead of the test compounds. A positive control doxorubicin (Mr = 579.9) was used as cytotoxic natural agent giving 100% inhibition. Dimethyl sulfoxide (DMSO) was the vehicle used for dissolution of tested compound, and its final concentration on the cells was less than 0.2%. All tests and analyses were done in triplicate, and the results were averaged.

Identification of promising isolates

The promising isolates which produced high amounts of EPS were identified based on biochemical, morphological, and physiological characteristics of the potential

producer as determined by adopting standard methods (Cappucino and Sherman 2004). The strains were confirmed with 16S ribosomal RNA gene sequence and compared with other bacterial sequences by using NCBI BLAST. Taxonomic affiliation of the sequences was retrieved from classifier program of ribosomal database project (RDP-II) (Maidak et al. 1997; Tamura et al. 2007). RDP-II hierarchy is based on the new phylogenetically consistent higher order bacterial taxonomy.

Production, separation, and purification of EPSs
The EPSs were produced from the five strains *Brevundimonas subvibrioides* MSA1, *Bacillus thuringiensis* E4, *Bacillus amyloliquefaciens* MGA2, *Pseudomonas fluorescens* SGA3, and *Advenella Kashmirensis* NRC-7. Therefore, the crude EPS was precipitated with 1,2,3,4-volumes absolute ethanol, and supernatant was recovered by centrifugation. EPS fractions named MSA1, E4, MGA2, SGA3, and NRC7 were obtained from the supernatant mentioned above by precipitating with ethanol. The main fractions were collected, dialyzed, dried, and used for further analysis.

Chemical structure of EPSs
Analysis of chemical composition
The EPS samples (30 mg) were hydrolyzed with 88% HCOOH (5 mL) at 100 °C in a sealed tube for 5 h. Excess acid was removed by flash evaporation on a water bath at a temperature of 40 °C and co-distilled with water (1 mL × 3) (Sudhamani et al. 2004). Then, the hydrolyzed monosugars were extracted with absolute ethanol. The purified hydrolysis monosugars were analyzed by HPLC (Agilate Pack, serics1, 200), equipped with Aminex carbohydrate HP-87C column (300 × 7.8 mm). Deionized water was used as the mobile phase at flow rate 1 mL/min. Chromatographic peaks were identified by comparing the retention times with the respective retention times of known standard reference material. Retention time and peak area were used to calculate sugar concentration by the data analysis of Agilat Packard (Randall et al. 1989). Total sugar content was determined by the phenol-H_2SO_4 method using glucose as

the standard (Dubois et al. 1956). Protein content was measured by the method of Bradford (1976). Sulfate ester content was measured using the turbidimetric method with sodium sulfate as standard (Therho and Hartiala 1971). Uronic acid content was determined by the carbazole-H_2SO_4 method using glucouronic acid as standard (Bitter and Muir 1962).

Fourier transform infrared spectroscopy (FTIR)
Fourier transform infrared was obtained by grinding a mixture of EPS sample with dry KBr and pressing in a mold. An IR spectrum was recorded on a Fourier transform infrared spectrophotometer Brucker Scientific 500-IR (Ray 2006).

Average molecular weight determination
The average molecular weight (Mw) of the EPSs was determined by high-performance liquid chromatography (HPLC, Agilent 1100 Series System, Hewlett-Packard, Germany) with refractive index (RI) detection. The EPS (10 mg) was dissolved in 2 mL of solvent and filtrated by filter 0.45, then the EPS solution was put in a GPC device (Jun et al. 2009). The polydispersity index (PI) was calculated from the Mw/Mn ratio (You et al. 2013). Number average molecular weight (Mn) and weight average molecular weight (Mw) were directly calculated according to the definition of Mn and Mw using molecular weight and RI signal values at each elution volume (You et al. 2013).

Results
Screening for production of EPSs
Twenty isolates of marine bacteria were isolated from Marsa-Alam, El-Ain El-Sokhna, and Sidi Bishr. The bacterial isolates gathered from different marine sediments and displaying mucous morphology on marine supplement agar medium were inoculated into 50 mL of nutrient broth under shaking. Marine bacterial isolates were screened for their ability to deliver EPS (Table 1). The most elevated yield of EPS (7.5, 8.2, 8.1, 7.6, 8.2, 9.0, 8.0, 8.5, 9.2, 7.9, 7.3, 8.0, and 9.1 g/L) was acquired by

Table 1 Production of EPS from isolates

Location	Code	EPS (g/L)	Location	Code	EPS (g/L)	Location	Code	EPS (g/L)
Marsa-Alam	M1	7.5	El-Ain El-Sokhna	E2	8.0	Sidi Bishr	S5	7.3
	M3	8.2		E4	8.5		S7	5.8
	M6	8.1		E8	4.8		S9	6.4
	M13	7.5		E10	9.2		S11	3.8
	M15	7.6		E14	4.5		S12	8.0
	M18	8.2		E16	5.8		S17	9.1
	M19	9.0		E20	7.9			

isolates M1, M3, M6, M15, M18, M19, E2, E4, E10, E20, S5, S12, and S17, separately.

In vitro cytotoxicity on HepG2 cell line

The EPSs from 20 isolates have been examined for their anticancer activity against hepatocellular carcinoma HepG2 cell line. This was done using the ability of viable cells to incorporate and bind the survival dye neutral red

in lysosomes. In an essential screening test, EPS (M1, M3, M6, M15, M19, E2, E4, E10, S5, S7, and S11) decreased the viability of HepG2 cell line in concentrations 125, 250, 500, and 1000 μg/mL (Fig. 1a–d and Table 2).

Identification of promising isolates

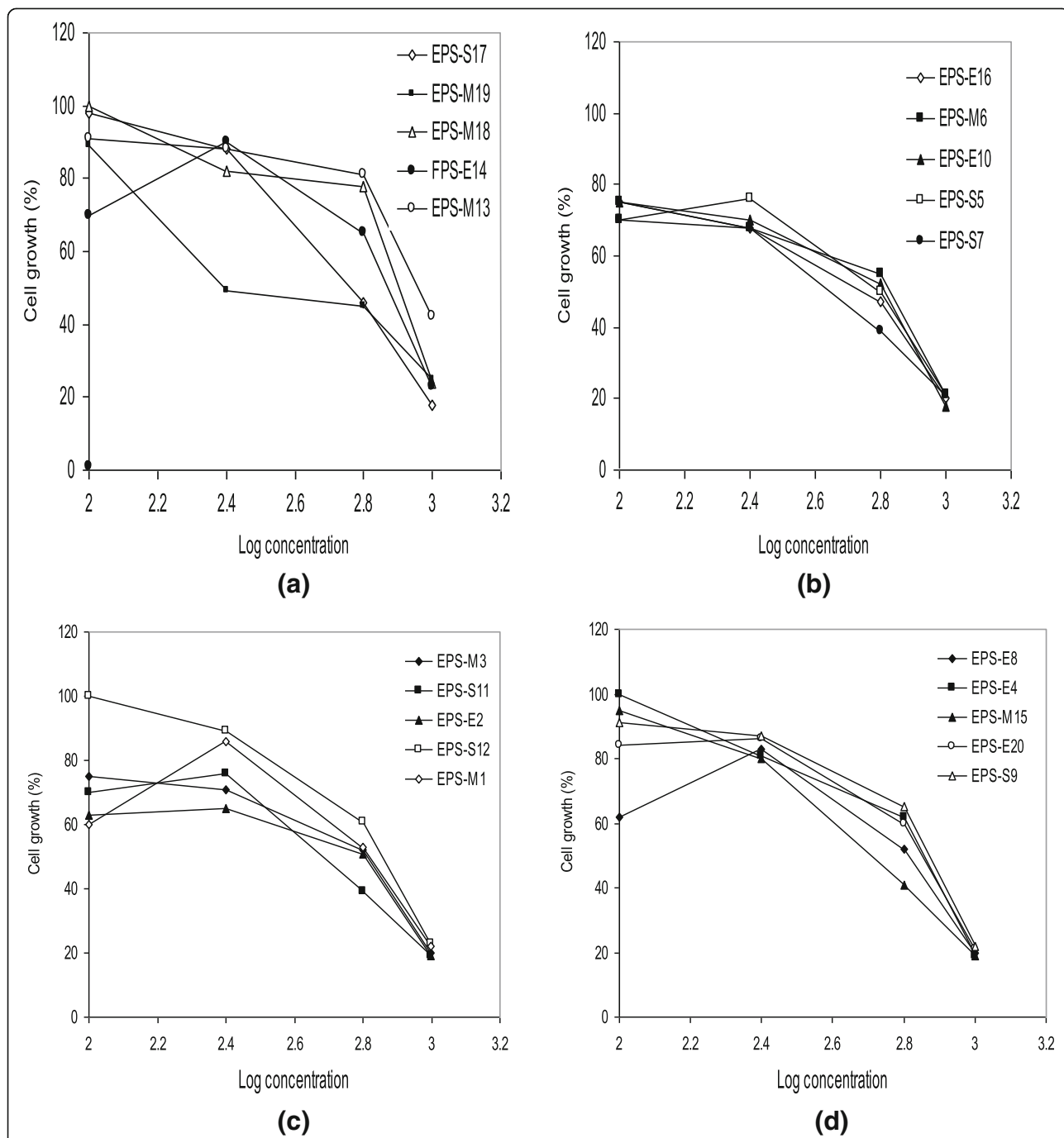The promising isolates which give high yield of EPSs and cytotoxicity against HepG2 (M19, E4, M6, E2, S7) were

Fig. 1 Effects of EPSs **a** S17, M19, M18, E14, and M13; **b** E16, M6, E10, S5, and S7; **c** M3, S11, E2, S12, and M1; and **d** E8, E4, M15, E20, and S9 on HepG2 cell line at 48 h

Table 2 In vitro cytotoxicity on HepG2 cell line

Code	IC$_{50}$ (µg/mL)	Code	IC$_{50}$ (µg/mL)	Code	IC$_{50}$ (µg/mL)
M1	524.0	E2	537.0	S5	436.0
M3	549.0	E4	630.9	S7	389.0
M6	562.0	E8	630.0	S9	676.0
M13	891.0	E10	549.0	S11	407.0
M15	501.0	E14	660.0	S12	707.0
M18	724.0	E16	467.0	S17	467.0
M19	309.0	E20	640.0	Drug	2.1

Table 4 Molecular weights and polydispersity of EPS

EPSs	Weight average molecular weight (*Mw*)	Number average molecular weight (*Mn*)	Polydispersity (*PI*) (*Mw/Mn*)
MSA1	2.51×10^4	2.13×10^4	1.18
E4	1.94×10^4	1.51×10^4	1.28
MGA2	1.18×10^5	1.05×10^5	1.12
SGA3	7.95×10^5	7.53×10^5	1.05
NRC7	2.48×10^4	2.32×10^4	1.07

distinguished in light of biochemical, morphological, and physiological characteristics (Table 3). The phylogenetic investigation of 16S rDNA exhibited that the bacteria had a place with the gamma subdivision of the Proteobacteria phylum and is firmly identified as *Brevundimonas subvibrioides* MSA1 (M19), *Bacillus thuringiensis* E4 (E4), *Bacillus amyloliquefaciens* MGA2 (M6), *Pseudomonas fluorescens* SGA3 (E2) and *Advenella Kashmirensis* NRC-7 (S7), with accession numbers KP064319, KP096416, KP064320, KP064321, and KM017000, separately.

Chemical structure of EPSs
Chemical analysis of EPS fractions
The yields of the EPSs from *Brevundimonas subvibrioides* MSA1, *Bacillus thuringiensis* E4, *Bacillus amyloliquefaciens* MGA2, *Pseudomonas fluorescens* SGA3, and *Advenella Kashmirensis* NRC-7 were 7.5, 8.5, 8.2, 9.0, and 5.8 g/L broth media, individually. The main fractions MGA2, MSA1, E4, SGA3, and NRC7 were purified by fraction with ethanol precipitation from the crude EPSs. The fractions MGA2, MSA1, E4, SGA3, and NRC7 were gathered for further investigation of structure and activity. MGA2, MSA1, E4, SGA3, and NRC7 had a negative response to the Bradford test. This outcomes demonstrate the absence of protein and/or nucleic acid. The average

molecular weights (*Mw*) and average molecular numbers (*Mn*) of MGA2, MSA1, E4, SGA3, and NRC7 were 2.51×10^4, 1.94×10^4, 1.18×10^5, 7.95×10^5, and 2.48×10^4 g/mol and 2.13×10^4, 1.51×10^4, 1.05×10^5, 7.53×10^5, and 2.32×10^4 g/mol, respectively (Table 4). Results from phenol-H_2SO_4 assay showed that MGA2, MSA1, E4, SGA3, and NRC7 contained 87.4, 90.2, 94.3, 97.5, and 97.1% carbohydrate; 1.9, 2.7, 8.6, 7.3, and 13.3% uronic acid; and 12.5, 9.6, 5.4, 2.1, and 2.8% sulfate, respectively (Table 5). These show that MGA2, MSA1, E4, SGA3, and NRC7 are acidic sulfated heteropolysaccharides. Concerning monosaccharide quantitative and qualitative determination, the use of HPLC is favored due to its sensitivity. MGA2, MSA1, E4, SGA3, and NRC7 are comprised of five distinct monosaccharides, including glucose, mannose, galactose, glucouronic acid, and mannouronic acid with molar ratios of 1.0:1.6:1.2:0.2:0.0; 2.3:1.0:0.0:0.2:0.0; 2.52:1.0:0.0:0.0:1.4; 3.6:0.0:1.0:0.5:0.4; and 1.0:2.5:0.0: 1.8:0.6, respectively (Table 5).

FTIR analysis of MGA2, MSA1, E4, SGA3, and NRC7
FTIR analysis of the EPS fractions MGA2, MSA1, E4, SGA3, and NRC7 has unmistakably demonstrated a number of peak characteristics of carbonyl compounds. The intensity of bands around 3431 cm^{-1} was assigned to υOH stretching frequency, and as expected, it was

Table 3 Morphology and biochemical characterization of promising isolates

Code	Gram staining	Shape form	Starch hydrolysis	V.P test	H$_2$S test	Citrate test	Indol test	Nitrate test
M1	Positive	Short rods	−	+	+	−	−	−
E2	Negative	Short rods	+	+	+	−	−	−
M3	Positive	Bacilli	+	−	−	+	−	−
E4	Positive	Short rods	+	−	+	−	−	−
S5	Positive	Bacilli	−	−	+	−	−	−
M6	Positive	Bacilli	+	+	−	+	−	−
S7	Positive	Short rods	−		+	+	−	+
E10	Positive	Bacilli	+	−	−	+	−	+
M15	Positive	Bacilli	−	+	+	−	−	+
M19	Positive	Bacilli	+	−	+	−	−	+
S11	Positive	Bacilli	+	−	−	+	−	+

Table 5 The uronic acid, sulfate percentage, and molar ratio of monosaccharides for MGA2, MSA1, E4, SGA3, and NRC7

EPS	Sulfate (%)	Uronic acid (%)	Molar ratio (MR)				
			Glucose	Mannose	Galactose	Glucouronic acid	Mannouronic acid
MSA1	12.5	1.9	1.0	1.6	1.2	0.2	0.0
E4	9.6	2.7	2.3	1.0	0.0	0.3	0.0
MGA2	5.4	8.6	2.5	1.0	0.0	0.0	1.4
SGA3	2.1	7.3	3.6	0.0	1.0	0.5	0.4
NRC7	2.8	13.3	1.0	2.5	0.0	1.8	0.6

broad. The weak intensity of band attributed to C–H group stretching ($\sim 2933\,cm^{-1}$) could also be noticed. Also, peaks at $1636\,cm^{-1}$ and $1428\,cm^{-1}$, for the asymmetric and symmetric stretching of carboxylate anionic groups, demonstrate the carboxyl groups in MGA2, MSA1, E4, SGA3, and NRC7. Absorption at $1429\,cm^{-1}$ was possibly due to non-symmetric CH_3 bending. The strong absorption at $1069\,cm^{-1}$ was dominated by the glycosidic linkage υ (C–O–C) stretching vibration contribution (Fig. 2a–f).

Discussion

Recently, EPSs have great potential as antitumor drug, as recent treatment strategies showed limitations of severe side effects and multidrug resistance occurred (Romanenko et al. 2008). Marine microbes have turned out to be well known and novel sources of EPSs, albeit numerous known marine bacteria can produce EPSs. Few of them are of biotechnological significance, so the search of EPSs that may have imaginative applications is still of potential intrigue (Casillo et al. 2018). While trying to get the marine bacterial strains, we separated from various areas and assessed based on the productivity of the EPSs. From the 20 marine EPSs, the promising IC_{50} of 11 EPSs (M1, M3, M6, M15, M19, E2, E4, E10, S5, S7, and S11) were 524.0, 549.0, 562.0, 501.0, 309.0, 537.0, 630.9, 549.0, 436.0, 389.0, and 407.0 μg/mL, individually. However, the remaining EPSs showed moderate anti-proliferative activities (Table 2). A few polysaccharides significantly inhibit the development of malignancy cells as illustrated by Huang (2013), while other polysaccharides demonstrated different functions. For instance, the polysaccharides from *Ganoderma lucidum* fundamentally decreased tumor cells in vitro (Zhao et al. 2010); on the other hand, polysaccharide from *Pleurotus* sp. mycelium demonstrated its antioxidant activities in vitro (Liu et al. 2010). Marine bacterial strain GWS-BW-H8hM was accounted for to hinder development of gastric cell line (HM02), liver cell line (HepG2), and bosom cell line (MCF7) (Bitzer et al. 2006). EPSs and sulfated polysaccharides from *Halomonas stenophila* sp. repressing hypersaline condition have likewise been accounted for their pro-apoptotic effects for T-leukemia cells (Ruiz-Ruiz et al. 2011; Xu et al. 2014). These show that MGA2, MSA1, E4, SGA3, and NRC7 are acidic sulfated heteropolysaccharides. MGA2, MSA1, E4, SGA3, and NRC7 are comprised of different monosaccharides including glucose, mannose, galactose, glucouronic acid, and mannouronic acid. A lot of marine bacteria are encompassed by EPSs, which may help bacterial groups to endure extremes of saltiness, temperature, and nutrient availability (Casillo et al. 2018). In view of the chemical and rheological properties of the EPSs generated by these bacteria, examinations were performed to test their potential applications in biotechnology and ecological protection (Nichols et al. 2005; Guezennec 2002). The item and nature of bacterial EPSs are exceptionally affected by the biological and dietary status (Kumar et al. 2007). Most EPSs created by marine microbes are heteropolysaccharides containing diverse unit of monosugars coordinated in a range of around 10 to compose repeating units (Decho 1990). For the most part, EPSs are straight, with molecular weight from 1 to 3×10^5 Da (Sutherland 1977). Numerous EPSs are neutral molecules, but the greater parts are polyanionic for the SO_3^-, PO_4^-, and uronic acid presence. The physical properties of EPSs are incredibly influenced by the method for the monosugars arranged and the accumulation of the one polymer chain (Vanhooren and Vandamme 1998). Biological activities of EPSs are depending on its chemical structure and the molecular weight. The presence of rate sugars as ribose and arabinose besides the uronic acid in the order of polygalacturonic, glucouronic, galacturonic acid, and polymers of low molecular weight are important indicators reflecting the antioxidant activity of the EPSs (Roca et al. 2015). Furthermore, in the FTIR spectrum of MGA2, MSA1, E4, SGA3, and NRC7, the characteristic band at $929\,cm^{-1}$ belonged to the β-anomeric configuration in MGA2, SGA3, and NRC7 (Synytsya et al. 2003; Zhbankov et al. 1997), and the band at $840\,cm^{-1}$ was ascribed to α-pyranoses in the MSA1 and E4 (Park 1971). Besides, absorption at $1271\,cm^{-1}$ was assigned to the stretching vibration of S=O indicating MGA2, MSA1, E4, SGA3, and NRC7 were sulfated polysaccharides (Percival and Wold 1963).
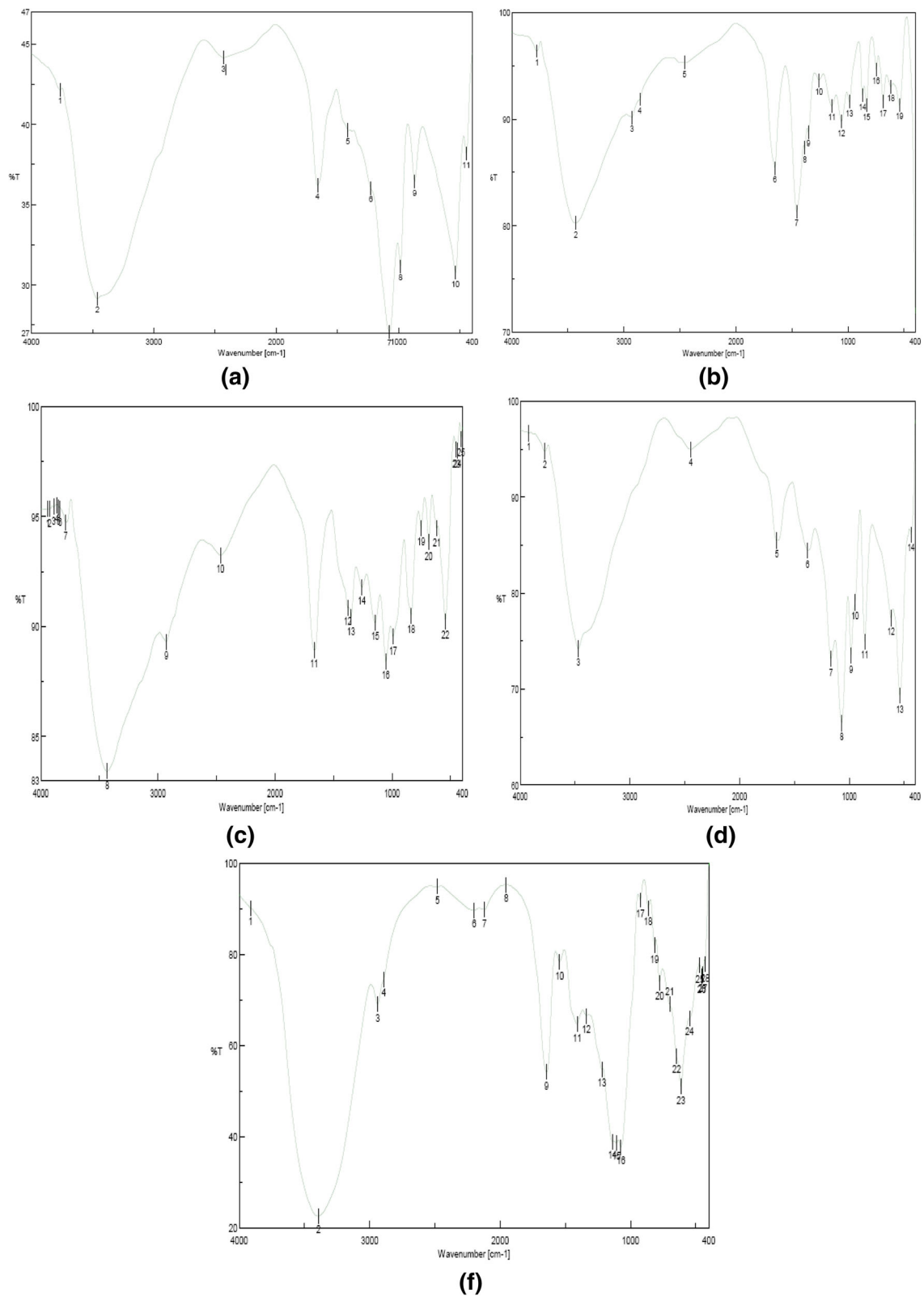
Fig. 2 Infrared of EPSs **a** MGA1, **b** MGA2, **c** E4, **d** SGA3, and **f** NRC7

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Conclusion

EPSs produced from marine bacteria are very promising for treating the hepatocellular carcinoma HepG2 cells.

Availability of data and materials
All data and material are available.

Authors' contributions
All the participant researchers contributed to do this work. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Consent for publication
All the participant researchers are consent for publication.

Competing interests
The authors declare that they have no competing interests.

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