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Isolation, characterization and biological activities of exopolysaccharide produced by *Bacillus marinus*

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ABSTRACT

The exopolysaccharide from Bacillus marinus is of study interest due to its wide spectrum bioactivity and high production (8.2g/L), so the strain isolated and identified based on morphological and characteristics. The EPS was obtained through the ethanol precipitation from fermentation broth and fractionated by anion-exchange and gel-filtration chromatography. The main fraction of EPS (BMEPS) was determined as homogenous with a molecular weight of 500 kDa. Its structural characteristics were investigated and elucidated by periodate oxidation, Smith degradation, HPLC, FT-IR. It was contained sulfate group (20.2%) and composed of glucose and glucouronic acid in a molar ratio of 3:1, respectively. Small amount of glycerol and large amount of liberated erythritol partially prove the presence of the β-(1−4) linkages between the monosaccharides in the backbone of the polysaccharide. The chemical analysis showed that the proposal structure could be as [Glc-β-(1−4)-Glc-β-(1−4)-Glc.A]$_n$. The biological activities of crude EPS and BMEPS as antiviral against MDCK and H1N1 virus and antioxidant were done. In addition, the antitumor against human breast adenocarcinoma cell line (MCF-7) and human alveolar basal epithelial cell line (A-549) was evaluated by MTT assay. The results suggested that F-6 could be considered as an effective natural antiviral, antioxidant and antitumor sources.

Keywords: *Bacillus marinus*, antiviral, antioxidant, antitumor, characterization

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INDRODUCTION

Marine microorganisms often produce exopolysaccharides (EPSs) with novel structures and diverse biological activities due to their specific marine environment [4, 50]. They have been used as an important class of bioactive natural products in the biochemical and medical applications due to their specific biological activities such as antioxidant activity [33], immunostimulating effects [59], antitumor effects [53] and antiviral activity [55]. In fact, most deep sea bacteria isolated were examined to produce EPSs under these restricted growth conditions [20]. There has been a growing interest in isolating new bacteria producing EPSs from marine environments [4]. Many marine bacterial EPSs with unique chemical compositions, structures and properties have been found to be suitable for potential applications [12, 15]. Analysis of EPS has shown, they have high antioxidant activity, and this may explain the pharmacological basis of polysaccharide prevention of anile, inflammation and atherosclerosis [63]. Cancer remains leading cause of death globally. IARC [28] recently estimated that 7.6 million deaths worldwide. Due to cancer with 12.7 million new cases per year were being reported worldwide. A significant proportion of this burden is borne by developing countries; 63% of cancer deaths are reported to be from developing countries. Cancer is a multigenic and multicellular disease that can arise from all cell types and organs with a multi-factorial etiology [18, 30]. The clinical treatment methods against cancer include: surgery, which is local excision of tumor; radiotherapy, which eliminate tumor by exposing to radiation; chemotherapy, which relies on drug targeting tumor cells; combined modality therapy, which includes all three former treatments together; and immunotherapy, which evokes an immune

response against tumor. Most tumor treatments incur side effects like complications and toxicity, thus patients have to suffer from the pain of treatments. The aim of the present study was to clarify the chemical characterization of exopolysccharide from marine *Bacillus marinus* and evaluate its biological activities

MATERIALS AND METHODS

Microorganism

Bacillus marinus was isolated from marine sediments of (Al-mangrove tree in Mars alm) Egypt using nutrient broth and maintained on nutrient agar slants containing (g/L): peptone 10, yeast extract 10, NaCl 5, Raffinose 20. Incubation was at 37°C for 48 h. The isolate was identified on the basis of morphological and biochemical characteristics [35].

Isolation, purification and fractionation of exopolysaccharide

A *Bacillus marinus was* grown aerobically for 96 h in a production medium at 37ºC and 150 rpm on a rotary shaker [48]. After incubation period the cells were removed by centrifugation at 5000 rpm for 10 min (Sigma-Laborzentrifugen, 2K15) to remove bacterial cells. Trichloroacetic acid was added (5%) and left overnight at 4ºC and centrifuged at 5000 rpm again to remove proteins. The pH of the clear solution was adjusted to 7.0 with NaOH solution and dialyzed three times. The supernatant was subjected to fractional precipitation by 1, 2, 3, and 4 volumes of absolute ethanol according to Whistler and Lauterbach [57], stirred vigorously and kept over night at 4°C. The precipitate from the ethanol dispersion was collected by centrifugation at 5,000 rpm (Sigma-Laborzentrifugen, 2K15) for 15 min, re-suspended in distilled water and lyophilized to afford the crude EPS. The crude polysaccharide fraction (EPS) was re-dissolved in deionizaed water and forced through a filter (0.45 mm), then applied to a column (1.5 × 70 cm) of DEAE-cellulose. After loading with sample, the column was eluted with gradient NaCl solution (0.2—3.0 M), and the procedure was monitored by the phenol-sulfuric acid method mentioned above. The collected fractions were further purified on a Sephadex G-200 column (2 × 80 cm) eluted with 0.1 M NaCl at a flow rate of 0.5 mL/min. Total sugar content of each tube was measured at 490 nm by Dubois's method, and protein absorption at 280 nm recorded for each fraction. One polysaccharide active fraction (BMEPS) was collected, dialyzed and lyophilized. BMEPS was used for activity assessment and structural analysis [5].

Molecular weight determination

The molecular weight of BMEPS was determined by gel permeation chromatography (GPC) on a Sephadex G-200 column (80 x 2.0 cm). Standard dextrans (40,000; 500,000 and 2000,000 Daltons, Fluka Chemical Co., Buchs, Switzerland) and glucose were used, and the elution volumes were plotted against the logarithm of their respective molecular weights. The elution volume of the purified polysaccharide was plotted in the same graph, and the molecular weight was determined [33].

Analysis of monosaccharide composition

The BMEPS was hydrolyzed with 88% formic acid 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 [49]. The monosaccharides contents were quantified by HPLC on a Shimadzu Shim-Pack SCR-101N column (7.9 mm × 30 cm), using deionized water as the mobile phase (flow rate 0.5 mL/min), as described by El-Sayed *et al*. [17]. Uronic acid content was determined according to *m*-hydroxydiphenyl method using glucuronic acid as standard [19].

Infrared Spectroscopy

The infrared (IR) spectrum of BMEPS was measured on a Bucker scientific 500-IR Spectrophotometer. The polysaccharide was mixed with KBr powder, ground and pressed into a 1 mm pellets for FTIR measurements in the range of 400-4000 Cm^{-1} [46].

Periodate oxidation and Smith degradation

The sample (30 mg) dissolved in 12.5 mL of distilled water was mixed with 12.5 mL of 30 mmol/L $NaIO_4$. The solution was kept in the dark at room temperature; 0.1 mL aliquots were withdrawn at 24 h intervals, diluted to 25 mL with distilled water and read in a spectrophotometer at 223 nm [34]. Periodate consumption was calculated on the basis of the change of the absorbance at 223 nm [6]. The solution of periodate product (2 mL) was used to assess the amount of formic acid by titration with 0.005 mol/L NaOH. Ethylene glycol (2 mL) was added, then the experiment of periodate oxidation was over. The solution of periodate product was extensively dialyzed against tap water and distilled water for 48 h, respectively. The content inside was concentrated and reduced with $NaBH_4$ (100 mg), and the mixture was left for 24 h at room temperature, neutralized to pH 6.0 with acetic acid (50 mL/100 mL), dialyzed as described above, and re-concentrated to 10 mL. One-third of the solution mentioned above was freeze-dried, fully hydrolyzed and analysis by HPLC [17]. Two-thirds of solution was added to the same volume of 1 mol/L sulfuric acid, kept for 40 h at 25 °C, neutralized to pH 6.0 with $BaSO_4$, and filtered for analysis by smith

degradation. The filtrate was dialyzed and the content out of dialysis bag was analyzed by HPLC; whereas the contents inside the dialysis bag was mixed with four volumes of absolute ethanol and centrifuged. The supernatant and precipitate were also analyzed by HPLC [60].

Biological activity

Radical scavenging activity (RSA) of BMEPS toward DPPH radical

The free radical scavenging activity of BMEPS was measured against 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radicals using the method of Asker *et al.* [6]. Five mL of DPPH ethanol solution (freshly prepare at a concentration of 0.1 mmol/L) was added to 1 mL of BMEPS solution of different concentrations (100 - 1500 µg) in water. After 30 min, absorbance was measured at 517 nm using Spectrophotometer UV-Visible 2401PC (Shimadzu, Japan). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity, which was analyzed from the graph (inhibition percentage plotted against concentration of compound). Ascorbic acid was used as positive controls. The experiment was carried out in triplicate and averaged. The capability to scavenge the DPPH radical was calculated using the following equation:

Scavenging ability (%) = $[(\Delta A_{517 \text{ of control}} - \Delta A_{517 \text{ of sample}}) / \Delta A_{517 \text{ of control}}] \times 100$.

The EC_{50} value is the effective concentration (µg) of BMEPS at which the DPPH radicals were scavenged by 50%.

Antitumor activity against A-549 and MCF-7 cells *lines*

Antiproliferative activity against various tumour cell lines human breast adenocarcinoma cell line (MCF-7) and human alveolar basal epithelial cell line (A-549) was estimated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, based on the cleavage of the tetrazolium salt by mitochondrial dehydrogenases in viable cells [22]. The relative cell viability was expressed as the mean percentage of viable cells relative to the respective DMSO treated cells (control).

Antiviral assay

The antiviral activity of the exopolysaccharide was determined against A/Puerto Rico/8/1934 (PR8) H1N1 virus. Stock solutions of the crude exopolysaccharide and BMEPS were prepared in DMSO. Cells grown to confluence in 96-well plates were infected with 100 µl of 100 $TCID_{50}$ viruses. After an adsorption period of 1 h at 37^{o}C, virus was removed and serial dilutions of the exopolysaccharide were added. Maintenance DMEM with 2% FBS was added (100 µl/well).The cultures were further incubated at 37^{o}C for 3 days, until complete CPE was observed in the infected and untreated virus control. The determination of the anti H1N1 virus activity of BMEPS was based on virus induced cytopathogenicity of H1N1 infected MDCK cells, measured at day 4 post virus infection by the MTT colorimetric method [44]. An absorbance of formazan was detected by a multi well plate reader at 540 nm with 620 nm reference wavelength. The results were expressed as the 50% effective concentration (EC_{50}). The 50% effective antiviral concentration (EC_{50}) was defined as the BMEPS concentration required for protecting 50% of the virus-infected cells against viral cytopathogenicity.

RESULTS AND DISCUSSION

Isolation and purification of the exopolysaccharide

The crude exopolysaccharide produced by *B. marinas* was isolated from the culture supernatant with fractional precipitation by 1, 2, 3, and 4 volumes of absolute ethanol. The fraction 1 obtained by 1volume of absolute ethanol showed high antitumor activity against Ehrlich Ascites Carcinoma Cells (86.38 % dead cells) comparing with other fractions 2 and 3 which produced 81.62 and 83.35% dead cells, respectively. Therefore, the fraction 1 was used for further purification studies. Te purification of polysaccharides produced by *B. marinus* after partial purification with ethanol was applied to DEAE-cellulose column and eluted with the deionized water and NaCl. The carbohydrate contents of each aliquot of elute (1mL/min) were determined. Any successive elute was not applied unless the last aliquot of the preceding one was devoid of carbohydrates. Distilled water should elute the almost natural fraction which does not attached to tertiary amine of the DEAE-cellulose, while the attached acidic or sulfated polysaccharides were eluted by different salt solutions with the proper polarity according to the degree of acidity of sulfated group on the polysaccharide molecules. The BMEPS obtained from the previous step DEAE-cellulose was subjected to the final purification on Sephadex G-200 and determination of their molecular weight. The elution diagrams of the polysaccharide are illustrated graphically in Figure (1&2). The molecular weight of the sub fraction exopolysaccharide was calculated from standard dextran marker by using Sephadex G-200 column as presented Figure (2). The molecular weight was found to be 5×10^5 for the BMEPS. The last BMEPS which gave the highest antitumor activity against EACC cells was further investigated for its molecular structure.

Infrared spectra of the sub fractions

The infrared spectra of BMEPS indicate that the polysaccharide have many fractional groups, stretching bands at 3500−3700 cm^{-1} are probably associated with the –OH group. The absorption at 1735 cm^{-1} revealed the presence of COO^{-}, which may be associated with the glucouronic acid and internal hydrogen bonds. The absorption band at 1250 and 1370 cm^{-1} indicated the presence of sulfate ester. The spectrum showed the characteristic S=O stretching vibration at 1240 cm^{-1} [29]. The absorption at 876 cm^{-1} indicated the β-glycosidic linkages of purified fractions. Spectrum of the desulfated sub fraction-6 indicates the presence of sulfate group. This means that no complete desulfation and the appearance of glucouronic acid by HPLC analysis and the absorption band at 1735 cm^{-1} of carboxylate [11, 1, 10, 51].

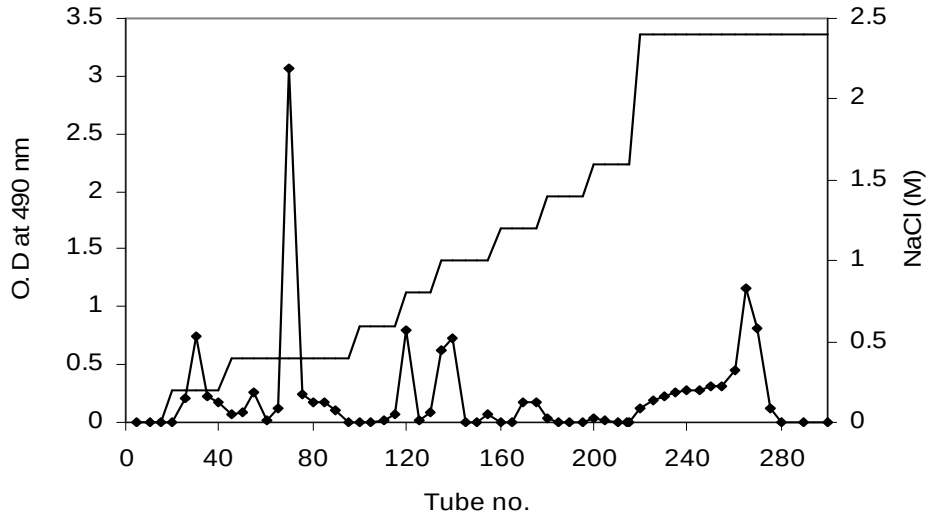


Figure (1). Elution curve of EPS from *B. marinas* over DEAE-cellulose column. The absorbance at 490 nm was that of the resulting reactive solutions of polysaccharides, phenol and sulfuric acid

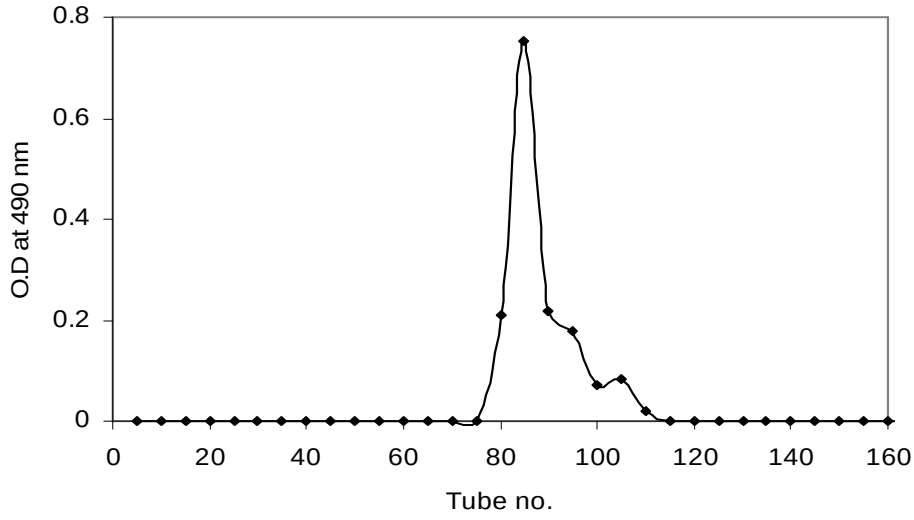


Figure (2). Purity of BMEPS on Sephacryl S-200 column eluting with 0.1M NaCl at a flow rate of 25 mL h^{-1}. The absorbance at 490 nm was that of the resulting reactive solutions of polysaccharides, phenol and sulfuric acid

Periodate Oxidation and Smith degradation

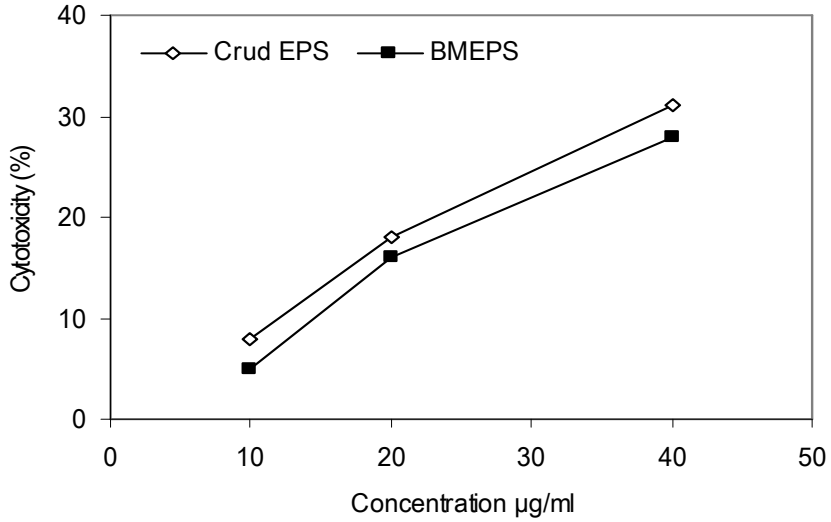
The results of periodate consumption and formic acid production of BMEPS and desulfated BMEPS show that. The BMEPS did not produce formic acid and consumed periodate by periodate treatment. This may be due to the one of the pair or all hydroxyl groups of the exopolysaccharide were sulfated. While, desulfated BMEPS consumed periodate (0.894 moles) to produce formic acid (0.000472 moles) per one mole of anhydrosugar. The produced polysaccharide from periodate treatment was reduced by sodium borohydride (Smith degradation). Small amount of glycerol and big amount of liberated erythritol partially prove the presence of the β-(1−−4) linkages between the monosugars in the backbone of the polysaccharide. At the same time, these results support the hypothesis of the presence of sulfate groups on the C_2 and/or C_3. Periodate consumption of desulfated BMEPS would be less than one

mole IO_4^-/mole anhydrosugar unit, because some of these units can not be affected. These results are reflected on the appearance of some glucouronic acid during the Smith's degradation experiment. At the end of the periodate oxidation process, the resulting polyaldehydes were reduced to the corresponding polyalcohols which were subjected to hydrolyses. The resulting polyalcohol hydrolyzates were then subjected to HPLC analysis. The sugar derivatives, *e.g*. glycerol, erythritol and erytheric acid in addition to glucouronic acid were separated and quantitatively determined. The molar ratios of these sugars are 3.01: 0.15:0.8:0.1 respectively. Erythritol was produced from C_3, C_4, C_5 and C6 of the (1−4) glycosidic linkages of glucose after hydrolysis of the backbone. While, erytheric acid was liberated from C_3, C_4, C_5 and C_6 of the (1-- 4)-glycosidic linkage of glucouronic acid. The presence of relatively small quantities of glycerol from the glucose units and erytheric acid from the glucouronic unit in the hydrolysis of polysaccharides, gave the information that glucose may be found at the non-reducing end. These interpretations are in a good agreement with Abdel-Akher *et al.* [2] and Danishefk *et al* [16]. The appearance of glucouronic acid as the unit indicated the presence of some sulfate groups on the C_2 and/or C_3 of some units of glucouronic acid or the presence of branches on some glucouronic acid units at C_2 and/or C_3. These interpretations are in a good agreement with Hussein *et al.* [24-26]. From the previous results, the proposal structure of BMEPS from *B. marinus* could be suggested as $[Glc\text{-}\beta\text{-}(1{-}4)\text{-}Glc\text{-}\beta\text{-}(1{-}4)\text{-}Glc.A]_n$.

Biological activities of the crude exopolysaccharide and BMEPS

The cytotoxicity against MDCK cell line

Results showed in Figure (3) demonstrate that the crude EPS and BMEPS displayed different, dose-dependent cytotoxicity in both cell culture systems employed. The cytotoxicity (%) of the crude EPS was 22.5, 39.8, and 77.5 at concentrations (µg/ml) 20, 40 and 80, respectively. On the other hand, the cytotoxicity of BMEPS was 21.8, 42.2 and 76.4% at concentrations20, 40 and 80µg/ml, respectively. From these observations, it is clear that the concentrations of the crude EPS and BMEPS which inhibited 50% (IC_{50}) were detected at 50.3 and 50µg/ml, respectively. It is clear that the cytotoxicity and IC_{50} of the crude EPS and BMEPS are nearly the same. This meant that the crude EPS can be use as antiviral without further purification.



Figure(3). Cytotoxicity of different concentrations of crude EPS and BMEPS

The inhibitory effect against H1N1virus

The inhibitory affects of the crude EPS and BMEPS at concentrations 10, 20, and 40µg/ml on H1N1 infection was investigated by plaque reduction assay. The obtained results in Figure (4) reveal that the inhibition effects of the crude EPS against HINI virus were 7.0, 16.6, and 32 % at 10, 20, 40 µg /ml, respectively. On the other hand, the inhibition effect of BMEPS against HINI virus was 6.0; 15.0 and 31.4 % at 10, 20, 40 µg/ml, respectively. In conclusion, the results reveal that the crude EPS showed better result than BMEPS against HINI virus, these results are in a good agreement with those of Marchetti *et al.* [38] who demonstrated that sulfated polysaccharides (including fucoidan) exhibited antiviral activities both *in vivo* and *in vitro*, of interest in view of their low cytotoxicity compared with other antiviral drugs currently used in clinical medicine. Furthermore, Asker *et al.* [6] isolated crude water-soluble sulfated polysaccharides (SP) from brown algae *S. latifolium* had antiviral activity against HSV-1 and HAV. The antiviral activities are depending on both the degree of sulfation and molecular weight. Also, polysaccharides containing high amount of uronic acid residues, show very little antiviral activity, which stands in contrast to other polyanionic compounds. The specific position of sulfate ester group appears to be

additionally important for antiviral activity of sulfated polysaccharides [38, 9, 13]. The influence of the distribution of sulfate group along polymer chain and the conformational flexibility of this chain for adopting a definite shape that might be required during the formation of polysaccharide virus complex [32]. The sulfated EPS are known to interfere with the absorption and penetration of viruses into host cell and to inhibit various retroviral reverse transcriptases [23, 39].

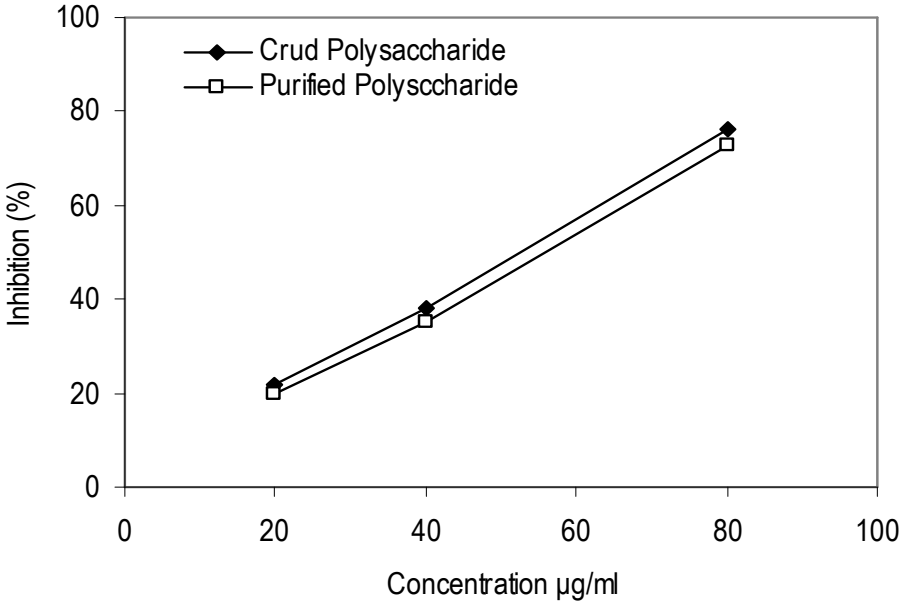


Figure (4). Antiviral activity of the crude and BMEPS against H1N1 virus

Antioxidant activity

The antioxidant activities of BMEPS at different concentrations (100, 300, 500, 1000 and 1500 µg /ml) were evaluated as free radical DPPH scavenging and their results are found in Figure (5). It is clear that the highest scavenging activity 79.10 % was found for BMEPS at concentration 1500 µg /ml followed by these of 1000 µg /ml (79.00 %), 500 µg /ml (78.32 %), 300 µg /ml (17.28 %), and 100 µg /ml (16.00 %) in decreasing order. In the *in vitro* antioxidant assay, the BMEPS was found to possess DPPH radical-scavenging activity, with an IC_{50} value of 400µg/ml. The results are similar to those of Zhang *et al.* [64] who found that the sulfated galactan fraction F1 (isolated from the red seaweed, *Porphyra haitanensis*) had significant *in vivo* antioxidant activity, Seng *et al.* [48], found that polysaccharide extracts from *Ganoderma tsugae* possessed good antioxidant properties except for their scavenging ability towards hydroxyl radicals and may be good candidates as a new dietary supplement and functional food, Asker *et al.* [6] evaluated the antioxidant activity of the CPS, CPSI and CPSII *in vitro* by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay (RSA). CPSI fraction showed the highest antioxidant activity among the three fractions, Asker and Shawky [7] isolated a water-soluble acidic EPS reaching a maximum concentration of 23.4 g/l growth medium, coded as BSMA, from the non-pathogenic soil bacteria *B. otitidis* BTS44, by precipitating with two volumes of ethanol. In the *in vitro* antioxidant assay, BSMA was found to possess DPPH radical-scavenging activity, with an IC_{50} value of 120µg/ml, Hu *et al.* [27] isolated two sulfated polysaccharide fractions from seaweed *Undaria pinnitafida* possessed good antioxidant properties, Chen *et al.* [14] obtained a homogeneous EPS, designated As1-1, from the culture medium of the mangrove entophytic fungus *Aspergillus sp.* Y16 and purified. As1-1 possessed good *in vitro* antioxidant activity and superoxide radicals and Kodali *et al.* [31] isolated an EPS from *B. coagulans* RK-02 and purified by size exclusion chromatography. *In vivo* evaluations showed that this EPS displayed significant antioxidant activity. Also, Agili and Mohamed [3] obtained the EPS from *Padina pavonia* by extraction had *in vitro* antioxidant activity. The antioxidant properties of EPS are mainly associated with monosaccharide component, molecular mass, structure, and conformation of polysaccharides [54, 50, 45]. Molecular mass of polysaccharides was one of the most important structure factors [65]. Porphyrans with different molecular weight showed different antioxidant activities.

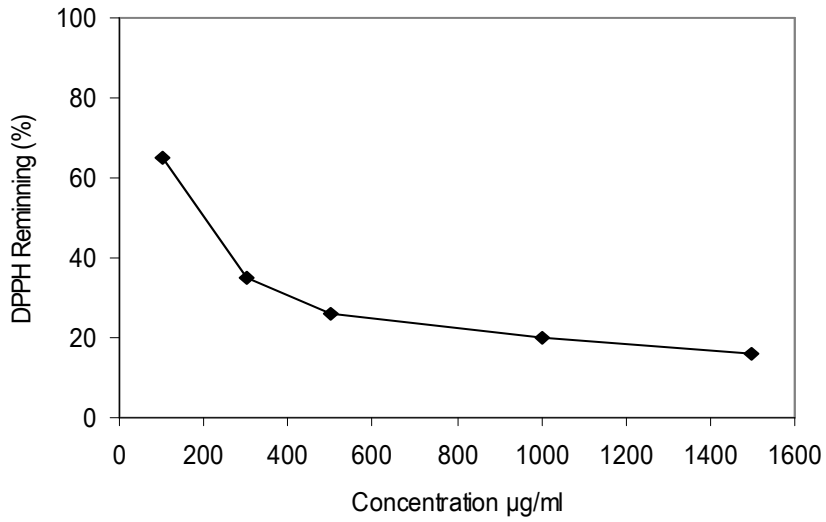


Figure (5). Radical scavenging activity (RSA) of different concentrations of BMEPS

Antitumor activity against A-549 and MCF-7 *cell lines*

Using MTT assay, the effect of BMEPS on the proliferation of MCF-7 and A-549 was studied after 48 h of incubation. Results in Figure (6) show that the viability of MCF-7 cells after incubation with BMEPS is 55 % at 100 µg/ml, 72 % at 50 µg/ml, 79 % at 25 µg/ml and 82 % at 12.5 µg/ml. The calculated IC_{50} for cell line MCF-was 118.0µg/ml for BMEPS. Results in Figure (6) show that the highest viability of A-549 cells (88 %) was found for BMEPS at concentration 12.5 µg/ml followed by those of 86% at 25 µg /ml, 79% at 50 µg /ml and 63% at 100 µg /ml. The calculated IC_{50} for cell line A-549 showed a weak cytotoxic level as concluded from the high calculated IC_{50} values–indicating low antitumor affinity to be 220µg/ml for BMEPS. Umezawa *et al.* [55] screened EPS of marine bacteria for their antitumor activity against sarcoma-180 solid tumor in mice, Tanaka *et al.* [52] isolated a sulfated polysaccharide peptidoglycans complex, DS-4152, from the culture supernatant of an *Arthrobacter* species inhibited angiogenesis and tumor growth. Also, Zhang *et al.* [62] reported that glycoproteins from *E. cloacae* showed antitumor effects on mice and Wu and Chen [58] suggested that sulfated polysaccharides could affect tumor cells directly. Sulfated polysaccharides could inhibit the metastasis and proliferation of tumor cells by binding to growth factors and cell adhesion molecules. Polysaccharides with antitumor action differ greatly in their chemical composition and configuration, as well as their physical properties. Antitumor activity is exhibited by a wide range of glycans extending from homopolymers to highly complex heteropolymers [43]. Differences in activity can be correlated with solubility in water, size of the molecules, branching rate and form. Although it is difficult to correlate the structure and antitumor activity of complex polysaccharides, some relationships can be inferred. It is obvious that structural features such as β-(1−3) linkages in the main chain of the glucan and additional β-(1--6) branch points are needed for antitumor action. β-Glucans containing mainly (1--6) linkages have less activity. High molecular weight glucans appear to be more effective than those of low molecular weight [40]. However, obvious variations in antitumor polysaccharides have also been noted. Antitumor polysaccharides may have other chemical structures, such as hetero-β-glucans, heteroglycans, β-glucan-protein, α-manno-β-glucan, α-glucan-protein and heteroglycanprotein complexes [65, 41, 42].

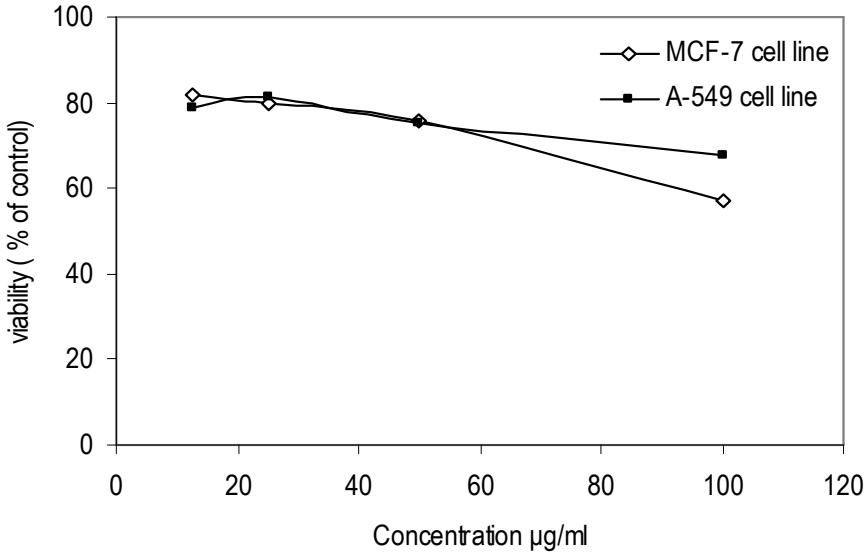


Figure (6). Antitumor activity of the BMEPS against MCF-7 and against A-549 cell line

CONCLUSION

The BMEPS from *B. marinus* with biological activities was source usefully obtained from the fermented broth. Based on the chemical and chromatographic analysis the main chain was characterized to consist of (1--4) glucose and glucouronic units. Posses a good scavenging ability on DPPH radical, antiviral and could be potential source of antituomer.

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