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In vitro evaluation of antioxidant, anticancer, and antiviral activities of exopolysaccharide from *Streptomyces hirsutus* NRC2018

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ABSTRACT

An exopolysaccharide Exopolysaccharide (EPSNC2) produced from marine *Streptomyces hirsutus* NRC2018 which was isolated from marine sediments at North Coast, Egypt with accession number MK050544. EPSNC2 was a β-type heteropolysaccharide contained uronic acid (72.73%) with no sulfate groups and overall average molecular weight (*Mw*) 4.25×10^5 g/mol. The monosaccharide composition was glucuronic:galacturonic:glucose:mannose:arabinose with molar ratio 1.2:0.6:0.1:0.2:0.1, respectively. EPSNC2 was subjected to antioxidant, anticancer, and antiviral *in vitro* tests, showed high 1, 1-diphenyl-2-picrylhydrazyl free radical scavenging activity whereas, the maximum antioxidant activity was 95.9% at 1,500 µg/ml after 120 minutes and the IC_{50} value was 158.5 µg/ml. Also, EPSNC2 had the ability to scavenge H_2O_2 and the maximum activity was 75.6% with an estimated IC_{50} value 501.2 µg/ml. Furthermore, EPSNC2 showed a reducing power activity as well as a metal chelating activity in a dose dependant manner and the activity reached 98.5% at 1,000 µg/ml. EPSNC2 had a significant and specific anticancer effect on CaCo-2 cell line without any effect on other cell lines and the IC_{50} was estimated to be 295.1 µg/ml. Our results regarding the antiviral activity against Herpes Simplex virus type 1 (HSV-1), Hepatitis A virus, and Coxsackie B-4 were 84.9%, 20.3%, and 45.4%, respectively at 125 µg/ml with no activity against adenovirus. Therefore, the EC_{50} value against the enveloped virus HSV-1 was 32.4 µg/ml.

INTRODUCTION

Exopolysaccharides (EPSs) are microbial primary metabolites biopolymers produced and transferred outside the cells. They are composed of monosaccharide units linked with α- or β-glycosidic linkage. EPSs can be classified into homo- or hetero- as well as neutral, basic, or acidic polysaccharides based on monosaccharide content, organic and inorganic substituents (Sutherland, 1997). Microbial EPSs showed diversity in physicochemical properties due to differences in monosaccharide composition and sequence, type of linkage and polymerization, and branching degree. This diversity of characteristics has drawn attention to the industrial values of EPSs in different fields (Patel *et al*., 2010).

In recent decades, marine bacteria have been an interesting field in biomedical research since they produce many novel EPSs of great valuable bioactivity such as anticancer (Sun *et al*., 2013), anti-inflammatory (Jones *et al*, 2014), immunomodulatory (Tang *et al*., 2015), antithrombotic, hypoglycemic, hypocholesterolemic, antiviral (Rincão *et al*., 2012), and antioxidant (El-Newary *et al*., 2017). *Streptomyces*, a Gram-positive bacterium, is well-known as an important industrial microorganism for its natural derived metabolites production as EPS production (Beshay *et al*., 2009; Manivasagan *et al*., 2013).

Cancer is a set of diseases involving cell growth abnormalities with the ability to spread through blood or lymphatic vessels and invade other parts (metastasis). Cancer is the second disease that causes mortality worldwide with about 15.7% deaths and about 14.1 million new cases occurring annually. Over 100 types of cancer had been diagnosed to affect humans. Colon cancer, also known as colorectal and bowel cancer, is an abnormal overgrowth of epithelial cells lining the colon or rectum. Every

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year more than 1 million people have colorectal cancer and it was the second most common type in women about 9.2%, the third in men about 10%, and the fourth most common causing cancer death globally (Forman and Ferlay, 2014). Treatment of cancer represents a large obstacle for scientists and physicians because these remedies lack specificity against cancerous cells leading to damage of healthy cells and a lot of serious side effects of patients. It is, therefore, necessary to discover and develop other types of highly efficient and specialized treatment for cancer cells to be safe on healthy ones (Sarkar *et al*., 2013).

Herpes simplex virus type-1 is an enveloped virus that belongs to the family Herpesviridae, subfamily alpha-herpesvirinae. Herpesviridae has been divided into alpha-, beta-, and gamma subfamilies with over 100 different members, eight of them are known as human pathogens. Herpes Simplex virus type 1 (HSV-1) mediates its entry into cells via interaction between viral envelope glycoproteins with cell surface receptors heparan sulfate, nectin-1,2 and herpesvirus entry mediator (Akhtar and Shukla, 2009). Herpesviridae family viruses cause diseases varying in severity between mild to more severe. Alpha-herpesvirinae member HSV-1 causes cold sores and encephalitis and it was reported that there is an indirect link between that virus and Alzheimer's disease while, HSV-2 causes genital disease, encephalitis, and infections of newborns (Whitley and Roizman, 2001). HSV-1 may persist latent in trigeminal ganglia, whereas HSV-2 persists in sacral ganglia and the reactivation occurs during immunodeficiency (Whitley and Roizman, 2001).

Therefore, the aim of this work was to explore the potential biological activities of a new safe microbial exopolysaccharide possessed by *Streptomyces hirsutus* NRC2018. Exopolysaccharide (EPSNC2) was evaluated for its antioxidant, anti-cancer, and antiviral activities.

MATERIALS AND METHOD

Sampling and isolation of streptomycetes

Samples were collected during summer 2016 from marine sediments from North Coast, Egypt. Samples were serially diluted according to Hayakawa and Nonomura (1987), and plated on starch nitrate agar medium containing (g/l): starch 10.0, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, KNO_3 2.0, $CaCO_3$ 2.0, $FeSO_4.7H_2O$ 0.01, and agar 20.0, which was dissolved in 750 ml seawater then topped to 1 L with 250 ml distilled water, pH adjusted to 7.0 (Waksman, 1961). The streptomycete isolate was selected and picked up based on the morphological features of colonies.

Identification of streptomycete isolate

The isolate was identified according to morphological, physiological, and biochemical features (Goodfellow *et al*., 1987; Kutzner, 1976; Shirling and Gottlieb, 1966; Szabó *et al*., 1975; Tresner and Backus 1963). Also, molecular identification was performed by 16*S* rRNA gene sequencing. The bacterial genome was isolated and 16*S* rRNA gene was amplified using polymerase chain reaction technique using the following primers F(5′ GAGTTTGATCCTGGCTCAG 3) and R(5′GGTTACCTTGTTACGACTT 3′) based on Gardes and Bruns (1993) method. Sequencing was done using kit of Big Dye terminator cycle sequencing (Applied Biosystems, USA). Products of sequencing were analyzed on a model Applied Biosystems 3730XL automated DNA sequencing system (Applied Biosystems, USA). The resulted 16*S* rRNA sequence was submitted to GenBank database and compared with the other sequences in https://www.ncbi.nlm.nih.gov/ using the BLAST program. The sequence of other bacterial strains with the most similarity to the 16*S* rRNA gene of our isolate was selected and aligned for making the suitable phylogenetic tree. Then, 16*S* rRNA sequence was deposited in the DDBJ/EMBL/GenBank databases with accession number MK050544.

EPS production

Streptomyces isolate was inoculated into production medium containing (g/l) Glucose 10.0, Tryptone 5.0, Yeast extract 5.0, K_2HPO_4 3.0, NaCl 3.0, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.5, $CaCO_3$ 0.5 dissolved in 750 ml seawater and topped to 1 L by distilled water and adjusted to pH 7 (Manivasagan *et al*., 2013) then incubated at 28°C for 5 days at 120 rpm. After incubation, the medium was centrifuged at 5,000 rpm for 30 minutes, the supernatant mixed with Trichloroacetic acid (TCA) (10%) and left overnight at 4 C. Then, centrifuged at 5,000 rpm for 20 minutes to remove the protein. The supernatant pH was neutralized to 7 with NaOH solution (Liu *et al*., 2010). The EPSs containing solution was collected and mixed with four volumes of ethanol (95%), left it overnight at 4°C. The precipitated EPSs were separated by centrifugation at 5,000 rpm for 20 minutes and redissolved in deionized water and dialyzed three times (1 L × 3) using dialysis tubing (MWCO 2000) for 48 hours. For purification and major fraction determination, absolute cold ethanol was added in 1, 2, 3, and 4 volumes gradually and the precipitated EPS was collected up to date. The major fraction obtained by one volume was dialyzed against distilled water for 72 hours, washed twice using acetone. Then, dehydrated by ether, dried at 40°C, and coded as EPSNC2 (Shene *et al*., 2008).

Homogeneity and molecular mass of EPSNC2

The mass average molar mass (*Mw*) of EPSNC2 was determined used high-performance gel permeation chromatography (HPGPC, Agilent 1100 Series System, Hewlett-Packard, Germany) with refractive index (RI) detection (You *et al*., 2013). The number average molar mass (*Mn*) was determined and polydispersity index (PI) was calculated from (*Mw/Mn*) ratio.

Monosaccharide composition analysis

EPSNC2 was subjected to complete acid hydrolysis with formic acid (88 %) at 100°C for 5 hours. High-performance liquid chromatography (HPLC, Agilent Pack, serics1, 200), equipped with Aminex carbohydrate HP-87C column (300 × 7.8 mm) was applied for monosaccharide composition and calculation of molar ratio (Randall *et al*., 1989).

Fourier-transform infrared spectroscopy (FTIR)

Dried EPSNC2 (2 mg) was mixed with 200 mg KBr powder then pressed to form a pellet of 1 mm. FTIR spectrum was measured in the range between 400 and 4,000 cm^{-1} using FTIR-UNIT Bruker Vector 22 Spectrophotometer (Brock-Neely, 1957).

Sulfate and uronic acid content determination

EPSNC2 was subjected to complete acid hydrolysis with formic acid (88%) at 100°C for 5 hours. The sulfate was estimated by the turbidity method using Barium Chloride-Tween 80 reagent according to Dodg=son and Price (1962). The content of uronic acid was determined using the *m*-hydroxybiphenyl colorimetric method at 525 nm (Filisetti-Cozzi and Carpita, 1991).

Evaluation of EPSNC2 antioxidant bioactivity

DPPH radical scavenging activity

The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was determined according to Brand-Williams *et al*. (1995). Different concentrations of EPSNC2 (100, 300, 500, 1,000, and 1,500 µg/ml) were added to 2 ml of DPPH solution. Then, left in dark for 10, 30, 60, and 90 minutes at 517 nm using UV-Visible spectrophotometer. The DPPH scavenging activity was calculated from the following equation:

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

Hydrogen peroxide scavenging activity

According to Ruch *et al*. (1989), different concentrations of EPSNC2 (200, 400, 600, 800, 1,000, and 1,500 µg/ml) were mixed with 40 mM hydrogen peroxide solution in 50 mM of phosphate buffer solution (pH 7.4). The results were measured at different periods (15, 30, 45, and 60 minutes) against phosphate buffer as a blank and at 230 nm using UV-Visible spectrophotometer. H_2O_2 scavenging activity was calculated as follows:

$$\text{Scavenging ability (\%)} = (A_{230\ \text{of control}} - A_{230\ \text{of sample}} / A_{230\ \text{of control}}) \times 100$$

Reducing power (RP) activity

EPSNC2 concentrations (400, 600, 800, and 1,000 µg/ml) were added to 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml potassium ferricyanide ($K_3Fe(CN)_6$) 1% w/v and incubated at 50°C for 20 minutes then trichloroacetic acid (2.5 ml) 10% w/v was added. Then, mixture was centrifuged at 3,000 rpm for 10 minutes and the supernatant mixed with 2.5 ml H_2O and 0.5 mL of $FeCl_3$ (0.1% w/v). The absorbance of colored complex was measured at 700 nm (Oyaizu, 1986).

Metal chelating activity

The chelating activity was detected using Dinis *et al*. (1994) method by mixing 0.1 mL of EPSNC2 solution with concentrations (400, 600, 800, and 1,000 µg/ml) with 0.5 ml of 0.2 mM ferrous chloride; then 0.2 ml of ferrozine (5 mM) was added and the mixture incubated for 10 minutes at room temperature until red color complex was formed and then measured at 562 nm. The activity of metal chelating was calculated according to the following equation:

$$\text{Metal chelating activity (\%)} = (A_{562\ \text{of control}} - A_{562\ \text{of sample}} / A_{562\ \text{of control}}) \times 100$$

Assessment of antitumor activity

Cell line and culture

Human colorectal adenocarcinoma cell line (CaCo-2), human hepatocellular carcinoma cell line (HepG2), human breast adenocarcinoma cell line (MCF-7), and human lung adenocarcinoma cell line (A-549) were obtained from Tissue Culture Unit, Holding Company for Biological Products and Vaccines (VACSERA) at Giza, Egypt. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% Glutamine, and 1% Penicillin/Streptomycin and incubated at 37°C in 5% CO_2 humidified incubator.

Cytotoxicity/Viability assay

The cytotoxicity of EPSNC2 was evaluated through the MTT viability assay (Mossman *et al*., 1983). The cancer cells incubated with different concentrations of EPSNC2 (7.8–500 µg/ml) in a 96-well micro-plate while, control was incubated with RPMI 1640 media without EPSNC2 for 24 hours at 37°C, with 5% CO_2. Cells were checked for any physical signs of toxicity, e.g. rounding, partial, or complete loss of the monolayer, cell granulation, or shrinkage. Then, 20 µl of MTT (BIO BASIC CANADA INC) solution (5 mg/ml dissolved in PBS) was added to the cells for 5 minutes then 200 µl of DMSO was added to dissolve formazan and then removed and the cells were washed. The absorbance was measured at 560 nm using a micro-plate ELISA reader. All tests were established in triplicate and averaged. The cell viability was calculated as follows:

$$[1 - (\text{O.D}_{\text{test}} / \text{O.D}_{\text{control}})] \times 100\ \%$$

Assessment of antiviral activity

Cell line and Cytotoxicity assay

African green monkey kidney epithelial (VERO) cell line was purchased from Tissue Culture Unit, VACSERA, Giza, Egypt. It was cultured in MEM-EARLE's medium (VACSERA, Egypt) enriched with 2% fetal bovine serum and incubated with serially diluted concentrations of EPSNC2 (15.65, 31.25, 62.5, 125, 250, and 500 µg/ml) for up to 48 hours in 5% CO_2 at 37°C. Cytotoxicity was determined using the MTT viability assay (Mossman *et al*., 1983). The half-maximal cytotoxic concentration (IC_{50}) and the minimum non-toxic concentration were calculated.

Viruses and evaluation of antiviral activity

The cytopathic effect of Herpes Simplex virus type 1 (HSV-1), Adenovirus, Hepatitis A virus (HAV), and Coxsackie B4 virus (Microbiology Department, Faculty of Medicine for girls, Al-Azhar University, Egypt) were tested on VERO cells and the half-maximal viral cytopathogenicity concentration was estimated using MTT method. Equal volume (1:1 v/v) of three dilutions of EPSNC2 (31.25, 62.5, and 125 µg/ml) were incubated with the half-maximal viral cytopathogenicity concentration of viruses for 1 hour. 100 µl of viral/sample suspension was added to 1×10^5 cells which were cultured in 200 µl MEM media per well in a 96-well micro-plate then incubated at 37°C with 5% CO_2 humidity for 24 hours until either viral cytopathic effect or antiviral activity of EPSNC2 was obtained. According to MTT viability assay (Pauwels *et al*., 1988), optical density was measured at 560 nm and the background was subtracted at 620 nm. The experiment was carried out in triplicate. The concentration which inhibits 50% of viral cytopathic effect (EC_{50}) was determined.

Statistical analysis

Graph Pad InStat version 2 software was used for analyzed of all results. All graphs were plotted using the Graph Pad Prism version 8. Results were considered statistically significant with p values < 0.05.

RESULTS AND DISCUSSION

Isolation and identification of EPS producing Streptomycetes strain

The streptomycete strain was isolated from the sediments of the Mediterranean Sea, North Coast, Egypt. The isolate was taxonomically identified morphologically, biochemically, and physiologically. Streptomycete strain was confirmed using 16*S* rRNA molecular identification. Table 1 shows the morphological, biochemical, and physiological features of the isolate. The isolate had a grayish color of spore mass with no melanin and diffusible pigment production. Also, it showed a variety in the utilization of different sugars as a carbon source. Whereas, the spore chain was spiral and spiny spore surface as obtained in Figure 1a and b. The partial analysis of NC2 isolate 16*S* rRNA gene and its comparison with the data included in GenBank proved that the isolate had 100% homology with *S. hirsutus* and gave an identification symbol NRC2018 with accession number MK050544.

The evolutionary history of the tree was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.00511422 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1,372 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

EPS production, purification, and chemical characterization

After 5 days of incubation in fermentation medium (Manivasagan *et al*., 2013), EPS 10 g/l of culture media was isolated and the major fraction was obtained by addition of one volume absolute ethanol and named EPSNC2. The novel EPSNC2 was subjected to chemical analysis which proved that it contains 72.73% uronic acid and no sulfate content. Furthermore, monosaccharide composition and molar ratio which were determined by HPLC showed that EPSNC2 consisted of glucuronic acid: galacturonic acid: glucose: mannose: arabinose with molar ratio 1.2: 0.6: 0.1: 0.2: 0.1, respectively, which meant that EPSNC2 is a highly acidic heterogeneous polysaccharide.

Table 1. Morphological, physiological, and biochemical characteristics of NC2 isolate.

Morphological and cultural characteristics								
Spore chain morphology		**Spore surface ornamentation**	**Color of spore mass**		**Pigmentation of substrate mycelium**		**Diffusible pigment**	
Spiral >20		Spiny	gray		grayish yellow		−ve	
Physiological and biochemical characteristics								
Melanin pigment production			**Degradation activities**			**Nitrate reduction**	**H2S production**	
peptone iron agar	**Tyrosine agar**	**xanthine**	**Elastin**	**arbutin**		+ve	+ve	
−ve	−ve	−ve	−ve	+ve				
Utilization of sugars								
D-fructose	**Sucrose**	**Rhamnose**	**D-mannitol**	**D-xylose**	**Raffinose**	**I-inositol**	**Galactose**	**L-arabinose**
+ve	+ve	+ve	−ve	+ve	−ve	−ve	+ve	+ve

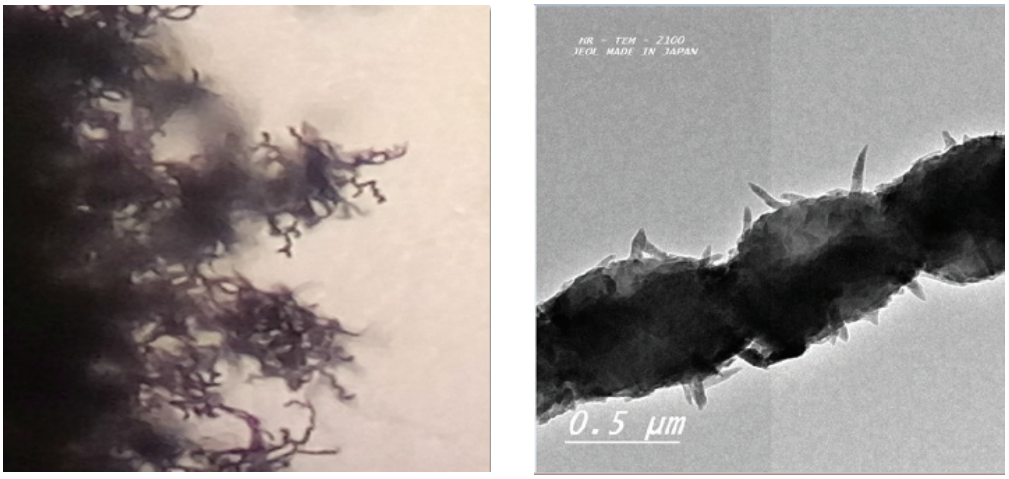

Figure 1. (a) Photomicrograph showing spiral aerial hyphae. (b) TEM micrograph showing spiny spore surface.

Molecular weight determination

The mass average molar mass (*Mw*) and the number average molar mass (*Mn*) were 4.25 × 10^5 g/mol and 2.71 × 10^5 g/mol, respectively, as shown in Figure 2. While the PI which was calculated by Mw/Mn ratio was 1.57. Therefore, it is noted that EPSNC2 has a high molecular weight. These results are consistent with what has been published that Mw of EPSs between 10 and 6,000 kDa (Xie *et al*., 2010).

FTIR spectroscopy analysis

FT-IR spectrum showed patterns which are confirmatory for EPSNC2, as shown in Figure 3. A broad peak at 3,430.74 cm^{-1} for OH stretching vibration (Kavita *et al*., 2014) and weak CH stretching bands were obtained at 2,961.16, 2,928.38, and 2857.99 cm^{-1}. The peak at 1,673.91cm^{-1} was attributed to C=O group stretching vibration as a result of the presence of the carboxylate group of uronic acid (Ahluwalia and Goyal, 2005; Lillo *et al*., 2014). While, the ß-pyranose structure was proved by the existence of 837.91 cm^{-1} band (Cheng *et al*., 2008) and 1,129.12 and 1,107.9 cm^{-1} peaks which were referred to C-O-C and C-O groups (Vijayabaskar *et al*., 2011).

Evaluation of antioxidant activity

DPPH free radical scavenging activity

The ability of EPSNC2 to scavenge free radical was estimated using DPPH as a source of a free radical. The determination of antioxidant activity could be based on two methods: single electron transfer and hydrogen atom transfer (Gulcin, 2012; Tan and Lim, 2015). Polysaccharides have many functional groups such as hydroxyl groups which can quench free radical by hydrogen donation. EPSNC2 showed high powerful free radical scavenging activity in a dose-dependant manner with IC_{50} value 158.5 µg/ml at 120 minutes, as shown in Figure 4. This antioxidant activity of EPSNC2 was due to presence of hydroxyl groups in addition to a high percentage of uronic acid (72.75%) containing carboxylic groups (Al-Sheraji *et al*., 2012; Wu *et al*., 2013). Ye *et al.,* (2012) isolated and purified an acidic EPS which belonged to β-type heteropolysaccharide and has a pyran group from marine *Pseudomonas* PF-6. This EPS showed antioxidant activity against DPPH. El-Newary *et al*. (2017) noted that BAEPS

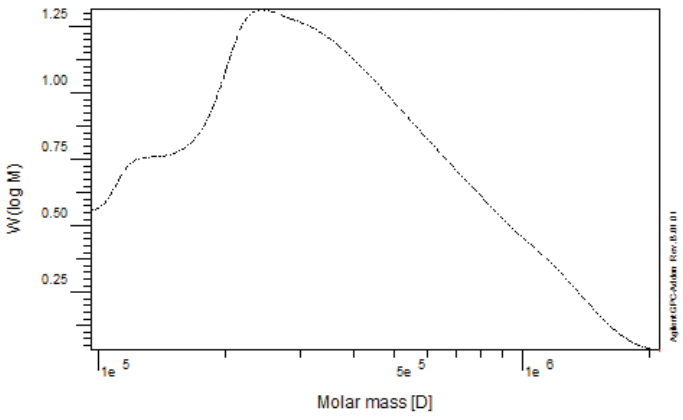

Figure 2. Mass average molar mass (Mw) of EPSNC2.

an EPS has been isolated from *Bacillus amyloliquefaciens* 3MS 2017 could scavenge DPPH free radicals and the maximum activity was 99.39% at 1,000 µg/ml. *Streptomyces carpaticus* produced an EPS which exhibited DPPH antioxidant activity with EC_{50} value 111 µg/ml (Selim *et al*., 2018).

Hydrogen Peroxide scavenging activity

Although the EPSNC2 showed antioxidant activity against hydrogen peroxide, its activity did not exceed 75.6% at 1,500 µg/ml after 60 minutes. s illustrates the antioxidant activity of EPSNC2 and IC_{50} value was estimated to be 501.2 µg/ml at 60 minutes. Besides the deleterious influences of H_2O_2, it is the source of hydroxyl free radical OH•, the most potent and reactive free radical inside cells via Fenton and Haber-Weiss reactions (Fenton, 1894; Haber and Weiss, 1934). BAEPS which had been isolated by El-Newary *et al*. (2017) could scavenge H_2O_2 and the scavenging ability was found to be 61.72% at 100 µg/ml and was elevated progressively to 92.17% at 1,000 µg/ml.

Ferric ion reducing power and ferrous ion metal chelating activity

It is supposed that the reducing power antioxidant activity of polysaccharides is due to the existence of free terminal electrophilic aldehyde and ketone groups which could reduce the metal ion. The Fe^{+3} of potassium ferricyanide was reduced to Fe^{+2} by EPSNC2 and Table 2 showed that RP increased in a dose-dependant manner. As observed by the metal chelating activity, 94.9% at 400 µg/ml and 98.5% at 1,000 µg/ml (Table 2). It is noteworthy that the hydroxyl and carboxyl groups found in EPSNC2 could interact with metal ions. Transition metal ions especially ferrous and cuprous ions are implicated in the formation of OH• via Fenton reaction and electron transferring via electron transport chain. *Streptomyces violaceus* MM72 producing an EPS showed strong metal chelating activities and moderate reducing activities (Manivasagan *et al*., 2013).

***In vitro* anticancer activity against colon cancer cells**

To assess the EPSNC2 cytotoxic effect on tumor cells, we observed the cancer cell lines after exposure to EPSNC2 for 24 hours. Interestingly, EPSNC2 showed a significant cytotoxic effect on CaCo-2 cell line without any effect on other cell lines (HepG2, MCF-7, and A-549) and the viability of CaCo-2 was remarkably decreased after EPSNC2 treatment in a dose-dependant manner (Fig. 6). Whereas there was no effect until 125 µg/ml, the cytotoxic effect increased to 44.8% and 75% at 250 and 500 µg/ml, respectively. IC_{50} was estimated to be 295.1 µg/ml as shown in Figure 7, which also showed that a strong correlation between the EPSNC2 concentration and the anticancer effect on CaCo2 cells (R^2 = 0.9536, p = 0.0043).

Many researchers found that polysaccharides had antitumor activity and their mode of action followed these mechanisms: i) stimulation and enhancement of immune system; ii) induction of apoptosis; iii) inhibition of angiogenesis; and iv) cell cycle arrest (Meng *et al*., 2016; Xu *et al*., 2016). EPS isolated from *Lactobacillus acidophilus* showed anticancer activity against two types of colon cancer cell lines HCT15 and CaCo-2 (Deepak *et al*., 2015). Zhou *et al*. (2017) reported that EPS116 from *Lactobacillus plantarum* NCU116 inhibits the

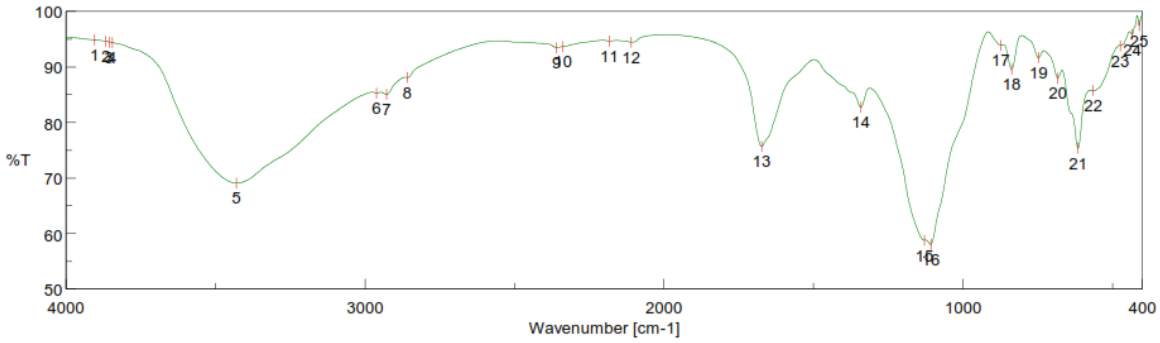

Figure 3. FT-IR spectrum of EPSNC2.

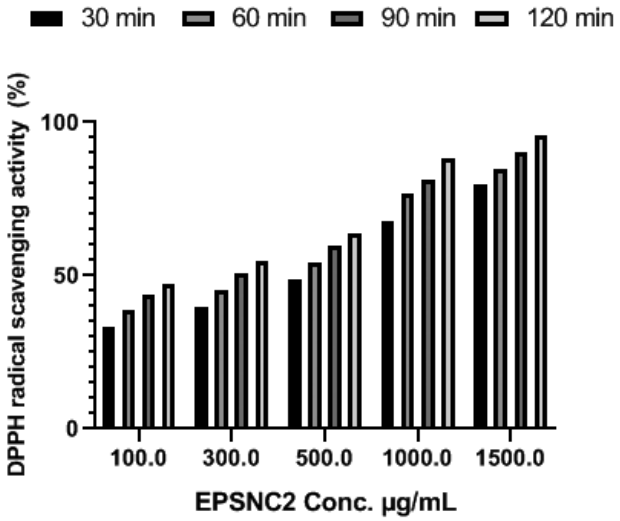

Figure 4. DPPH free radical scavenging activity of EPSNC2.

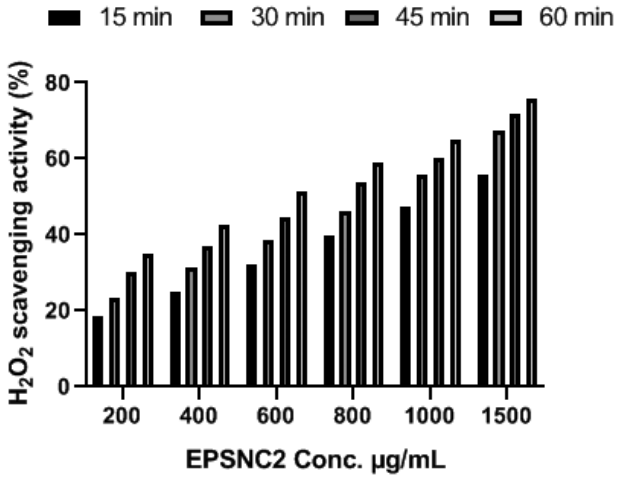

Figure 5. H_2O_2 scavenging activity of EPSNC2.

proliferation and survival of mouse colorectal carcinoma CT26 cells through induction of apoptosis via c-Jun, Fas/FasL pathways. Furthermore, the anticancer activity of EPS116 may be toll-like receptor (TLR)-2 dependent.

Table 2. RP and metal chelating activity of EPSNC2.

EPSNC2 conc. (µg/ml)	RP activity	Metal chelating activity %
400	0.192	94.9
600	0.2549	95.5
800	0.2967	96.6
1,000	0.3709	98.5

EPSNC2 has high molecular weight with monosaccharide composition diversity so it is believed to exhibit more interactions with many different cell surface receptors especially on macrophages and dendritic cells such as TLRs, mannose-binding lectins, and C-type lectins which have carbohydrate recognition domain enhancing innate immune system and complement system against tumor (Brown and Gordon, 2005; Willment *et al*., 2005). High molecular weight polysaccharides cannot enter the cells but, instead they bind with cancer cell receptors controlling cell signaling and cellular transduction.

Antiviral activity and EC50 determination

Before assessment of antiviral activity, cytotoxicity of EPSNC2 was evaluated against African green monkey normal kidney epithelial cell line (VERO). The half-maximal cytotoxic concentration (CC_{50}) was 387.19 µg/ml, while the maximum non-toxic concentration was 125 µg/ml. The antiviral activity of EPSNC2 was 84.9%, 20.3%, and 45.4% against HSV-1, HAV, and Coxsackie B4, respectively, at the non-toxic concentration 125 µg/ml. However, there was no antiviral activity against adenovirus at used EPSNC2 concentrations as shown in Figure 8. Figure 9 shows that the relationship between the logarithm of EPSNC2 concentration and its antiviral activity and the concentration which prevented the half-maximal viral cytopathic effect (EC_{50}) of HSV-1 was estimated to be 32.4 µg/ml. It has been reported that the antiviral activity of polysaccharides follows one or more of these mechanisms: i) Direct Virucidal Action, ii) Inhibition of Viral Adsorption, iii) Inhibition of Virus Internalization and Uncoating, iv) Inhibition of Virus Transcription and Replication, and v) Improvement of Host Antiviral Immune Responses. Carlucci *et al*. (1997) reported that the sulfate-containing λ-carrageenan could bind with HSV virion inhibiting its replication and change the structure of the glycoproteins of HSV (Carlucci *et al*., 2002). Also, they demonstrated that λ-carrageenan and cyclized µ/ι-carrageenan could interact with HSV-1 and HSV-2 cell surface receptors preventing their adsorption and entry

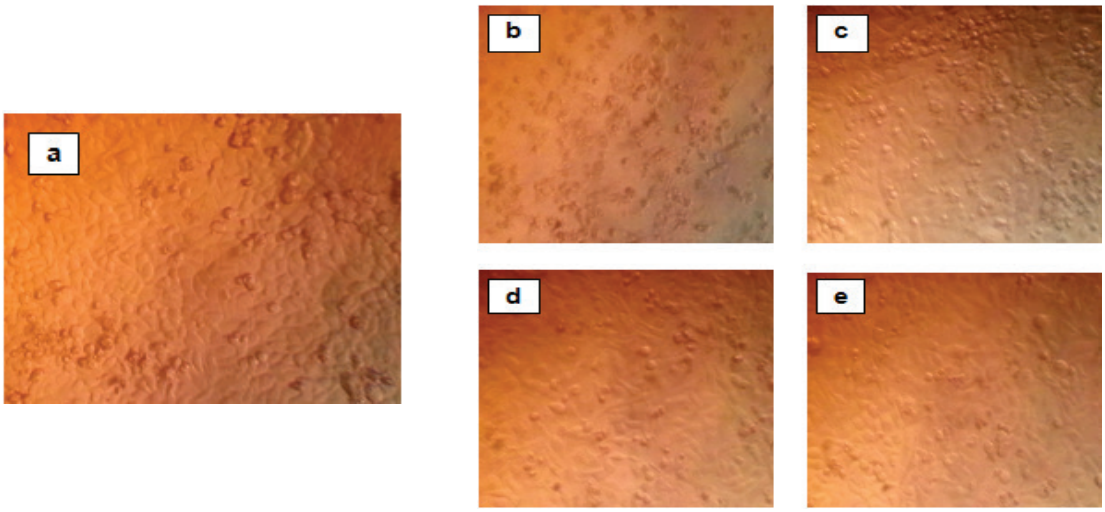

Figure 6. Anticancer effect against Caco-2 cell line. (a) Control; Caco-2 treated with EPSNC2 (b) 500 µg/ml; (c) 250 µg/ml; (d) 125 µg/ml; (e) 62.5 µg/ml. Partial or complete loss of the confluent monolayer, rounding, shrinkage, and cell granulation were observed.

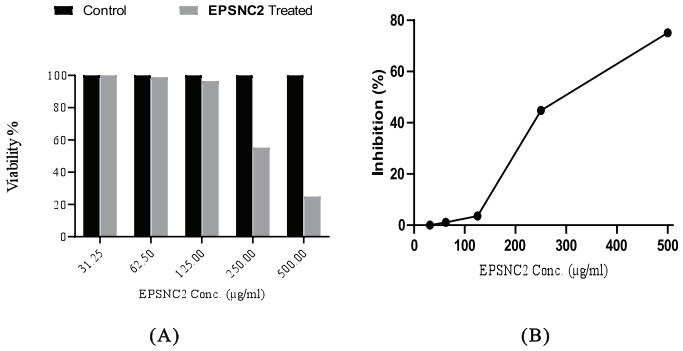

Figure 7. (A) Cytotoxicity (%) of EPSNC2 against CaCo-2 colon cancer cell line, (B) Correlation of Anticancer Activity. Significance: *p* value = 0.0076**.

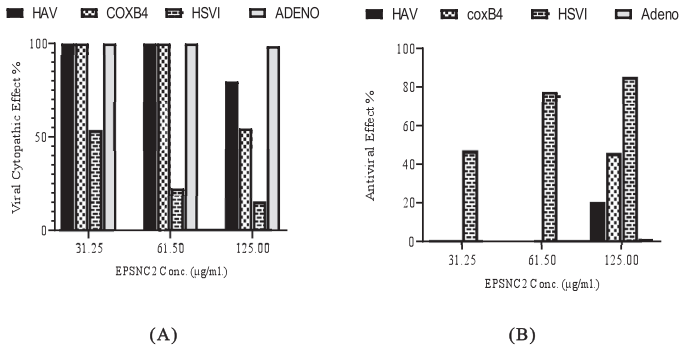

Figure 8. (A) Viral Cytopathic effect. (B) Antiviral activity of EPSNC2 against HAV, COXB4, HSV1, and ADENO viruses. Significance: *p* value = 0.0048**.

(Carlucci *et al*., 1997). Without any doubt, polysaccharides have potent antiviral activity against enveloped viruses rather than non-enveloped and EPSNC2 consistent with what was published previously (Ahmadi *et al*., 2015). Obviously, EPSNC2 contains a high content of glucuronic and galacturonic so; it can block the viral cell surface receptor preventing adsorption or interaction with HSV gC and/or gB glycoproteins preventing HSV gC and/or gB-heparan sulfate interaction via mimicking heparan sulfate proteoglycans.

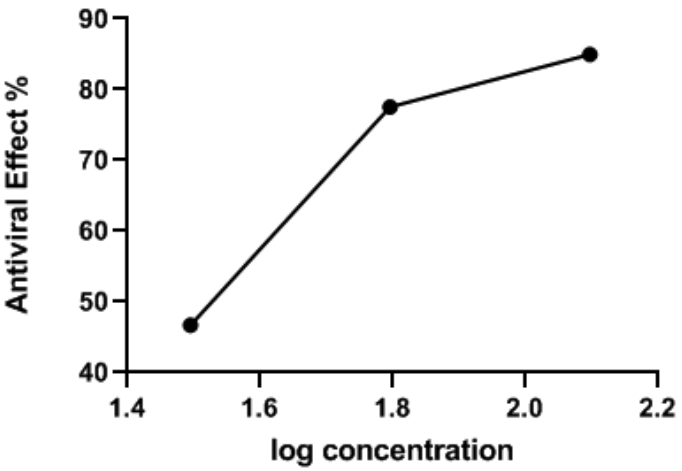

Figure 9. EC_{50} of EPSNC2 on HSV-1.

FINANCIAL SUPPORT

None.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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