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Characterization of the exopolysaccharides from *Rhodotorula minuta* IBRC-M 30135 and evaluation of their emulsifying, antioxidant and antiproliferative activities

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RESEARCH ARTICLE

General Note

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ABSTRACT

Some microorganisms such as yeasts can produce high molecular weight and valuable polymers that known as exopolysaccharides (EPSs). Given the functional properties of yeast exopolysaccharides (EPSs) and a few numbers of studies in this field, the present study was aimed to characterize the EPSs from *Rhodotorula minuta* (IBRC-M 30135) and to evaluate their emulsifying, antioxidant and antiproliferative activities. *R.minuta* excreted 1.2±0.1 g of the EPSs per 1000 ml of fermentation medium. Glucose, mannose and rhamnose were found as main monosaccharides of the EPSs (49, 38 and 13 mol%, respectively) through gas chromatography-mass spectrometry (GC-MS) analysis. In addition, three EPS fractions were obtained with the molecular weights of 356, 500 and 220 kDa. The water retention capacity of the EPSs was 342±27%. The EPSs exhibited relatively good oil-emulsifying activity and the enhanced synergism between the *R.minuta*'s EPSs and commercially used hydrocolloids (guar gum and sodium alginate) was observed. In vitro, antioxidant activity was investigated against DPPH, hydroxyl and superoxide free radicals. At a concentration of 10 mg/ml, the DPPH, hydroxyl and superoxide free radicals scavenging activities were 21.8±0.7%, 24.6±1.4% and 12.1±0.4%, respectively; and the scavenging activities against all were higher than hyaluronic acid. In addition, the treatment of human breast adenocarcinoma (MCF-7) and Madin-Darby Canine Kidney (MDCK) cell lines with 20-1000 µg/ml of the EPSs caused no significant differences in the cells proliferation (P>0.05). These results indicated the promising potential of the EPSs from *R.minuta* as non-toxic and biocompatible compounds for using in food, cosmetic and pharmaceutical fields.

Keywords: Exopolysaccharides, *Rhodotorula minuta*, antioxidant activity, emulsifying activity, antiproliferative activity.

1. INTRODUCTION

Polysaccharides are high molecular weight polymers, which can be obtained from various sources such as plants, microorganisms, algae and animals (Badel et al., 2011). These compounds have drawn increasing attention to their different and interesting biological activities as well as their industrial application properties in the last few decades (Wang et al., 2017). Compared to other sources, microbial exopolysaccharides (EPSs), which are located in the extracellular medium without any covalent bonds with microorganism membrane, exhibit many noticeable properties including easy production, cost effectiveness and diversity in the structures (Sutherland, 2001; Badel et al., 2011). Among the microbial EPS producers, bacteria and fungi are well-known for their ability to synthesize EPSs (Mahapatra and Banerjee, 2013). So far, a considerable number of yeasts were known as fungal strains producing EPS; however, many of them have not yet been investigated or are under exploration (Mahapatra and Banerjee, 2013; Gientka et al., 2015). According to Peterson et al, the isolation procedure of EPSs produced by yeasts is easier and faster than bacterial EPSs which is considered as an advantage in their production (Petersen et al., 1989).

More recently, yeast EPSs have received increasing attention due to their various bioactivities and several possible applications in the food and pharmaceutical industries (Mahapatra and Banerjee, 2013; Gientka et al., 2015). Yeast EPSs possess a wide range of functional properties including immunomodulatory (Gientka et al., 2015), antioxidant properties (Ghada et al., 2012), antiviral effects (Van Bogaert et al., 2009; Ghada et al., 2012), antimutant (Van Bogaert et al., 2009), antitumor (Ghada et al., 2012) and emulsifying activities (Kuncheva et al., 2007). These functional properties are affected by factors which are associated with the EPSs structures, such as their physico-chemical properties (monosaccharide composition, functional groups and molecular weight) (Mahapatra and Banerjee, 2013; Gientka et al., 2015). The microbial EPSs derived from different origins have different structures and their physical and chemical properties are the reasons for their unique properties (Mahapatra and Banerjee, 2013); however, various studies are required to examine EPSs and their functional properties.

Up to now, several strains of *Aureobasidium*, *Bullera*, *Candida*, *Cryptococcus*, *Debaryomyces*, *Lipomyces*, *Pichia*, *Pseudozyma*, *Sporobolmyces* and *Rhodotorula* were reported as yeast EPSs producers (Gientka et al., 2015).

Rhodotorula minuta is a pink glistening yeast that is commonly isolated from air, fresh water and sea water all over the world. *Rhodotorula minuta* BIOTECH 2178 was previously isolated from Laguna Lake in the Philippines and it was shown that this yeast is capable of producing biocompatible EPS (Ramirez, 2016), but its characterization was preliminary.

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The aims of this study were to figure out and analyze the physico-chemical properties of EPSs from *Rhodotorula minuta* (IBRC-M 30135), their water retention capacity and possible application of the EPSs as emulsifier and antioxidant. The possible antiproliferative activity against human breast adenocarcinoma cell line (MCF-7 cells) was evaluated as well.

2. METHODS AND MATERIALS

Microorganism and growth conditions

Rhodotorula minuta (IBRC-M 30135) was purchased from the Iranian biological resource center (IBRC). The basal medium for EPSs biosynthesis contained: 30 g/l glucose, 2.5 g/l $(NH_4)_2SO_4$, 1 g/l KH_2PO_4, 0.5 g/l $MgSO_4.7H_2O$, 0.1 g/l NaCl, 0.1 g/l $CaCl_2.2H_2O$ and 3 g/l yeast extract (Ghada et al., 2012). The initial pH of the medium was adjusted to 6. The cultivation was carried out in 1000 ml Erlenmeyer flasks containing 250 ml of the medium inoculated with 8% (v/v) 48 h inoculum (for 96 h at 28 ºC and 180 rpm).

Isolation and purification of EPSs

The obtained cultures were centrifuged at 7900 ×g, at 4 ºC for 15 min to separate the biomass from the supernatant. The EPSs in the supernatant were precipitated with two volumes of cold 96% ethanol at 4 ºC for 24 h, washed twice with cold 96% ethanol and centrifuged at 7900 ×g for 15 min at 4 ºC. The precipitated EPSs were dissolved in distilled water, dialyzed against distilled water for 24 h and stored at -80 ºC freezer. The frozen EPSs were freeze-dried using a Christ lyophilizer (Alpha 1-2 LDplus, Germany) to obtain constant weight of the EPSs and then the lyophilized EPSs were weighed using a precision balance. The carbohydrate and protein contents of the EPSs were tested by the phenol-sulphuric acid (Dubois et al., 1956) and Bradford (Bradford, 1976) methods using glucose and bovine serum albumin (BSA) as standards, respectively.

Fourier transform infrared (FT-IR) spectroscopy

The infrared spectrum of the EPSs was measured using a Perkin Elmer spectrum two FT-IR system (USA). The lyophilized EPSs (3 mg) were dispersed on the universal attenuated total reflectance (UATR) for FT-IR analysis in the frequency range of 400-4000 cm^{-1}. The analysis of IR spectra was carried out using spectrum ES software.

Scanning electron microscope (SEM)

Surface morphology of the EPSs was studied under field emission scanning electron microscope (TESCAN MIRA3, Czech Republic) after gold coating.

Molecular weight determination

Molecular weight of EPSs was measured using HPLC (Agilent 1100 Series) - steric exclusion chromatography system equipped with refractometer (RID). The analytical columns were TSKGEL PW-XL 3000 (10 µm, 7.8 x 300 mm) and TSKGEL PW-XL 5000 (10 µm, 7.8 x 300 mm) that preceded by a TSKgel PWXL pre-column (12 µm, 6.0 x 40 mm). The mobile phase was acetic acid 0.15M + sodium acetate 0.1M solution at a flow rate of 1ml/min, and the columns temperature was 30 ºC. Pullulan standards (Sigma-Aldrich) with different molecular weights (1.3 kDa to 800 kDa) were used. The EPSs and pullulan standards were injected (20µl) at 10g/l in mobile phase.

Monosaccharide composition analysis

Monosaccharide compositions of the EPSs were determined using gas chromatography-mass spectrometry (GC-MS) (Agilent 6890 Series GC System coupled to an Agilent 5973 Network Mass Selective Detector) according to the methods of Benaoun et al. (Benaoun et al., 2017). Briefly, ten milligrams of the EPSs were hydrolyzed with 1 ml of 2 M trifluoroacetic acid (TFA) at 120 °C for 90 min in a dry water bath. Then, the hydrolyzed product was evaporated under a stream of nitrogen. Trimethylsilylation derivatization was performed according to Pierre et al (Pierre et al., 2014). Sugar identification was done by comparing it with standard L-Rha, L-Fuc, L-Ara, D-Xyl, D-Man, D-Gal, D-Glc, D-GlcA, D-GalA, D-GlcN and D-GalN.

Water retention capacity

The water retention capacity of the EPSs was measured according to the methods described by Niknezhad et al (Niknezhad et al., 2018).

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Emulsifying activities

The emulsifying activities of the EPSs (0.5, 1 and 1.5 % w/v) on sun flower oil substrates were measured according to Priyanka et al (Priyanka et al., 2014) for 24 h. The emulsifying activity was calculated as the percentage of the height occupied by the emulsion layer respect to the total height after 24h. Guar gum and sodium alginate were used as controls at concentration of 0.5 % w/v. The synergistic effect of the EPSs in relation to emulsifying activities was investigated in mixtures with guar gum and sodium alginate.

Antioxidant activities

Different concentrations of the EPSs (0.1, 0.25, 0.5, 1, 2.5, 5 and 10 mg/ml) were used to assess their antioxidant activities. The free radicals (DPPH•, •OH, O_2^-•) scavenging activities were measured as the indicators of antioxidant activities of the EPSs based on the methods of Niknezhad et al (Niknezhad et al., 2018). The hyaluronic acid was used as a positive control to scavenge the radicals because of its ability (Sato et al., 1988).

Cell culture

Human breast adenocarcinoma cell line (MCF-7 cells) and Madin-Darby Canine Kidney cell line (MDCK cells) were purchased from Pasteur Institute of Iran (Tehran, Iran) and cultured in RPMI-1640 (Gibco, Karlsruhe, Germany) and Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Karlsruhe, Germany) media, respectively, at 37 ºC in humidified air containing 5% CO_2. All media were supplemented with 10% fetal bovine serum (Gibco, Karlsruhe, Germany), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, Stockholm, Sweden).

MTT assay

MTT assay is a colorimetric assay to assess cell viability. In present study, MTT assay was used to evaluate the EPSs activity on the cells proliferation. The cells (4×10^3) were allowed to attach overnight and then treated with different concentrations of the EPSs (20, 50, 100, 250, 500 and 1000 µg/ml in FBS free media) for 24 and 48 h. MTT was then mixed the cells at 37 ºC and incubated for 4 h in a humidified CO_2 incubator at 5% of CO_2. The formazan crystal was dissolved in dimethyl sulphoxide (DMSO) and optical density (OD) was then measured on an ELISA plate reader (Bio Tech Instruments, USA) at 570nm. Data were normalized to a control group (cells that were only treated with the media).

Statistical analysis

All of the measurements were performed in triplicate and the results were expressed as the mean±SD. One-way analysis of variance (One-way ANOVA) followed by a post hoc Tukey's test was used to analyze the data ($P< 0.05$) using SPSS software version 16.

3. RESULTS AND DISCUSSION

Rhodotorula minuta (IBRC-M 30135) produced 1.2 ± 0.1 grams of lyophilized EPSs powder per liter of the fermentation medium. The total carbohydrate and the total protein contents of the EPSs were 75% and 0.9%, respectively. The EPSs exhibited high carbohydrate content similar to other studies. They contained low protein content and had good purity considering that the EPSs did not undergo any costly purification processes such as microfiltration or diafiltration. The purity of the EPSs was better than the EPS produced by *Rhodotorula minuta* BIOTECH 2178 (59-64% of carbohydrate and 17% of protein content) (Ramirez, 2016).

The FT-IR spectrum of the EPSs from *Rhodotorula minuta* (IBRC-M 30135) is shown in Fig. 1 which displays the functional groups in the 400-4000 cm^{-1} region. The peak at 3430.09 cm^{-1} was corresponded to –OH (hydroxyl) stretching and the weak band at 2932 cm^{-1} was due to the aliphatic C-H (sp^3) stretching (Wang et al., 2017). The bands in the regions of 1643 cm^{-1} and 1376 cm^{-1} could be attributed to the C=O stretching (Garza et al., 2016). In 1447.8 cm^{-1}, there was a signal corresponding to the presence of C-O-C (for typical structure of glucose in pyranose form)(Ramirez, 2016). The peaks at 1097.6 cm^{-1}, 1123.6 cm^{-1} and 1198 cm^{-1} were corresponded to the C-O stretching vibrations (Zhang et al., 2013).

The microstructures of the EPSs are presented by FE-SEM (as shown in Fig 2). The micrograph of EPSs showed porous microstructure.

The GC-MS and HPLC-SEC methods were applied to investigate monosaccharide compositions and molecular weight distribution of the EPSs. As shown in Table. 1, the EPSs were consisted of glucose, mannose and rhamnose (49%, 38% and 13%, respectively) and they had three fractions with different molecular weights (356, 500 and 220 kDa). These results suggested that the obtained biopolymer was a hetero-polysaccharide. Previously, Ramirez carried out only the preliminary characterization of the EPS produced by *Rhodotorula minuta* BIOTECH 2178 using FT-IR spectroscopy (Ramirez, 2016) and no more details have been reported

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on its monosaccharide compositions or molecular weight. The monosaccharide compositions of EPSs produced by some species of *Rhodotorula* have been described; for example, EPSs produced by *Rhodotorula acheniorum* MC were hetero-polysaccharides and were composed of mannose and glucose (with molecular weights of 310 and 249 kDa) (Pavlova et al., 2005); and the EPSs produced by *Rhodotorula mucilaginosa* CICC 33013 were constituted by neutral sugars (galactose, glucose, mannose and arabinose) (Ma et al., 2018). The presence of rhamnose in our EPSs indicated that they were the novel types of *Rhodotorula*'s EPSs, because there has not been reported any rhamnose in EPSs produced by *Rhodotorula* genus (Gientka et al., 2015).

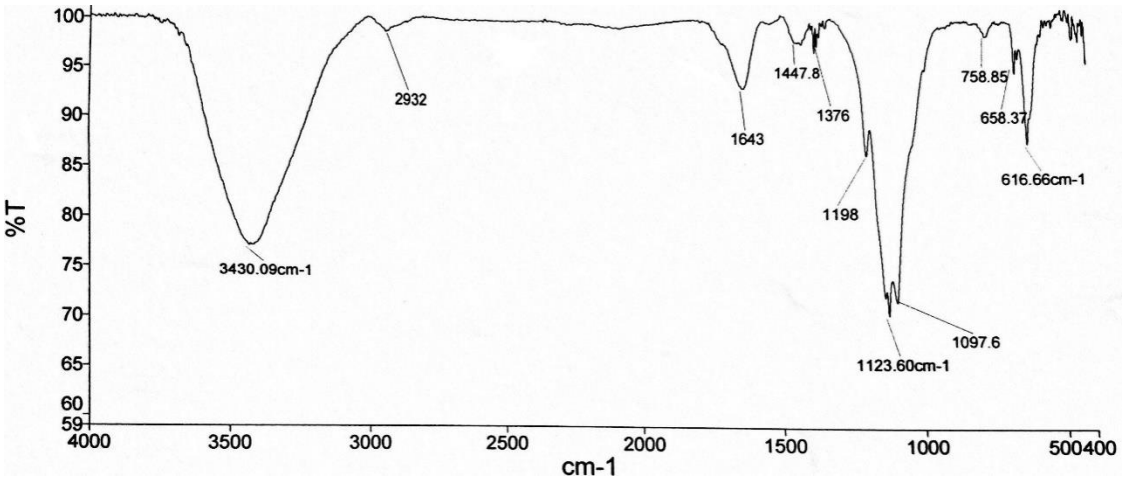

Figure 1 FT-IR spectrum of the EPSs produced by Rhodotorula minuta IBRC-M 30135.

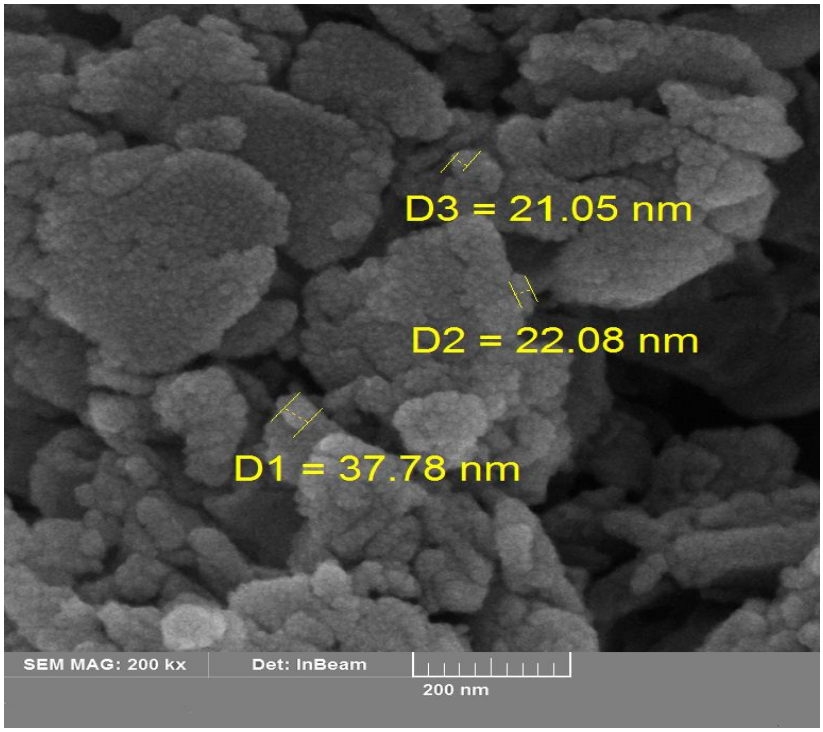

Figure 2 FE-SEM micrograph of the EPS from *Rhodotorula minuta* IBRC-M 30135

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Table 1 Monosaccharide composition and molecular weight distribution of the EPSs extracted from *Rhodotorula minuta* IBRC-M 30135

Monosaccharides (mol %)				Mw (g/mol)
Glc	Man	Rha	Glc/ Man	3.56×105, 5×105, 2.2×105
49	38	13	1.3	

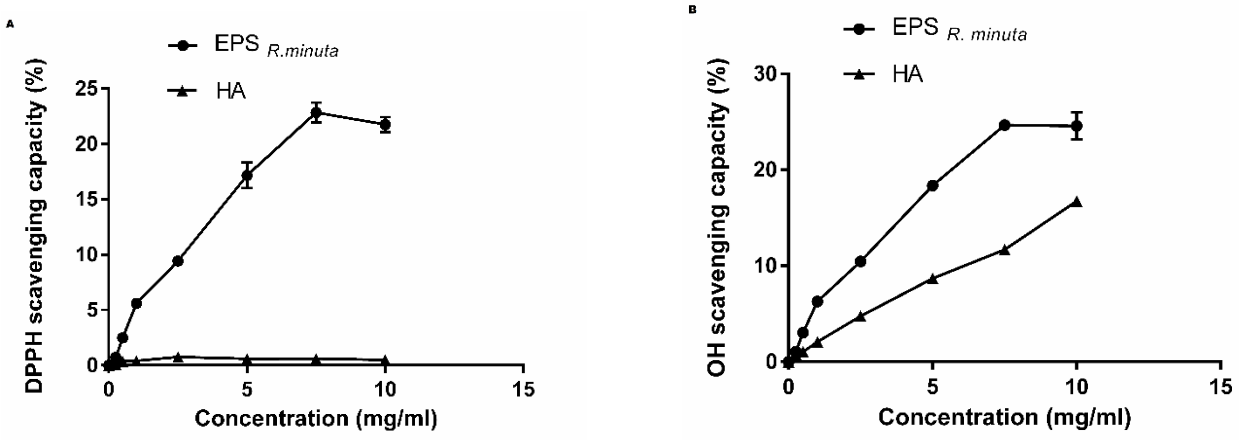
The water retention capacity of the EPSs was 342±27% and it was greater than that of EPS produced by *Weissella cibaria* YB-1 and *Leuconostoc lactis* KC117496 (287.84 and 117% respectively) (Saravanan and Shetty, 2016; Ye et al., 2018). The EPSs with high water retention capacity are good choices to use in the food and pharmaceutical industries (Ramirez, 2016).

The emulsifying activities of the EPSs are shown in Table 2. The results showed that the EPSs were capable of emulsifying mixtures of sun flower oil and water; the emulsifying activities were enhanced when the concentration of EPSs increased. Also, using sodium alginate and guar gum, the synergistic effect of the added commercial hydrocolloids on emulsifying activity reached 58.3±0.6% and 81.7±1.5%, respectively. Many of the EPSs were capable of generating oil in water emulsions; according to Llamas et al, the protein moieties of the EPSs may increase their emulsifying activities (Llamas et al., 2012). Similarly, the positive effects of commercial hydrocolloids on the emulsions produced by EPSs have been reported (Kuncheva et al., 2013; Radchenkova et al., 2014).

Table 2 Emulsifying activities of the EPSs synthesized by *Rhodotorula minuta* IBRC-M 30135 and commercial hydrocolloids

Emulsifiers origin	**Emulsifying activity (%)**
0.5% EPSs	40.3±1.5
1% EPSs	44.3±0.6
1.5% EPSs	45.7±0.6
0.5% sodium alginate	42.3±1.2
0.5% guar gum	72.3±1.5
0.5% guar gum + 1% EPSs	81.7±1.5
0.5% sodium alginate + 1% EPSs	58.3±0.6

Antioxidant activities of the EPSs were estimated using three different methods, namely DPPH•, •OH and O_2^-• scavenging activities. Hyaluronic acid, which is largely used in the cosmetic industry, was used as a positive control. As shown in Fig 3A-C, the antioxidant activities of the EPSs were increased due to their concentrations and were higher than that of hyaluronic acid. At a concentration of 10 mg/ml, the DPPH•, •OH and O_2^-• scavenging activities of the EPSs were 21.8±0.7%, 24.6±1.4% and 12.1±0.4%, respectively. According to Wang et al., EPSs can quench free radicals by donating hydrogen atom or transferring single electron and the presence of functional groups such as –OH, -O- and C=O are in favor of free radicals scavenging activities (Wang et al., 2016). In addition, antioxidant activities of EPSs were considered to be dependent on several physical and chemical factors (such as molecular weight and chemical composition) (Zhu et al., 2018) and are not easily predictable. Nevertheless, the antioxidant mechanisms of EPSs are not systemically disclosed (Wang et al., 2016).



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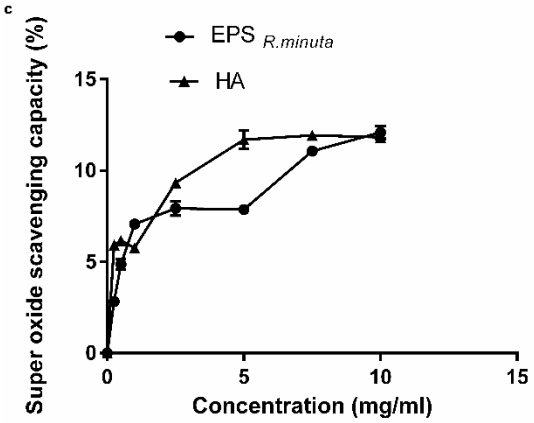

Figure 3 The DPPH radical (A), hydroxyl radical (B) and superoxide anion radical (C) scavenging activities (capacities) of the EPSs from Rhodotorula minuta IBRC-M 30135 with hyaluronic acid as the positive control

MTT assay was performed to evaluate the effects of the EPSs on the proliferation of MCF-7 and MDCK cells over 24 and 48 h exposures. As shown in Fig 4A-B, the cells viabilities were not significantly different compared to the control (0 µg/ml) and also there was no significant difference between the time duration of the treatments under the exposures to each of the matched EPSs (P>0.05).These findings were in concordance with the results of other studies (Kianpour et al., 2018; Hamidi et al., 2019). According to the results, the EPSs are non-toxic and biocompatible as natural biopolymers and the biocompatibility is one of the favorable features for using in formulation of food and pharmaceutical products.

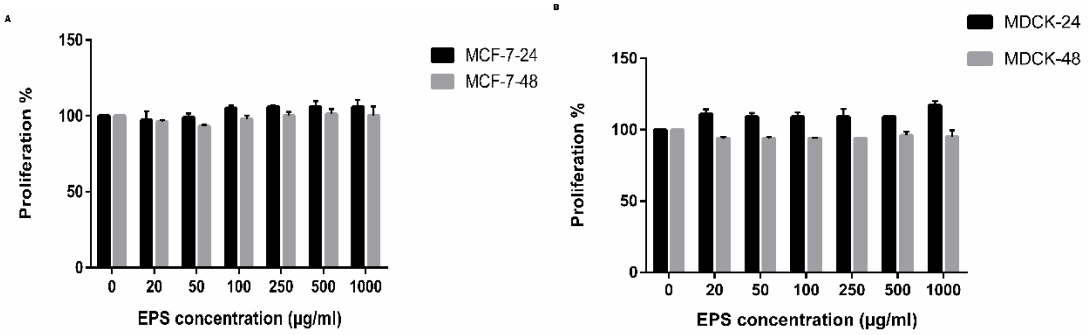

Figure 4 Cytotoxicity assay of the EPSs on MCF-7 cell line (A) and MDCK cell line (B) in various concentrations after 24 and 48 h exposure. Each value is expressed as mean ± SD (n=3)

4. CONCLUSION

Rhodotorula minuta (IBRC-M 30135) excreted 1.2 ± 0.1 g/l of EPSs. The EPSs were composed of glucose, mannose and rhamnose; and had three fractions with different molecular weights. The EPSs from *Rhodotorula minuta* (IBRC-M 30135) exhibited considerable emulsifying and antioxidant activities; and had a good water retention capacity. The EPSs can be applied as non-toxic and biocompatible polymers in food and cosmetic fields.

Ethical approval

All authors hereby declare that all experiments have been approved by the appropriate ethics committee from Baqiyatallah University of Medical Sciences and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki (IR.BMSU.REC.1397.179).

Conflict of Interest

The authors of this study have declared no conflict of interest.

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Disclosure statement

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