



Nano-Exopolysaccharide From The Probiotic *Weissella Paramesenteroides* MN2C2: Production, Characterization And Anticancer Activity

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Abstract

Exopolysaccharides nanoparticles (EPS-NPs), especially from probiotics, became one of the most innovative approaches for intelligent cancer therapy due to their stability and biocompatibility. In a previous study performed by the research team, a polysaccharide, produced by *Weissella paramesenteroides* MN2C2 was identified as a sulphated hetero-polysaccharide. In this study, this exopolysaccharide was partially purified and the cultivation period, pH and temperature were optimized. Nano-forms of crude and partially purified EPS were synthesised by sonication and characterized by Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS). The particles were platelet-like and their size diameter ranged from 37 to 105 and from 45 to 204 nm for crude and partially purified EPS-NPs respectively. Both EPS-NPs were effective and highly selective against Colon Caco-2, Liver HepG-2 and Breast MCF-7 malignant cells. They were also found to have potent DPPH antioxidant activities dependent on their concentration. The producing probiotic strain showed high sensitivity towards Tetracycline, Fusidic acid and Ampiciline with no haemolytic activity. And it was tolerant to low pH, bile salts and pancreatic juices, and exerted antimicrobial activity against *Pseudomonas aeruginosa* and *Bacillus subtilis*. These findings suggest that nano-EPS from the probiotic *W. paramesenteroides* possess excellent characteristics for potential medicine applications.

Keywords: Nano-EPS, characterization, probiotic, *Weissella paramesenteroides*, safety, anticancer, antioxidant.

1. Introduction

In recent years, EPS nanoparticles (EPS-NPs) have been developed to overcome many problems, including targeting and efficiency, so they can be applied as pharmaceutical and nutraceutical products due to their stability, biocompatibility, renewability, biodegradability, unique properties and low toxicity [1]. EPS-NPs have different physicochemical characters; such as morphology, particle size, loading capacity and surface charge distribution; which influence their mode of action and *in vivo* lifetime [2].

Lactic Acid Bacteria-EPSs have varying composition and applications, therefore, they are used as anti-cancer, anti-inflammatory, anti-ulcer, antiviral [3] and antimicrobial agents [4]. In addition, their structure is highly related to its application [5] for example, sulfated EPS has gained more attention due to its unique structure, properties and activities so it is widely applied in modern biotechnology [6].

The production conditions, especially the medium nutrients as well as certain physical parameters, affect strongly the secretion process of various extracellular products by microorganisms [10]. In the current study, the probiotic bacteria, which are safe and useful bacteria for their hosts [11] were applied as producer microorganism. Recently EPS are considered as therapeutic agents for the treatment of different diseases such as allergy, gastrointestinal diseases, urogenital infections and they also increase the resistance of the body to infectious diseases [12]. Moreover, they were also found to participate in the prevention of cancer, and increase the body response to different medical treatments [13]. The true probiotic is defined as the strain which can exist in harsh environments; such as low pH, presence of bile salts or gastric juices; as well as having the ability to adhere to gut epithelia [14]. Furthermore, their use by human beings requires that they must fulfill some

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special safety properties including their susceptibility toward certain standard antibiotics, their inability to cause hemolysis activity, in addition to their production of aggregation substances [11]. Probiotics are the most famous example of lactic acid bacteria (LAB) such as *L. Brevis* LB63, *L. rhamnosus* and *L. delbrueckii*, [3] and *Weissella* strains [15] which are considered one of the most potential examples of LAB bacteria as they were found to produce medically relevant compounds. These bacteria have a beneficial association with human hosts because they can reduce the incidence of serious human diseases [16] through the production of functional exopolysaccharides (EPS) [5].

This study aimed to investigate the probiotic potentiality of *Weissella paramesenteroides* MN2C2 and its safety aspects. Also, a nano-EPS was produced, prepared and characterized then evaluated for its application as a stable biocompatible anticancer and antioxidant drug.

2. Experimental

2.1. Microorganism and culture conditions

The strain was previously isolated by our research team from buffalo colostrums after 48 h of delivery and identified as *Weissella paramesenteroides* MN2C2 under the accession number of MK530206 in gene bank [17]. The organism (10^6 CFU/ml) was inoculated in DeMan-Rogosa-Sharpe (MRS) broth medium and incubated aerobically at 37°C for 24 h at pH 6.5 in static incubator. At the end of the incubation period, the culture was mixed to a solution of 40% glycerol and the resulting suspension was stored at -80°C until needed [18].

2.2. Probiotic properties

The following major criteria were chosen for the *in vitro* determination of probiotic properties of *W. paramesenteroides* MN2C2.

2.2.1. Tolerance to acidity and bile salts

W. paramesenteroides MN2C2 cultures were grown overnight in MRS broth at 37°C, then subcultured in 10 ml broth of the same medium adjusted at different pH values (1.5, 3, 5 and 9) using HCl and NaOH. Also, the oxgall bile (0.3 % and 0.7 % from Sigma, St. Louis, MO) was added to the cultures and then all samples were incubated at 37°C for 3 and 6 h. The survival percentages of the tested samples compared to controls were detected by serially diluting the cultures then 0.1 ml of each dilution was plated onto MRS agar plates and incubated for 24 h [19]. The survival rate (%) was then calculated using the

following formula: Survival rate (%) = [Biomass at time (t)/Biomass at initial time (0)] × 100.

2.2.2. Tolerance to pancreatic fluid

The cultures were inoculated in MRS broth for 24 h at 37°C, then 30 µl were transferred into the micro titer plates each containing 270 µl of test medium (150 mM NaHCO₃ and 1.9 mg/ml pancreatin (Sigma); pH 8). The cultures were incubated in shaking incubator at 37°C, and then the survival rate was determined at different time intervals of 0, 3 h and 6 h by plating into MRS agar plates which are then re-incubated for 24 h [20].

2.2.3. The viability in a simulated gastrointestinal solution

To simulate the stomach conditions, a sterilized solution of NaCl (0.9% w/v) adjusted at pH 2.0 was prepared then pepsin (0.3%) (purchased from Science Lab.Texas, USA) was added. After 2 h of strain exposure to stomach solution, the small intestine conditions were simulated by adding; 0.3% bile salts; 0.083 KCl; 0.65 NaCl; 0.138 NaHCO₃; 0.022 CaCl₂ and 1 pancreatin (purchased from BIOBASIC INC, Canada) then sterilized and the pH was adjusted to 7.0. The survival rate was calculated at 0, 3 and 6 h of incubation as mentioned above (modified method from ElSayed et al., 2014 [21]).

2.2.4. Hydrophobicity

Overnight culture was collected by centrifugation then washed twice and resuspended in phosphate buffer solution to a final OD of 0.5 at 600 nm (this absorbance result was designated as A0). After that, 3 mL of this suspension was added to 1 mL of toluene then mixed for 90 s and left to stand for 1 h at 37°C to allow phase separation. The toluene phase was removed and the aqueous phase was measured at 600 nm to obtain A1 reading [22]. The decrease in the absorbance of the aqueous phase after exposure to toluene was calculated to determine the cell surface hydrophobicity percentage according to the following equation:

$$\text{Hydrophobicity (\%)} = [1 - (A1/A0)] \times 100$$

2.3. Safety assessment

2.3.1. Susceptibility to antibiotics

Antimicrobial susceptibility discs were obtained from bioMerieux (Marcy l'Etoile, France). The susceptibility of the tested strain to six different antibiotics (including kanamycine, ampicilline, fucidic acid, tetracycline, vancomycine and

erythromycin) was tested using the disk diffusion method. The obtained inhibition zones diameters were measured and the results were categorized as: resistant (R), intermediate (I), and susceptible (S), according to the cut off levels proposed by NCCLS (2002). Inhibition zone diameters were measured in mm and the diameters ≥ 21 mm was referred as sensitive and ≤ 15 mm as resistant; whereas the intermediates fell between 16-20 mm [23].

2.3.2. Hemolytic activity

To test the hemolytic activity, the strain was subcultured on MRS agar plates as mentioned above, and then streaked on Columbia agar plates containing sheep blood (5%). The plates were anaerobically incubated at 37°C for 48 h. The colonies that formed green-hued zones or didn't have any effect on the blood plates were considered non hemolytic whereas, the colonies that showed blood lysis zones were classified as hemolytic thus having β -hemolysis activity [24].

2.3.3. Antimicrobial activity

Agar well-diffusion assay was used to assess the antimicrobial activity of the strain against five indicator microorganisms as shown in Table 1 [25]. Briefly, the nutrient agar medium, containing the indicator strain of approximately 10^5 cells/ml, was poured into plates. Wells were then cut using a sterile cork borer and 100 μ l of overnight MRS culture filtrate was added in each well. Plates were incubated at 37°C for 48 h. After incubation, the antimicrobial activity was evaluated by measuring the inhibition zones diameters around holes in millimeter.

Table 1: Indicator microorganisms

Strain type	Indicator organism	Source
Gram positive	<i>Bacillus subtilis</i>	ATCC 6633
	<i>Staphylococcus aureus</i>	ATCC 6538
Gram negative	<i>Escherichia coli</i>	ATCC 35218
	<i>Pseudomonas aeruginosa</i>	ATCC 9027
Fungi	<i>Candida albicans</i>	ATCC 10231

ATCC; American Type Culture Collection.

2.4. Production conditions of EPS

For EPS production, the LAB under test was inoculated in MRS broth, in the concentration of 2%, in triplicate flasks. The inoculated flasks were then incubated under static aerobic conditions. Different incubation periods (6-72h), different pH (5-8) and different temperature degrees (25-45°C) were studied. The EPS production was checked as mentioned by Zisu and Shah (2003) [26].

2.5. Extraction of EPS

To extract EPS from the culture medium, the collected supernatant was neutralized to pH 6.8 and heated for 30 min in a boiling water bath then centrifuged at 4°C for 20 min to discard cells. An equal volume of cold ethanol was added to the supernatant then stored at 4°C for 18 h. The precipitated EPS was centrifuged and washed with distilled water then dialyzed against deionized water at 4°C for 24 h; freeze-dried and weighed [26].

2.6. Determination of EPS concentration

The amount of produced EPS in the fermentation medium was estimated according to the method of Du Bois *et al.* [27], as follows: In an ice cold water bath equal volumes of 1ml, of sample and of 5% phenol, were mixed, then, 5 ml of concentrated H₂SO₄ (35.7 N) were carefully added. After 20 min of incubation, the intensity of the developed orange color was estimated by measuring its absorbance at 490 nm. The amount of EPS present in the sample was eventually calculated from a previously performed standard curve using different fructose concentrations ranging from 0 to 500 μ g from a 1 mg/ml fructose stock solution.

2.7. Partial purification of EPS

About 5 liters of MRS broth, inoculated with 2% inoculum of *W. paramesenteroides* MN2C2 (10^8 CFU/ml), were used for large scale production of EPS. Then Trichloroacetic acid (TCA) solution (10%) was added to remove proteins. The supernatant was dialyzed as mentioned above and freeze-dried. Then the partially purified EPS was dried at 50°C and weighted [26].

2.8. Characterization of partially purified EPS

In a previous study, performed by our research team, the spectroscopic and chemical analyses of the partially purified EPS were done using Ultraviolet-visible spectroscopy, Fourier-transform infrared (FTIR), Nuclear magnetic resonance (NMR) and High Performance Liquid Chromatography (HPLC) as well as, Scanning Electron Microscopy (SEM), Energy Dispersive X-Ray (SEM-EDX) and Mapping analysis [17].

2.9. Preparation of EPS-NPs

The nanoparticles of both crude and partially purified EPSs (EPS-NPs) were prepared according to Taurozzi *et al.* (2012) [28] method with some modifications. The powdered EPSs were dissolved in distilled water and the produced suspension was sonicated for 5 min on ice bath using horn sonicator using 45% power (Cole-parmer instruments, CPX750, USA). Then the produced solution was filtered using a filter membrane (0.45mm) and lyophilized.

2.10. Characterization of EPS-NPs

2.10.1. Transmission Electron Microscopy (TEM)

The morphological characteristics of the formed nanoparticles were detected by Transmission Electron Microscopy (TEM). The imaging was carried out using Electron probe micro-analyzer (JEM-1400-USA). The film samples prepared on the TEM grid and allowed to dry then the images of the nanoparticles were taken as described by Chen and Subirade (2005) [29].

2.10.2. Dynamic light scattering (DLS)

To measure the size distribution of the formed nanoparticles; the dynamic light scattering (DLS) (NICOMP 380 ZLS, PSS, Santa Barbara, CA, USA) was used [30].

2.11. In vitro anticancer activity

The cytotoxicity assay was evaluated in the Embryology and Cell Culture Lab, Faculty of Agriculture, Cairo University Research Park (CURP). Lung cancer (A549), Breast cancer (MCF-7), Colorectal adenocarcinoma (Caco-2), Hepatocellular carcinoma (HepG-2), and Human Lung normal (Wi-38) cell lines were used. A concentration of 10^3 cell/well was inoculated in 96-well microtiter plates containing Roswell Park Memorial Institute (RPMI 1640) medium then incubated at 37°C for 24 h under 5% CO₂ incubator. Then a fresh, serum free, medium was added followed by the addition of different concentrations of the tested samples (0.5-10.0 mgml⁻¹) then the plates were incubated for further 48 h against negative control (cells alone). After that, 40 µl of 2.5 µg ml⁻¹ MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) were added to each well and incubated at 37°C for 4 h. Amount of 200 µl of sodium dodecyl sulphate (10% SDS) was added to each well and incubated overnight to stop the reaction. Doxorubicin standard (50 µg ml⁻¹) was prepared to give 100% lethality (positive control). The absorbance was measured at 540 nm using a microplate multi-well reader [31]. Viability % = [Reading of extract/Reading of negative control - 1] × 100

Selectivity index (SI)

High SI values (> 2) indicate selective toxicity of the tested samples towards cancer cells, while low SI values (< 2) reveals that the tested substances are broadly toxic to normal cells [32]. Each SI value was calculated by: SI = IC₅₀ normal cell/IC₅₀ cancer cell.

2.12. Antioxidant activity using DPPH

Radical scavenging assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method was applied to detect the activity of EPS-NPs samples [33]. Different concentrations of both crude and partially purified EPS-NPs samples (40, 80, 120, 160, 200, 240 µg/mL) were prepared in methanol. After that, 100 µl of each sample were added to 900 µl of DPPH and mixed well then incubated in the dark for 30 min. The decrease in absorbance was measured at 517 nm against a control without sample.

% Free radical scavenging = [(A control - A sample)/A control] × 100

Where A Sample is the absorbance of DPPH solution with sample and A control is the absorbance of DPPH solution in methanol without sample.

2.13. Statistical analysis

All of the determinations, reported in this study, were performed in triplicate, and the results were presented as mean values.

3. Results and discussion

3.1. Tolerance of the probiotic strain to acidity, bile salts as well as pancreatic and gastrointestinal juices

In vitro assessment of probiotic tolerance under the conditions present in the gut such as low pH, bile salts and pancreatic fluid was tested due to its importance in many applications [34]. The tolerance of the tested strain to acidic/alkaline pH values (1.5, 3.0, 5.0 and 9) was studied after 3 and 6h of incubation and the strain was found to be resistant to very low pH (1.5) for 6h as it recorded a survival rate that reached 52.2 (Table 2). By increasing the pH to 3, 5 and 9, the survival rate increased to 72.5, 95.6 and 97.1% respectively, indicating that the strain is considered as a potential probiotic that can survive in stomach-like medium for long time [35].

On the other hand, the strain was tolerant to 0.3% bile salts giving the highest viability percentage of 78.9% after 3h. However, at 0.7% bile salts, a high reduction in the cell count was observed and this could be attributed to their effect on the cell membrane of the microbe which depends on the microorganism species [36]. The viability of the strain was maximum (86.8%) after 3h of incubation in the pancreatic juice. Similar results were obtained by Ślizewska et al., 2020 [37] who recorded a high survival rate of about 90% for different LAB isolates grown under acidic conditions and bile salts. The ability of MN2C2 to survive while passing through

the gastrointestinal tract, therefore under simulated GIT conditions, was determined by El-Sayed et al., (2014) [21]. The surviving ability of MN2C2, after 2 h of cultivation under stomach conditions, was only 56% of the initial colony count. On the other hand, a good survival rate was detected when the strain was subjected to intestinal juices conditions which reached 91% after 6h. Similarly, Gharbi et al., (2019) [38] found that probiotic strains can survive under high levels of bile salts as well as stomach and intestinal juices conditions.

3.2. Hydrophobicity

The ability of bacteria to adhere to the intestinal mucosa layer is a very important matter, not only for the natural intestinal colonization, which is usually an exclusive property of pathogens microbes, but also to allow immune-modulation and the synthesis of valuable bacterial metabolites [39]. The hydrophobicity level of the strain under test was 57% suggesting that it has a good ability to adhere to the host cells and this criteria is considered as a good probiotic property as mentioned by Pabari et al., 2020 [4].

Table 2 Tolerance to pH, bile salts, and pancreatic enzymes

Test	3h.		6h.	
	CFU×10 ⁷	Survival %	CFU×10 ⁷	Survival %
pH 1.5	160±0.2	78.9±0.3	105±0.2	52.2±0.2
pH 3	192±0.2	94.7±0.2	147±0.2	72.5±0.3
pH 5	198±0.3	97.4±0.2	197±0.3	95.6±0.4
pH 9	160±0.2	97.4±0.4	124±0.2	97.1±0.2
Bile salt 0.3%	160±0.3	78.9±0.3	1240.3	60.9±0.1
Bile salt 0.7%	27±0.4	13.4±0.2	26±0.4	12.9±0.3
Pancreatic fluid	176±0.2	86.8±0.3	173±0.2	85.5±0.2

3.3. Safety assessment (microbial susceptibility, hemolytic and antimicrobial activities)

The strain was examined for its ability to suppress the growth of some pathogenic bacteria, specified in Table 1, as one of the main factors to evaluate its probiotic potentiality [40]. The strain was found to have an antimicrobial potency against *B. subtilis* and *P. aeruginosa* as inhibition zones of 25 and 19 mm were recorded respectively (Fig. 1.). Likewise, Pabari et al., 2020 [4], reported that *Weissella paramesenteroides* isolated from fruits, had antimicrobial activity against *E. coli* and *Staph. aureus*. Furthermore, these results are in agreement with those of Dinoto et al., 2018 [41] who found that *W. paramesenteroides*, isolated from eel intestines,

had broad spectrum antimicrobial potential against *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus agalactiae*, *Aeromonas hydrophila*, and *Salmonella enterica* via the secretion of different antimicrobial agents [42].



Fig. 1. Antimicrobial activity of *W. paramesenteroides* MN2C2

Table 3 elucidates the microbial susceptibility to six different antibiotics. The organism was highly sensitive to tetracycline, fusidic acid and ampicillin but resistant to vancomycin and kanamycin. These results were previously reported by Samedi and Charles, 2019 [43] who stated that almost all probiotics were interpreted as sensitive to tetracycline, ampicillin and chloramphenicol, while resistant to vancomycin [44] and, according to Pabari et al., 2020 [4], this is a widespread characteristic for known lactobacilli with no haemolytic activity. Similar data were also showed for *W. cibaria* and *W. confusa* strains isolated from a traditional Korean fermented vegetables food [45]. Furthermore, the strain had no hemolytic activity on blood agar plates, so it is recognized as a safe microorganism. This result was confirmed by Garai et al., 2007 [46] who proved that *Weissella* strains doesn't show haemolytic activity which is a characteristic associated to pathogenic virulence that causes toxicological effects upon its accumulation in large quantities.

Table 3 Antibiotic susceptibility profile of *W. paramesenteroides*

Antibiotic	Antibiotic conc. (µg)	I.Z.* (mm)	Sensitivity
Ampicillin	10	21	S
Vancomycin	30	11	R
Kanamycin	30	7	R
Tetracycline	30	25	S
Fusidic acid	10	22	S
Erythromycin	15	16	I

*Inhibition zone

3.4. Optimization production of EPS

The results in Fig. 2 revealed that EPS production was growth associated since the later was detected in the medium after only 6 hours of incubation and was found to follow the standard growth curve of the strain very closely. The highest EPS yield (1110 mg/L) was reached after 24 h of incubation which more or less coincide with the end of the logarithmic phase of growth of the producing microorganism. These results are in accordance to those of *De Vuyst et al.*, (1998) [47] who reported that the EPS production occurs at the onset and during the stationary phase of LAB growth. However, an obvious decrease in the EPS production curve was detected after 48 hr of incubation. According to *Zisu and Shah* (2003) [26], this decline was attributed to the enzymatic degradation of the produced EPS.

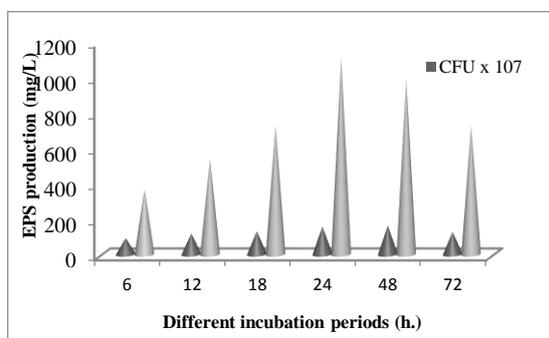


Fig. 2. Effect of different incubation period on EPS production

The results also show that, however the growth of the strain after 24 h of incubation was more enhanced when the initial pH was adjusted within a slightly acidic range (5.5-6.5), the EPS production increased from 1200 to 1700 mg/L when the pH was shifted from 6.5 to 5.5, respectively (Fig. 3). This result coincides with those obtained by *Vanangelgem et al.*, (2004) [48] and *El Shafei et al.*, (2011) [49]. The former observed that the viscosity of the medium, which result from the significant production of EPS, apparently increases when the pH was dropped below 5.75 during the fermentation of *S. thermophiles*, and the later recorded the pH of 5.5 to be the optimum pH for EPS production by *Lb. bulgaricus*. Furthermore, the different incubation temperatures were studied as well and the recorded results proved that the maximum EPS yield of about 1900 mg/L was reached at the optimum temperature of 35°C and this result was previously confirmed by *El Shafei et al.*, (2011) [49].

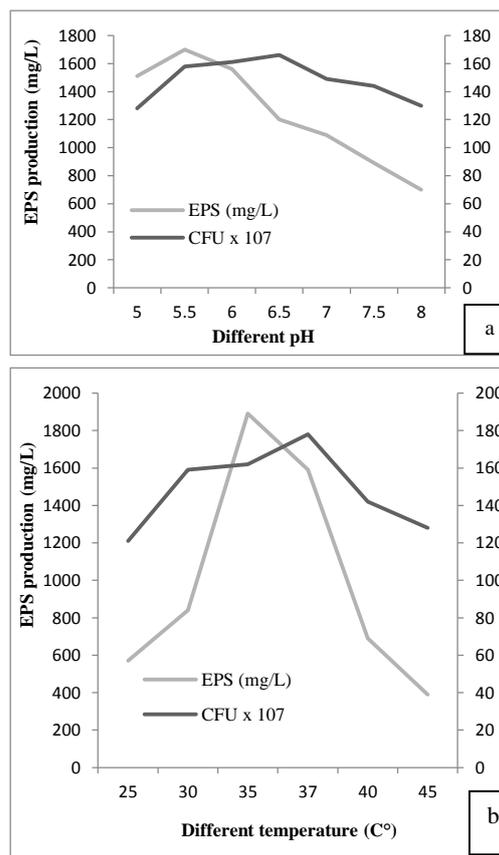


Fig. 3. Effect of different a) pH and b) temperature on EPS production

3.5. Key properties for characterization of nano-EPS using Dynamic light scattering (DLS) and TEM analyses

DLS is the most common sizing technique in pharmaceutical technologies. When applied in this study, the DLS results revealed that the mean particle sizes of crude and partially purified EPS-NPs were 210.1 nm and 480.3 nm, respectively (Fig. 4 a, b). However, when comparing these results with those retrieved from TEM analysis, it was observed that smaller particle sizes, ranging from 37.3 nm to 105 nm for crude EPS-NPs (Fig. 5a), and from 45.7 nm to 204 nm for partially purified EPS-NPs, were obtained (Fig. 5b). Accordingly, a clear variation in the mean diameters results between the two analyses techniques was observed. This variation could be explained by the fact that the DLS analysis measures the hydration layer and polymer shells of the particles thus recording larger particle sizes. In addition to, the scattering of light by large particles strongly overlay that of the small ones producing misleading results for samples having different particle sizes and shapes [50]. Moreover, the sample features also affect the

DLS measurements consequently the different results between DLS and TEM sizes were found [51].

According to that, the TEM analysis results of particle sizes are considered more reliable and therefore the above mentioned nano-EPSs sizes proves that they are very suitable for biological and pharmaceutical applications as they are consequently in a highly optimum size range since different investigators, such as Carvalho et al., 2018 [52], reported that the NPs have size-related properties which can affect their mechanism of action and their *in vivo* lifetime. Furthermore, according to Hoshyar et al., 2016 [53], the ideal nanoparticles (NPs) diameter should not exceed 200 nm to avoid their rapid leakage into blood capillaries and not less than 10 nm to escape capturing by fixed macrophages. In that issue, Raveendran et al., 2013 [54] prepared EPS based nanoparticles produced from *Halomonas Maura* with particle diameter ranging from 30 to 200 nm. Also, the clear variations in the mean diameters and particle distribution between crude and partially purified EPS-NPs were confirmed by Scala et al., (2019) [55].

Moreover, the TEM image revealed the morphological characteristics of the produced nano-EPS, where the NPs have platelet-like shape with different sizes. In this context, Scala et al., 2019 [55] obtained similar results with EPS T14-Ag NPs from marine bacteria.

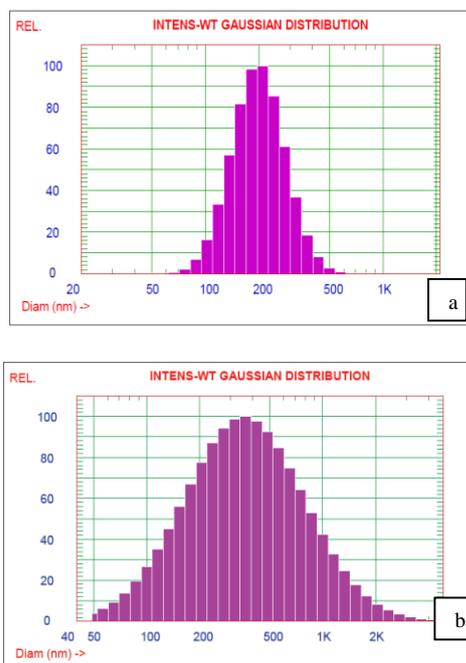


Fig. 4. The mean particle size diameter of a) crude and b) pure EPS-NPs using DLS technique

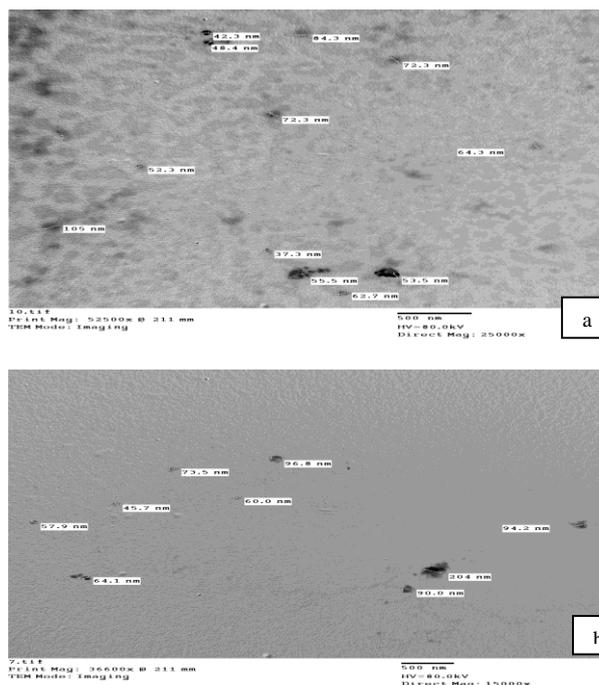


Fig. 5. TEM observation for particle size diameter of a) crude and b) pure EPS-NPs

3.6. Different applications of nano-EPS

3.6.1. Anticancer activity

The stability, biodegradability and compatibility of nano-EPS gave them great features to be applied in cancer therapy and diagnosis [54]. In this study, different anticancer activities were observed when crude and partially purified EPS- NPs, in different concentrations ranging from 0.5 to 10 mg/ml, were tested against colorectal (Caco-2), breast (MCF-7), lung (A549) and hepatocellular (HepG-2) carcinoma cell lines as well as human lungs normal cell lines (Wi-38) (Table 4). The results showed that the highest selectivity index of 6.9, with a significantly low IC_{50} concentration of 1.3 mg, was observed when crude EPS-NPs were applied on cell line MCF-7 indicating the sensitivity of breast cancer to these produced nano particles. Moreover, satisfactory selectivity indexes ranged between 3 and 4.2, were also obtained when either crude or partially purified EPS-NPs were tested on cell lines of Caco-2 and HepG-2 proving their anticancer effect on both these malignant cells.

Interestingly, the anticancer activity of these prepared nano-EPS was superior to the previously obtained anticancer activities of the same full size EPS which was previously produced from *W. paramesentroides* MN2C2, and tested on the same cancer cell lines [17].

The results therefore clarify that both tested, crude and partially purified, EPS-NPs were safe towards normal cell lines (Wi-38) and this was confirmed by their high selectivity toward most of cancer cell lines (SI >2) [56]. However, the highest selectivity index of the crude EPS-NPs may be attributed to their optimum sizes as mentioned before [53]. These results therefore strongly suggest that these prepared EPS-NPs could participate in the treatment of certain types of cancer cells. The replacement of the currently used chemotherapy agents, which have adverse effects against normal cells, by non-toxic and highly selective nanomedicines to improve cancer treatment efficiency was also recommended by an array of investigators such as Calzoni et al., 2019 [57]. In this context, Raveendran et al., (2013) [54] prepared a successfully stable sulfated-EPS based nanoparticles, extracted from *Halomonas Maura*, which are safe to normal cells and toxic to certain cancer cells including breast adenocarcinoma MCF7 and fibro blast cells cancer.

The structure of any EPS is related to its application [58] and its mode of action, which is not only by the induction of apoptosis of cancer cell lines [59] but also by affecting the regulation of host immunity [60]. As previously mentioned, this tested EPS is a hetero-polysaccharide containing high ratios of carbon, nitrogen, oxygen, phosphorous and sulfur [17]; which means that it is a sulfated EPS and the later are known for their unique properties and their high activities due to their stability and biodegradability [61].

Finally, the distinct morphology and different sizes of the produced EPS-NPs, as well as the presence of reactive functional groups, that permits a wide range of modifications, allow their possible application in the drug delivery system as suggested by Debele et al., 2016 [62].

Table 4. *In vitro* anticancer activity of both crude and pure EPS-NPs

Cell lines	Nano-crude EPS		Nano-pure EPS	
	IC ₅₀ (mg/ml)	Selectivity Index (SI)	IC ₅₀ (mg/ml)	Selectivity Index (SI)
Caco-2	2.7	3.3	1.9	3.3
HepG-2	2.1	4.2	2.1	3.0

MCF-7	1.3	6.9	3.4	1.9
A549	0	0	5.8	1.1
Wi-38	8.9	1	6.4	1

3.6.2. Antioxidant activity by DPPH Radical Scavenging method

Microbial EPSs which contain active groups such as phosphoryl, acetyl or benzyl always have anticancer and antioxidant activities [63]. The DPPH antioxidant activity of the tested EPS-NPs was detected. The results shown in Table 5 clarify that the antioxidant activity of the tested EPS-NPs depends on their concentrations. The results also show that both crude and partially purified EPS-NPs resulted in effective IC₅₀ that reached 39.8 and 40.0 µg/ml respectively. These results are actually better than our previously reported results of the DPPH test performed for normal size EPS (127.9 crude and 128.7 µg/ml partially purified EPS) (Amer et al. 2021 [17]). In this context, Abdel-Fattah et al., (2012) [9] recorded a great antioxidant activity for EPS (levan) from *B. subtilis* using DPPH test. That means EPSs which possess this property can be applied as therapeutic agents to prevent many human diseases such as immune system decline, cancer and heart disease [64].

Table 5 Antioxidant activity of crude and partially purified EPS-NPs

Sample	Conc. (µg)	DPPH %	IC ₅₀ (µg/mL)
Nano crude EPS	40	50.3	39.8
	80	52.6	
	120	53.2	
	169	53.9	
	200	56.5	
	240	58.0	
Nano pure EPS	40	50.0	40.0
	80	52.8	
	120	53.5	
	169	54.0	
	200	56.8	
	240	58.8	

Conclusion

According to these results, it could be concluded that these prepared EPS-NPs could be considered to be potentially used as a much safer alternative in cancer treatment than the currently used chemotherapeutic

substances, which are found to be accompanied by extensive and complicated life aggressive side effects that can be regarded to be even worse than the tumor itself. Moreover, the results of their antioxidant activity also suggest that these EPS-NPs could participate in cancer prevention and in the general boosting of the body innate immune system.

Furthermore, the study performed on the producing microorganism proved that this strain possesses all the characteristics of probiotics and can therefore play a highly significant role in medical therapy as well as a prophylaxis through increasing the microbial resistance of the body against a great number of infectious diseases if properly administrated, on a regular basis, as food additives or as dietary supplements.

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Authors' contributions

Mai Amer contributed to conceptualization, formal analysis, funding acquisition, methodology, resources, and writing the original draft. Eman Elgammal and Nagwa Atwa contributed to conceptualization, formal analysis, supervision, writing the original draft, review, and final editing. Ahmed Eldiwany, Ferial Rashad and Insaf Dawoud, contributed to conceptualization, formal analysis, and supervision.

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Arabic abstract

تعد جزيئات عديدة السكاريد النانوية (EPS-NPs) وخاصة المستخلصة من البروبيوتيك ، واحدة من أكثر الأساليب المبتكرة و المتطورة لعلاج السرطان نظراً لتميزها بدرجة عالية من الثبات بالإضافة الى توافقها الحيوي. في هذه الدراسة تم عزل عديدات السكريات الكبريتية المتغايرة السابق تعريفها من *Weissella paramesenteroides* MN2C2 و تنقيتها بشكل جزئي. تم تحسين و ضبط بعض الظروف المستخدمة في عملية الانتاج لضمان كفاءة انتاج عاليه من بيئة الانتاج . تم تكوين الجزيئات النانويه من عديدات السكريات الخام و المنقى جزئياً باستخدام جهاز الالتراساوند و التأكد من انتاجها باستخدام الميكروسكوب الالكتروني النفاذ (TEM) و تشتت الضوء النفاذ (DLS). تميزت الجزيئات بشكل يشبه الصفائح وتراوح قطرها من 37 إلى 105 نانومتر للمركب الخام و 45 إلى 204 نانومتر للمركب المنقى جزئياً. و قد اظهر كلا المركبين فعاليه ضد خلايا سرطان القولون Colon Caco-2 و الكبد Liver HepG-2 و الثدي Breast MCF-7. كما أظهر كلاهما نشاطاً قوياً مضاداً للأكسدة يعتمد على تركيزهما. أظهرت السلالة المنتجة حساسية عالية تجاه التتراسيكلين وحمض الفوسيديك و الأمبيسيلين مع عدم وجود نشاط لتحلل خلايا الدم. بالإضافة الى درجة تحمل عاليه لانخفاض درجة الحموضه و املاح العصاره الصفراويه و عصاره البنكرياس و علاوة على نشاط مضاد للميكروبات ضد *Pseudomonas aeruginosa* و *Bacillus subtilis*. تشير هذه النتائج إلى أن هذا المركب يتميز بخصائص ممتازة لتطبيقات الطب المحتملة.