Role of exopolysaccharides (EPSs) as anti-Mir-155 in cancer cells

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1. Introduction

Micro-ribonucleic acids (miRNAs) are a class of small non-coding RNAs that regulate cellular gene expression. MiR-155 overexpression has been implicated in many types of cancer. Besides, miR-155 appears to help tumor invasion and migration and works as a moderator of epithelial-to-mesenchymal transition (EMT). Exopolysaccharides (EPSs) are a large group of natural heterogeneous polymers of sugars with a biologically antitumor effect. Herein, we test a hypothesis that EPS might promote its anti-tumorigenic effect via regulating miR-155 expression and its target pathways. Expression of miR-155 and a panel of targeted genes were investigated by real-time PCR. In our study, we have succeeded in the extraction, purification of exopolysaccharide with great cytotoxicity to different cancer cell lines, HepG II, Caco-2, and MCF-7. We reported that EPSs have a suppression effect on the oncogenic miR-155. In conclusion, this work clarifies a new possible mechanism for the anti-tumorigenic effect of EPSs in cancer cells and provides insights into the biological pathways through which EPSs act. Moreover, it paves the way for new prospective cancer therapeutics as anti-miRNA.

MiR-155 dysregulation has been implicated in many types of cancer. Besides, miR-155 has been appeared to improve tumor invasion and migration and works as a moderator of epithelial-to-mesenchymal transition (EMT). MiR-155 has participated in epithelial cell adhesion molecule-positive cancer cells in Hepatocellular Carcinoma (HCC) [6]. The overexpression of miR-155 may be related to the high degree of Hepatocellular Carcinoma HCC tumorigenicity and invasion [7]. The most essentially dysregulated miRNAs in breast cancer are miR-125h, miR-145, miR-21, and miR-155 [8]. Moreover, miR-155 levels are overexpressed in human breast cancers with Breast cancer 1 (BRCA1) mutation. This proposes that miR-155 is firmly related to BRCA1 and can promote the essential functions of miR-155 in breast cancer progression [8]. Besides, MiR-155 may be involved in the progression of colorectal cancer and its overexpression has been related to poor prognosis of colorectal malignancy and pancreatic cancers [9].

Exopolysaccharides (EPSs) are a large group of natural heterogeneous polymers of sugars biologically produced by many organisms that are either weakly bound to the cell surface or released into the surrounding environment [10]. Many studies proved the antitumor effect EPSs synthesized by Lactobacillus a strain, which is influenced by monosaccharide composition, molecular weight, the structure of polymeric backbone and side...
chain, and the number of branching points. Between the existence of mannose and glucose residues and branching points in repeating units in EPSs structure impacts on raising their antitumor action [11]. However, the biological pathways through which EPSs promote its antitumor effect remain non-obvious.

Herein, we hypothesize that EPSs might promote its anti-tumorigenic effect via regulating miR-155 expression and its target pathways. To test this hypothesis, this work has been done to investigate the biological activity of natural EPSs synthesized by Lactobacillus sp. bacterial strains on different cancer cell lines; hepatocellular carcinoma cell line (HepG II), breast cancer cell line (MCF-7), and colorectal carcinoma (Caco2) and its proposed effect on miR-155 expression and miR-155 target genes. Moreover, this study has highlighted miR-155 as a potential therapeutic target in different malignancies.

2. Material and methods

2.1. Bacterial exopolysaccharides extraction

EPSs extracted from Lactobacillus acidophilus bacteria (DSM20079), obtained from the Microbiological Resource Center, Faculty of Agriculture, Ain Shams University, Egypt. Extraction was performed according to the method described previously by Vidhyalakshmi and Vallinachiyar. Briefly, the segregates were inoculated independently in Nutrient Broth provided with 1 g of glucose and incubated at 35 °C for 3–4 days in a shaking incubator. The inoculated broths were centrifuged following 3–4 long periods of incubation for 10 min at 10,000 rpm to discharge the cell pellets. The blend was shaken while adding alcohol to preclude locally high concentration of the precipitate and left at 4 °C overnight. EPSs pellets were gathered by centrifugation at 7000 rpm for 20 min. After centrifugation, the precipitate was gathered in Petri plates and dried at 60 °C [12].

2.2. HPLC analysis of extracted EPSs

To test the purity of the extracted EPSs, the isolated EPSs were analyzed with a high-performance liquid chromatography (HPLC) system (LC 1110) equipped with a C18 column and eluted with a mobile phase composed of a mixture of acetonitrile:dist. Water (75:25) at a flow rate of 1.2 ml/min at 20 °C. The separated components were monitored by an ultraviolet (UV) detector at a wavelength of 254 nm.

2.3. Cell lines and culture

Human breast adenocarcinoma cell line (MCF-7), human colon (large intestine) adenocarcinoma cell line (CaCo-2), and human liver cancer cell line (HepG II) were obtained from Holding Company for Biological Products & Vaccines (VACSERA), Egypt. The cells were maintained in Eagle's Minimum Essential Medium (EMEM) (LONZA, USA) provided with 10% FBS (SIGMA, Germany), Penicillin 10,000 Units/mL, Streptomycin 10 mg/mL, Amphotericin B 25 μg/mL (LONZA, USA) in a humidified atmosphere of 5% CO2 at 37 °C.

2.4. Cell viability assay

The impact of EPSs was determined by incubating the cell lines (5 × 10^3) with different concentrations (200 μg/ml, 400 μg/ml, 600 μg/ml, 800 μg/ml, and 1000 μg/ml) of EPSs and screening for their viability after 48 h by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The formazan crystals formed were dissolved in Dimethyl sulfoxide (DMSO), and the absorbance at 570 nm (A570) gained was compared with that of the control. The concentration required for 50% inhibition of viability (IC50) was determined and was utilized for advanced experiments. The effect of different concentration EPSs on the proliferation of different cancer cell lines is expressed as the % cell viability with the formula: % cell viable = A570 of treated cells/ A570 of control cells × 100.

2.5. Apoptosis and cell cycle assay by flow cytometry

A flow cytometer was used to analyze cells’ behavior with the presence of isolated EPSs. The treated and untreated cells were collected and washed with cold PBS. An aliquot [105 cells/100 μl] of cell suspension was added with 1 μl fluorescein Isothiocyanate (FITC)-conjugated annexin-V and 2.5 μl Propidium Iodide PI:250 μg/ml to measure DNA content by fluorescence-activated cell sorting (FACS) analysis. The percentages of cells in each phase of the cell cycle (G1, S, and G2) were determined.

2.6. Real-time quantitative PCR (RT qPCR)

Expression (before and after EPSs cell line treatment) of various messenger RNAs (mRNAs) of miR-155 and different target genes of different biological pathways (Apoptotic, anti-apoptotic, autophagy, proliferative, and self-renewal genes) were determined by real-time quantitative PCR (RT qPCR) studies. Basally and after EPSs treatment, total RNA from four cell lines; MCF-7, Caco2, and HepG II were extracted using (RNeasy® Mini Kit, Quiagen, Cat. No. 74104) while the extraction of miRNA was performed using (miRNeasy® Mini Kit, Quiagen, Cat. No.217004). The extracted RNA (Total RNA and miRNA) was converted to complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit and then was stored at -20 °C to be used for additional studies. The qPCR reaction mixture (total 20 μl), performed triple, essentially contained 2μl (10 pmol/μl) of forward and reverse primers of various studied genes, 2μl of cDNA template, and 10 μl SYBR green PCR master mix (Thermo Scientific, Lithuania). Each reaction was amplified for 40 cycles. Primer sequences for different studied genes are designed using primer designing tools (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?INPUT_SEQUENCE=A513262.1&LINK _LOC=nucore) presented in Table 1. For normalization of the cycle threshold (Ct) values for each target gene, the Ct value of the control gene was used, namely glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The Expression of various target studied genes was determined using the comparative ΔΔCT method. Relative expression was calculated basally (without EPSs treatment) and after EPSs treatment with EPSs of various cell lines.

2.7. Statistical analysis

IC50 values acquired from MTT cell Proliferation assay and fold changes in gene expression dictated by RT-qPCR. The results were exposed to one way ANOVA test utilizing Graph Pad Prism programming. For examination of significance between groups, Tukey’s multiple comparison post-test was applied after ANOVA. The mean differences were seen as significant at the 0.05 level.

3. Results

3.1. EPSs purity

The isolated EPSs from the broth media were estimated at 0.35 ± 0.12 gm/L and the HPLC analysis showed that the isolated EPSs were pure and appeared at retention time 3 min 53 s.

3.2. Cytotoxic effect of EPSs

A comparative analysis of proliferation inhibition on different cancer cell lines by the isolated EPS showed that the isolated EPSs showed cytotoxicity to the cells with different inhibition percentages. As shown in (Figure 1), the inhibition effect increased significantly (P < 0.05) along
with the increased concentrations (200 μg/ml, 400 μg/ml, 600 μg/ml, 800 μg/ml and 1000 μg/ml).

3.3. Cell cycle

The increased growth rate is well known as one of the most prominent features of cancer cells. Therefore any agent by which cancer cell cycles can be halted represents an effective anti-cancer substance. The effects of EPSs on the cell cycle phase distribution were tested by flow cytometry (Figure 2). As shown in Table 2, when cells treated with the IC50 concentration of EPS on HepG II, MCF-7 & Caco-2 cells, it was found that the G0/G1 phase was significantly increased in all cells (P < 0.05) (64.6 %, 74.9 % & 83.0 %) respectively, while the S phase was significantly decreased in all cells (P < 0.05) (22.7 %, 2.6 % & 0.8%) respectively indicating that the highest percentage of G0/G1 phase appears significantly (P < 0.05) in HepG II cells with a significant decrease of S phase (P < 0.001).

3.4. Apoptosis and necrosis

Annexin V-FITC PI staining method was employed and apoptosis and necrosis analyses were carried out to determine the apoptosis induction effect of EPSs on different cells. As illustrated in Figure 3. As illustrated in Table 3, it was shown that when cells treated with the IC50 Concentration of EPS on HepG II, MCF-7, and Caco-2, it was found that the percentage of apoptotic cells was (53.4%, 62.0% & 54.3%) respectively while the percentage of necrotic cells was (2.6%, 4.9% & 2.2%) respectively indicating that the highest rate of apoptosis induced by EPSs has been found in MCF-7 (P < 0.001) cells, in contrast, the lowest apoptosis rate in HepG II cells (P < 0.01). The highest necrosis induction by EPSs has been shown in MCF-7 (P < 0.01) cells then HepG II and Caco-2 cells, respectively.

### Table 1. Primer sequences for different studied genes.

| Gene                     | Primer Sequences | PCR Product Size |
|--------------------------|------------------|------------------|
| miRNA155                 | Forward: TTAATGCTAAATCGTGATAGGGGT | 55 bp            |
|                         | Reverse: ATATGTAGGAGTCAGTGGGAGGC  | 107 bp           |
| BRCA1                    | Forward: ACAGCTGTGGTGGTGCCTTGTTG | 202 bp           |
|                         | Reverse: ATAGCTGTGCTGCTGCAAGGGATT  | 214 bp           |
| SOCS1                    | Forward: CACTCGCAGCATCGTGTTC   | 172 bp           |
|                         | Reverse: AGGCCATCTACAGGCTTAAAGG  | 160 bp           |
| MLH1                     | Forward: TGGTGGCAATACAGGGGACAGAT | 119 bp           |
|                         | Reverse: CAGGTGGAACATTGCTGGTATG  | 180 bp           |

**Apoptotic & Anti-Apoptotic genes**

| TP53                     | Forward: CATAGTGTGGGTGGCCCTATGAG | 84 bp            |
|                         | Reverse: CAAAGTGTTGCTGGCCGAGTAGA  | 91 bp            |
| AIFM1                    | Forward: CAGAAAAGGAGGGCCTATCT   | 143 bp           |
|                         | Reverse: ATACACTAGTACCCTGGGCC   | 82 bp            |
| Bcl2                     | Forward: CTGGACCTAGGGCCCTTCACC | 119 bp           |
|                         | Reverse: CACATGGGCCGAGGTAATGAG  | 172 bp           |
| BAX                      | Forward: CCGGGTTCGCTGCTTTTCTA   | 156 bp           |
|                         | Reverse: CGGCTCACGCAGGTGTTGATG  | 224 bp           |
| Caspase-9 (CASP 9)       | Forward: TTCCCGAGGGTTGTGCTGTG   | 81 bp            |
|                         | Reverse: CCCTTTACCGAAGAAGCATT   | 150 bp           |

**Autophagic genes**

| m-TOR                    | Forward: GCCCAGGGCGAGATGGCTCTAT | 84 bp            |
|                         | Reverse: GCAGTAATATGAGTGGTATGAG  | 91 bp            |
| LC3A                     | Forward: GCCCTCTCTTCTGGGGTGAAAC  | 88 bp            |
|                         | Reverse: AGGCGGCTGCTGCTCTCTC    | 150 bp           |

**Proliferative genes**

| c-myc                    | Forward: AGGGAGATCCGGAGGGAATAA | 156 bp           |
|                         | Reverse: GTCCCTGTGCTGGGGTGTTGA  | 88 bp            |
| Ki-67                    | Forward: GTGGTGTCGACGGACCTTCCT | 224 bp           |
|                         | Reverse: ACCCCCTCCAAAGAAGCAGG  | 150 bp           |
| K-Ras                    | Forward: GCCCTCCTGAAATGATGTAATATA | 81 bp            |
|                         | Reverse: TTAGCGTATGGCGAGGACTC  | 150 bp           |

**Self-Renewal genes**

| β-Catenin (CTNNB1)       | Forward: CCAGGTTGGGTGTAATAGAG | 88 bp            |
|                         | Reverse: CTGGGAGAAAGGCTATGAG  | 150 bp           |
| BMI-1                    | Forward: TCCCTAACAGGTCGATGTAATACC | 87 bp           |
|                         | Reverse: CACAGTTCTTCACTATTTCCA | 87 bp           |

**House Keeping gene**

| GAPDH                    | Forward: TGCAGACACCTAGGTCTTGAGC | 87 bp            |
|                         | Reverse: GCCATGGACTGTGGTATGAG  | 87 bp            |
3.5. EPSs effect on gene expression level

The relative gene expression level of different genes to estimate the anti-tumor effect of the isolated EPSs is shown in (Figure 4). An important result of EPSs treatment of different cancer cell lines is the changes in the gene expression of many target genes in different biological pathways. MiR-155 is upregulated in all cancer cells, HepG II, MCF-7, and Caco-2, in which the first and the most important result is the effect of isolated EPS on the expression of oncogenic miR-155 which is decreased significantly through EPS treatment to all cancer cell lines, HepG II (P < 0.001), MCF-7 (P < 0.001) & Caco-2 (P < 0.001) and consequently provide a significant effect on the studied genes, apoptotic, autophagic, proliferative, self-renewal and some target genes, as follows (Figure 4a).

For the apoptotic genes, expressions of total protein 53 (TP53), the apoptosis induced factor M1 (AIPF1), and the retinoblastoma susceptibility gene (Rb1) were increased significantly in HepG II (P < 0.001) and MCF-7 (P < 0.001) cancer cells with more evident effects in HepG II while their expression was decreased in Caco-2 but Caspase-9 and Bax expression level is decreased in MCF-7 and Caco-2 cancer cell lines but increased significantly (P < 0.001) and slightly increased in HepG II respectively. For the anti-apoptotic genes, Bcl2 expression is decreased significantly in all cancer cells in which the highest effective level on Caco-2 (P < 0.001) (Figure 4b).

For Autophagic genes, the mammalian target of rapamycin (mTOR) and Microtubule-associated protein (LC3A) are important autophagic genes in which our results show that both genes expression is decreased in all cancer cells with different levels except LC3A expression in HepG II cells that is increased significantly (P < 0.001). The down-regulation of mTOR and LC3A was clearer in Caco-2 cells (Figure 4c).

For Proliferative genes, the nuclear protein KI-67 gene, proto-oncogene c-myc and proto-oncogene KRas, their expression level through the effect of EPSs are decreased significantly (P < 0.01) in all cell lines except for KI-67 in MCF-7 is increased and nearly no effect for KRas in HepG II cell line (Figure 4d).

For Self-renewal genes, β-catenin, an oncogene that its over expression seems to play an important role in several types of cancer, is decreased significantly in HepG II (P < 0.05) and Caco-2 (P < 0.01) by the effect of EPS and hence negatively affect the continuous proliferation (self-renewal) of cancer cells while BMI-1 is increased in HepG II and decreased significantly (P < 0.01) in MCF-7 and with a more significant decrease in Caco-2 (P < 0.001) (Figure 4e).

As regards miR-155 targeted genes, BARCA-1, SOCS-1 & MLH-1, Breast cancer type 1 susceptibility protein (BARCA-1) is a tumor suppressor gene normally expressed in breast cells and helps repair damaged DNA or destroy cells if DNA cannot be repaired. Notably, EPSs treatment did not significantly affect the BARCA-1 expression level in all studied cancer cells, MCF-7, HepG II, and Caco-2 (Figure 4f). Suppressor of cytokine signaling 1 (SOCS1) is considered a tumor-suppressor-like role in hepatoc-cellular carcinoma (HCC), hence SOCS1 expression level in HepG II cells increased significantly (P < 0.001) by the addition of EPSs as shown in (Figure 4f). Similar results were obtained for MCF-7 (P < 0.01) and contrary results were obtained for Caco-2 indicating its tumor suppressor effect that was more evident in HepG II cells (Figure 4f). Mutl homolog 1 (MLH1) is one element of DNA mismatch repair system proteins that work coordinately in sequence to start the repair of DNA mismatches in humans. However, in our study, when adding EPSs, we found that its expression level increased significantly (P < 0.01) in Caco-2. However, for both MCF-7 and HepG II cells, there is nearly no significant effect after the addition of EPSs (Figure 4f).

Collectively, the treatment of cancer cell lines with EPSs leads to suppression of expression of miR-155, anti-apoptotic genes, autophagic genes, proliferative genes, and self-renewal genes. Meanwhile, EPS leads to the up-regulation of the expression of tumor suppressor genes. These results indicated the anti-tumor effects of EPSs in different cancer cell lines, HepG II, MCF-7 & Caco-2 by different mechanisms all through the inhibition of miR-155 proving that EPSs act as anti-miR155 to inhibit cancer cell growth.

4. Discussion

Cancer cells are well known for their ability to create chemotherapy resistance during treatment. In this way, there is a constantly developing interest in novel anti-tumor drugs. Numerous investigations have demonstrated that miR-155, a tumorigenic miRNA, is a target for treatment [13]. There are many particular targets of miR-155, including c-Myc and CCAAT/enhancer-binding protein beta (C/EBPb) [14]. MiR-155 upregulation leads to nuclear accumulation of β-catenin and concomitant increment in cyclin D1, c-Myc, and survivin [15].

Jiang, Zhang, et al. found that miR-155 mimics significantly boosted the proliferation of MDA-MB-231 cells and activated MCF-7 cells to grow more (>3-fold) and larger colonies on soft agar Conversely,
anti–miR-155 in MDA-MB-231 cells significantly decreased cell proliferation and lead to 2.5-fold fewer colony foci [16]. BARCA1 functional loss leads to miR-155 up-regulation. These results were summarized in human cell lines, where insufficient BARCA1 cells have 50 fold higher miR-155 levels compared to those with normal functional BARCA1 [17] wherein our study; the down-regulation effect of EPSs to miR-155 has nearly no significant effect on BARCA1 and in case of HepG II and Caco-2 cells they have no significant effect (Figure 4f). Ghafouri-Fard, Oskooei, et al. found that SOCS-1 expression is down-regulated in breast tumoral tissues than adjacent non-cancerous tissues which are in accordance with the results obtained (Figure 4f) in which its expression is down-regulated in MCF-7 cells and when adding EPSs, its expression increased. but in Caco-2 cells, its expression decreased on adding EPSs which was confirmed by Beaurivage, Champagne, et al. who found SOCS-1 expression is over expressed in colorectal carcinoma (CRC) tissues [18]. Also, the expression of MLH-1 was increased when Caco-2

Table 2. Cell Cycle Phases distribution on HepG II, MCF-7 & Caco-2 cells upon treatment with The IC50 concentration of EPS.

|           | HepG II | MCF-7 | Caco-2 |
|-----------|---------|-------|--------|
|           | Control | Treated | Control | Treated | Control | Treated |
| G0/G1 Phase | 49.5 %  | 64.6 % | 64.0 %  | 74.9 %  | 63.7 %  | 83.0 % |
| S Phase    | 30.0%   | 22.7%  | 17.7%   | 2.6 %   | 16.9%   | 0.8 %  |
| G2/M Phase | 10.5%   | 4.2 %  | 0.9 %   | 10.3 %  | 0.5 %   | 0.8 %  |

Figure 2. Effect of EPSs on the cell cycle progression of different cells. (I) Representative Histogram showing cell cycle distribution phases in the cells treated with EPS. (a) Cells without EPS (Control), (b) Cells treated with EPS. (II) Columns show cell cycle phases percentage in the cells treated with EPS. *P < 0.05, **P < 0.001.
cells were treated with EPS. This was in accordance with a study that demonstrated that miR-155 targets MLH1 and MSH2 and reported an inverse relationship between the miR-155 expression and MLH1 or MSH2 proteins expression in human colorectal cancer [19].

Zadeh, Motamed, et al. found that the expression levels of miR-21 and miR-155 had significantly decreased after 48 h silibinin of treatment and the expression levels of four genes after 48 h; CASP-9, p53, and BID (BH3 interacting domain death agonist) levels were upregulated [20]. Similar results were obtained in the present study but the expression level of CASP-9 was upregulated in HepG II cells only (Figure 4b). Venkatadri, Muni, et al. found that the natural phenol resveratrol has a significantly decreased Bcl-2 expression [21] which was in agreement with the results obtained in MCF-7 cells. Liu, Wang, et al, found that the expression level of RB-1 is increased in HepG II cells while using anti-miR-1297 [22]. Similarly, RB-1 expression was increased while adding EPSs (Figure 4b).

MiR-155 incited by hypoxia can stimulate autophagy by focusing on various genes in mTOR signaling, including RHEB, RICTOR, and RPS6KB2 [23]. M-TOR expression was decreased in all cancer cell lines in which are mostly decreased in Caco-2. On the other hand, LC3A expression was increased in HepG II and decreased orderly in Caco-2 and MCF-7 (Figure 4c). Similar findings were reported by Qi, Xue, et al, who found that LC3-I was significantly increased in HepG II cells [24]. Another report demonstrated that the expression of LC3A upregulated in colorectal and operable breast carcinoma patients [25]. Similarly, its

![Flow cytometry analysis with Annexin V-FITC/PI. (a) Representative Histogram showing cells treated with medium used as control (I) and cells treated with EPS (II). (b) Columns show apoptosis and necrosis percentage in the cells treated with EPS.*P < 0.001, **P < 0.001. Note: Upper right represent Necrosis while lower right represents Apoptosis.](image)

![Table 3. The Total apoptotic and necrotic death rates on HepG II, MCF-7 & Caco-2 cells upon treatment with The IC50 concentration of EPS.](table)

|          | HepG II |          | MCF-7 |          | Caco-2 |
|----------|---------|----------|-------|----------|--------|
|          | Control | Treated  | Control | Treated  | Control | Treated |
| Total Apoptotic Rate | 8.5 % | 53.4% | 5.5 % | 62.0 % | 8.2 % | 54.3 % |
| Necrotic Rate | 8.2 % | 2.6 % | 13.3 % | 4.9 % | 10.1% | 2.2 % |
expression was upregulated in Caco-2 and MCF-7 cells, but when cells treated with EPSs the expression levels decreased.

Exopolysaccharides (EPSs) are neutral, non-toxic, and biodegradable polymers that have a great effect on different biological mechanisms. It has been suggested that EPSs with anticancer activity can be used as one ideal substitute for cancer treatment [26]. In our study, we have applied EPSs to number cancer cell lines, HepG II, Caco-2, and MCF-7. El Ghany, et al. showed the antioxidant and anticancer effects of Lactobacillus acidophilus P185 strain in which EPSs from Lactobacillus acidophilus P185 could suppress the growth of Ehrlich ascites carcinoma (EAC) cell line with 93–99% [27]. Subsequent anti-proliferation assay of purified EPSs from the bacteria confirmed their antiproliferation effects on HepG II, MCF-7 and, Caco-2 cells. Nehal M. El-Deeb, et al, showed that the presence of not matched cellular responses to LA-EPS-200790 treatment based on different cell death mechanisms and their sensitivity to the therapy [28]. Almost the same finding was reported by Nami, et al. [29], where they found that Enterococcus-lactis IWS5 secretions had no toxic effect on normal cells, with 95% of the cells grew normally.

Cell proliferation is firmly regulated by the cell cycle: S phase for DNA synthesis, M phase for mitosis, G0/G1, and G2 phase. The G1/S transition is an important checkpoint in the development of the cell cycle and responsible for the beginning and finishing of DNA replication. By using FACS analyses we found that the inhibitory effect of the isolated EPS on HepG II, MCF-7, and Caco-2 cell proliferation was related to the prevention of G1 to S transition (Figure 2). Almost the same results were obtained by Weis DI, et al. where they reported an anti-proliferative effect of crude and acidic EPS on HT-29 cells with the inhibition of the transition of G1 to S phase [26]. Naxin Sun, et al. found that the isolated fraction EPS-3a could stimulate HepG II cell apoptosis and cell cycle arrest at the G0/G1 phase by increasing incubation time [30]. Also Nehal M. El-Deeb, et al. reported that the purified EPS induces G0/G1 and increased with incubation compared to normal cells and S and M phases are decreased [28].

The effect of isolated EPS induces apoptosis and necrosis with different ratios as reported by Parisa Mojibi, et al. The extract of L. Brevis bacteria was capable of apoptosis induction at both 48 and 72h and found that the bacterial extract of L. Brevis is more potent than that of L. paracasei [31]. Guodong Wang et al. reported that Exopolysaccharide from Trichoderma pseudokoningii induces the apoptosis of MCF-7 cells with different proportions and also reported that the increase of cell inhibition may result from apoptosis induction by EPS [32]. For apoptotic genes, we have found that most apoptotic genes are upregulated especially in HepG II cells. R Vidhyalakshmi, et al. found that the decreased expression level of the proto-oncogene Bel-2 and increased expression level of TP53 for Human breast cancer cell line MCF-7 by the effect of extracted bacterial EPS [12].

Similar results demonstrated by Oh et al that the decreased expression level of the anti-apoptotic gene Bcl-2 [33] was in agreement with the results obtained in MCF-7. Mahgoub M Ahmed, et al, demonstrated that the effect of Exopolysaccharide from Marine Bacillus velezensis MHM3 on MCF-7 was that the expression level of TP53 and Bax genes were increased while Bel-2 was decreased [34] but in the present study Bax was decreased (Figure 4b), while Oh et al. who demonstrated the expression level of Bel-2 and Bax on HepG Iicelell line reported that Bax was increased and Bcl-2 was decreased [35] which are in agreement with the results of the present study where the expression level of Bax was elevated while Bcl-2 was decreased (Figure 4b). Regarding Bcl-2, Caco-2 cells treated with capsaicin leads to reduced levels of Bel-2 expression [36]. Similar findings were observed in the present study (Figure 4b).

Takahisna, Inoue, et al. had studied the effect of resveratrol on apoptosis of different cancer cell lines in which they demonstrated the effect of resveratrol on the expression level of K-Ras on HepG II and MCF-7, they found that resveratrol has no appreciable effect on K-Ras expression [37] which was in agreement with the results in our study for its expression in HepG II but not for MCF-7 where its expression decreased. β-catenin overexpression was related to CRC development [38]. This was in agreement with the present study where its expression in the cells treated with EPS β-catenin expression was decreased in Caco-2 cells (Figure 4e). Also, Ki-67 was reported to be overexpressed in patients with colorectal cancer and related to living survival where high expression of Ki-67 was significantly shorter disease-free survival compared to low Ki-67 expression and is also highly expressed in breast cancer tissues. In the present study, it was found that EPSs decreased Ki-67 expression in both Caco-2 and HepG II cells (Figure 4d). Lin, Xia, et al, demonstrated that β-catenin is implicated in breast cancer progression [39]. This finding confirmed our study in which when cells are treated with EPS, the expression of β-catenin is decreased (Figure 4e). Alajez showed that there is a significant up-regulation of BMI-1 in CRC patients [40]. This was in agreement with the present study where EPS down-regulated its expression in Caco-2 cells (Figure 4e).

To confirm this effect of EPS and to clarify the biological pathways through which EPS works, gene expression of miR-155 and different genes representing different pathways in the cell (apoptotic, autophagic, proliferative, and self-renewal) were investigated in our work.

5. Conclusion

Collectively, in all cancer cell lines, EPSs leads to suppression of expression of miR-155 and hence effect on apoptotic genes, anti-apoptotic genes, autophagic genes, proliferative genes, and self-renewal genes with
different pathways on the different cancer cell lines that each cell line was affected by EPSs through different pathways. All these results indicate the more preferable cytotoxic effect of EPSs on HepG2 HCC cancer cell line which was affected through increasing apoptotic genes expression, decreasing anti-apoptotic genes expression, acting through autophagy through LC3A, decreasing mostly all proliferative genes, and also decreasing beta-catenin expression, a self-renewal gene, while in Caco-2 which was less affected by EPSs. It was affected by decreasing proliferative and self-renewal gene expression. This may open a new area of research for new effective therapeutic agents of cancers notably hepatocellular carcinoma.

To the best of our knowledge, this is the first work investigating the potential EPSs effect on miR-155 expression and the possibility that EPSs promote its anti-tumorigenic effect via regulating miR-155 expression and its target pathways. We reported that EPSs have an inhibitory role for the oncogenic miRNA 155 (that represents a key cellular regulator in different pathways) in all cancer cell lines and inhibit cancer growth in all cancer cells with different mechanisms. Finally, this work provides a new possible explanation for the biological anti-tumorigenic effect of EPS in cancer cells.

Further analysis of gene expression of different pathways in cancer cells is recommended to confirm the results of the present study and to provide insights on the biological pathways through which EPSs act.

Declarations

Author contribution statement

Ahmed A. Emam: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Salwa M Abo-Elkhair: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Mohamed Sobh: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.
Ahmed M. A. El-Sokkary: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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