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Inhibition of Cancer Stem Cells Growth with Silibinin Encapsulated in Nanoparticles with Deregulation of miR-34a, miR-221, and miR-222

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Abstract

Background: Colorectal Cancer (CRC) is the most common malignant gastrointestinal cancer. Cancer stem cells (CSCs) are the major cause of cancer recurrence and cancer drug resistance. Silibinin, as an herbal compound, has anticancer properties.
Objectives: The present study aimed to evaluate the antiproliferative effects of silibinin on HT29 stem-like cells (spheroids).
Methods: In this study, antiproliferative and apoptotic properties of Silibinin encapsulated in Polymersome Nanoparticles (SPNs) were evaluated by MTT assay, propidium iodide (PI) /AnnexinV assay, cell cycle analysis, and DAPI (4',6-diamidino-2-phenylindole) staining. The expression of some miRNAs and their potential targets was evaluated by real-time reverse transcription-polymerase chain reaction (qRT-PCR).
Results: IC50 of SPNs was determined at 28.13±0.78µg/ml after 24 h. SPNs (28µg/ml) induced apoptosis by 32.36% in HT29 cells after 24 h. DAPI staining indicated a decrease in stained nuclei after SPNs induction. SPNs treatment increased the expression of miR-34a, as well as P53, BAX, CASP9, CASP3, and CASP8. The downregulation of miR-221 and miR-222 was observed in SPNs treated cells. Moreover, SPNs decrease the expression level of CD markers in HT29 spheroids (cancer stem cells) compared to untreated spheroids. Spheroids were completely destroyed after 72 h treatment with SPNs (28µg/ml).
Conclusion: As evidenced by the obtained results, SPNs can be used as an effective anticancer agent in multi-layer (cancer stem cells) and mono-layer cancerous cells with the upregulation of tumor suppressive miRs and genes, as well as downregulation of oncomiRs and oncogenes.

Keywords: Cancer stem cells, miR-34a, miR-221/222, Silibinin, SPNs

1. Background

Colorectal cancer (CRC) is the third most common malignancy and the second cause of cancer-related death in men and women aged over 65. Nonetheless, due to increasing risk factors, such as obesity, bad nutritional habits, and smoking, in recent decades, the rate of this disease has risen in younger people (1-3). In addition to physical and environmental issues, genetic factors play a significant role in this cancer (4). Deregulation of miRNAs as an important group of non-coding RNAs contributes to tumor initiation and progression (5). miRNAs have oncogenic and tumor-suppressive activities in different cells (6). These small RNAs inhibit gene expression by binding to the 3′non-coding region of their targets (7).

Due to the importance of cancer in life quality, with more studies on CRC, the heterogeneous population cells were found with specific surface CD markers than common CRC cells, such as CD44, CD24, epCAM, ALDH1, and CD133 (8,9). These exclusive cells, called Cancer Stem Cells (CSCs), have individual features and are responsible for self-renewal, producing differentiated cancer cells, initiating tumor growth, and lost regulated proliferation (2).

Nowadays, one of the most current treatments is chemotherapy which is recommended before or after surgery (10). Due to the multiple side effects of chemotherapies, researchers try to find new methods with more benefits and no adverse effects, such as native drugs (herbals) (11). The use of natural drugs has a long history and can be suggested as a candidate for cancer therapy (12,13) . Silibinin is a polyphonic flavonoid with a wide range of properties (14,15), such as hepatoprotective activity, antioxidant (5), immunomodulatory, antiviral properties, and anticancer (14,15). Due to low solubility in water, silibinin has a deficiency in absorption into the cell. These complications cause low bioavailability and poor cellular uptake of the drug (16).

Nowadays, different nanocarriers with individual features are applied, such as liposomes (17), dendrimers (18), micelles (19), nanoemulsions (20), and polymersomes (21). Polymersomes are a group of self-assembling polymers that are highly flexible, and more stable than liposomes, with long-time blood circulation (22). Therefore, this is compatible with the maintenance of persistent drug concentration in the blood for a long time, with no application to further doses. Moreover, with various compounds which can be loaded in polymersomes, it will have several capabilities in many applications in nanomedicine.

Accordingly, many lipophilic anticancer drugs, amphiphilic dyes, transgenes, and membrane proteins could be assembled within this nanocarrier without changing their activity. These advantages of polymersome make it one of the remarkable supramolecular structures (6,23) with a size of 5nm-5μm (24) for numerous applications in nanomedicine and nanobiology (6,23).

2. Objectives

In light of the aforementioned issues, the present study aimed to investigate the apoptotic effects of Silibinin-loaded polymersome nanoparticles (SPNs) on HT-29 cancer stem-like cell lines and on HT29 cells after sphere forming with the hanging drop method. Furthermore, the effect of SPNs on the expression of miR-34a, miR-221, and miR-222 and their potential target genes were evaluated by real-time reverse transcription-polymerase chain reaction (qRT-PCR).

3. Methods

3.1 Silibinin encapsulation in nanoparticles

In the first stage, oleoyl chloride (3.01 g, 0.01 mol) and poly ethylene glycol$_{400}$ (20 g, 0.01 mol) were mixed and in the solvent of triethyl amine (1.2 g, 0.012 mol) and chloroform were subjected to esterification reaction at 25°C for 4 h. At the purification stage, the triethylammonium chloride salt was cleansed, the chloroform was evaporated, and as a result, Polyethylene glycol $_{400}$-oleate (PEG$_{400}$-OA) was obtained. To use the PEG$_{400}$-OA as a nanocarrier in the preparation of Nanoparticles, 300mg of PEG$_{400}$-OA and different concentrations of Silibinin (Sigma-Aldrich, Germany) were dissolved in acetone solution. Thereafter, the acetone was evaporated, and the prepared product with different weight/weight ratios of Sil/ PEG400-OA (1:6) was stored at 4°C in a dark condition as described by Tahmasebi Mirgani et al. (25).

3.2. Determination of cell viability by MTT assay

HT-29 cells were cultured in a 96-well plate (1× 10^4 cells/well). The cultured cells were treated by the SPNs (0 to 200μg/ml) for 16-72 h. To evaluate cell viability, the treated cells were washed with PBS, and 100μL of fresh medium containing 10μL of MTT (5mg/ml) was added to each well and incubated for 3 h at 37ºC in a 5% CO2 humidified atmosphere. Finally, 100μL of dimethyl sulfoxide solution (DMSO) was added to each well. The optical density was measured at 570 nm using a microplate reader (ELx800, BioTek, USA).

3.3. Evaluation of cell death after SPNs induction

For apoptosis analysis, we used an Annexin V-FITC kit (Miltenyi Biotech, Germany), DAPI staining, and PI staining. HT29 cells (1×10^4 cells/well) were treated with SPNs (14, 28, 40, and 50 μg/ml), evaluated by Annexin V-FITC, and analyzed using a flow cytometry instrument (BD FACS Calibur™, USA). The SPNs treated cells (28μg/ml) were stained with DAPI and PI, and after 24, 48, and 72h, they were evaluated using Nikon inverted microscopy and flow cytometry instruments, respectively.

3.4 Preparation of Multicellular Tumor Spheroids

In brief, 15-20 drops of harvested cells up to 30μL containing 0.5×10^6 cells/ml were deposited on a 10cm dish lid. Following the inversion of the tray over the petri dish with 10 ml of PBS and incubation at 37°C for 3-10 days, the cells accumulated and gradually appeared in the form of a single MCTS. As a result, a large amount of sphere-forming cancer cells was obtained and incubated in untreated 6-well plates at 37°C for more experiments.

3.5 Flow cytometry analysis of CD surface markers

The percentage of CD surface markers in SPNs (28μg/ml) treated and untreated HT29 spheroids (after hanging drop) were evaluated. The cells were incubated with conjugated monoclonal antibody (Carlsbad, CA, USA) for CD133 PE (Phycoerythrin), CD24 APC (Allophycocyanin), and CD44 FITC (Fluorescein isothiocyanate) surface markers. After 1 h at 4°C, the final volume of the sample was adjusted to 1000μL with PBS and centrifuged at 300g for 5m at 4°C. Finally, the supernatant was removed, the cell pellet was fixed by adding 500μL of 1% cold paraformaldehyde (Sigma, USA), and the sample was analyzed by flow cytometry instrument (BD FACS Calibur™, USA).

3.6 Expression level of miRNAs after SPNs induction

Total RNA was extracted using the iNtRON kit (iNtRON Biotechnology, Korea) from SPNs (28μg/ml) treated and untreated HT29 cells (non-spheroids) after 24 h. cDNA synthesis was performed with BON-miR high sensitivity miRNA 1st-Strand cDNA Synthesis Kit (Stem cell Technology Research Center, Tehran, Iran). In brief, poly (A) polymerase at 37°C, and poly (A) tail was added to miRNAs for 30 min. Subsequently, the RNA poly(A) tail was mixed with a BON-RT adaptor (primer,10μM) over 5 min incubation at 75°C. Thereafter, RT enzyme, dNTPs, and RT buffer were added, and cDNA was synthesized at 25°C for 10 min, at 42°C for 60 min, and at 85°C for 5 min. SYBR®Premix Ex TaqTM II (Takara Bio, Japan) in Applied Biosystems StepOne™ instrument (Applied Biosystems, USA) was used for Quantitative Real-Time PCR(QRT-PCR) at 95°C for 30 sec, followed by 40 cycles at 95°C for 30 sec and 60°C for 30 sec. The $2^{-\Delta\Delta Ct}$ method was applied to evaluate the expression level of miRNAs relative to SNORD 47 (U47) as the internal control. The primers were purchased from the Stem Cell Technology Research Center in Tehran, Iran. The experiments were performed in triplicate and

repeated at least three times.

3.7 Prediction of potential targets of miR-34a, miR-221, and miR-222

The potential targets of miR-34a, miR-221, and miR-222 in intrinsic and extrinsic apoptotic pathways were evaluated with *in silico* analysis with several algorithms, such as TargetScan (https://www.targetscan.org/vert_80/) and miRWalk (http://mirwalk.umm.uni-heidelberg.de/). These algorithms were supposed to have several parameters, such as complimentary sites of miRNAs in 3´-UTR of different mRNAs and the minimum binding energies, to determine the thermo-dynamical stability of miRNA-mRNA bindings and predict potential targets of miRNAs (26).

3.8 Quantitative analyses of potential targets of miRNAs

The expression level of several potential targets of the abovementioned miRNAs in apoptotic pathways was evaluated by RT-qPCR. PrimeScript™ RT reagent Kit (Takara Bio) was used for cDNA synthesis, and RT-qPCR was carried out using SYBR®Premix ExTaqTM II (Takara Bio, Shiga, Japan) Applied Biosystems StepOne™ instrument (Applied Biosystems, Foster City, USA). The primers were obtained from the Stem Cell Technology Research Center (Tehran, Iran) (Table 1). The $2^{-\Delta\Delta Ct}$ method was performed for the assessment of the relative expression of potential targets, and the *HPRT1* gene was used as the internal control gene.

Table 1. Sequence of primers used for RT-qPCR analyses

Genes and miRNAs	Oligo-sequences (5´-3´)
P53-F	5´-GGAGTATTTGGATGACAGAAAC-3´
P53-R	5´-GATTACCACTGGAGTCTTC-3'
BAX-F	5'- CAAACTGGTGCTCAAGGC-3'
BAX-R	5'-CACAAAGATGGTCACGGTC-3'
CASP9-F	5'- AGGGTCGCTAATGCTGTTTCG-3'
CASP9-R	5'- TCGTCAATCTGGAAGCTGCTAAG-3'
BCL2-F	5'- GATAACGGAGGCTGGGATG-3'
BCL2-R	5'- CAGGAGAAATCAAACAGAGGC-3'
CASP3-F	5'-CACAGCACCTGGTTATTATTC-3´
CASP3-R	5'-TTGTCGGCATACTGTTTCA-3´
CASP8-F	5'-GGATGATGACATGAACCTGCTGGA-3´
CASP8-R	5'-TTGTTGATTTGGGCACAGACTCTT-3´
HPRT1-F	5'- CCTGGCGTCGTGATTAGTG-3´
HPRT1-R	5'- TCAGTCCTGTCCATAATTAGTCC-3´
SNORD47-F	5'-ATCACTGTAAAACCGTTCCA-3´
SNORD47-R	5'- GAGCAGGGTCCGAGGT-3´´
miR-34a-F	5'-ATGGTGGCAGTGTCTTAGC-3´
miR-34a-R	5'- GAGCAGGGTCCGAGGT-3´
miR-221-F	5'-ATTCAGGGCTACATTGTCTG-3´
miR-221-R	5'- GAGCAGGGTCCGAGGT-3´
miR-222-F	5'-ACGATGCCAGTTGAAGAAC-3´
miR-222-R	5'- GAGCAGGGTCCGAGGT-3´

3.9 Statistical analysis

Data were analyzed using one-way ANOVA followed by Newman–Keuls multiple comparison test or Student's t-test. A *P*-value of 0.05 was considered statistically significant. All Statistical analysis was accomplished in Prism 7 software (GraphPad Software, Inc, La Jolla, CA, USA).

4. Results

4.1 Effect of SPNs on HT29 cells viability

The MTT assay analysis demonstrated that SPNs inhibited cell proliferation in a dose/time-dependent manner. The half-maximal inhibitory concentration (IC50) of SPNs was 28.13±0.78µg/ml after 24 h of treatment. In addition, SPNs in concentrations of more than 40µg/ml led to a decrease of less than 20% in cell viability (Figure.1).

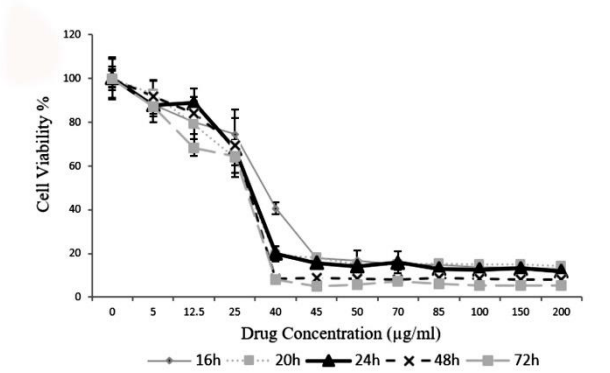

Figure 1. Effect of SPNs on HT-29 cell proliferation. Cell viability of HT-29 cells after treatment of SPNs (0-200µg/mL) for 16 to 72h

4.2 Apoptosis induction by SPNs

After 24 h of treatments, the population of late apoptotic cells (Annexin V+/PI+) increased significantly to 25.5% in SPNs treated cells at IC50 (28µg/ml) compared to untreated cells (2.26%). Apoptosis was stimulated by 32.36% in HT29 cells treated with 28 µg/ml SPNs. Moreover, 40 µg/ml and 50 µg/ml SPNs induced apoptosis in treated cells by 39.5% and 76.73%, respectively (Figure 2). Our DAPI staining analysis illustrated a significant decrease of stained nuclei in HT29 cells after treatment with SPNs in IC50 concentration compared to the control group (Figure 3).

4.3 Cell cycle analysis

Flow cytometric analysis pointed out that the percentage of cells in the sub-G1 phase changed from 7.71% in the control group to 11.32 at 28µg/ml (IC50) of SPNs-treated cells after 24 h. In addition, a significant decrease ($P<0.05$ to $P<0.01$) was observed in the cell population in the G0/G1 phase from 66.83% in the control cells to 60.38%, 59.84%, and 50.69% at 14 µg/ml, 28 µg/ml, and 40 µg/ml of SPNs-treated cells, respectively (Figure 4A). Compared to

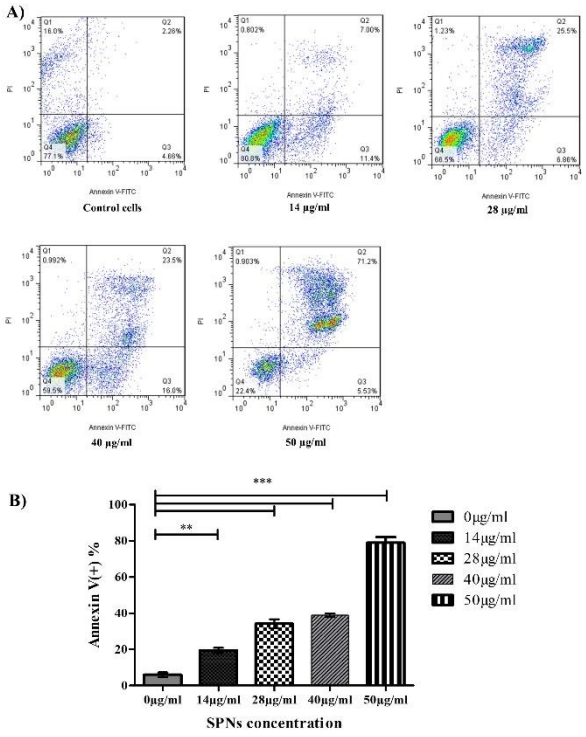

Figure 2. **A)** Flow cytometry analysis of AnnexinV/PI staining of treated cells with SPNs (0-50µg/ml). **B)** percentage AnnexinV(+) cells after treatment with SPNs. Data were expressed as mean±SD. ***P*<0.01; ****P*<0.001

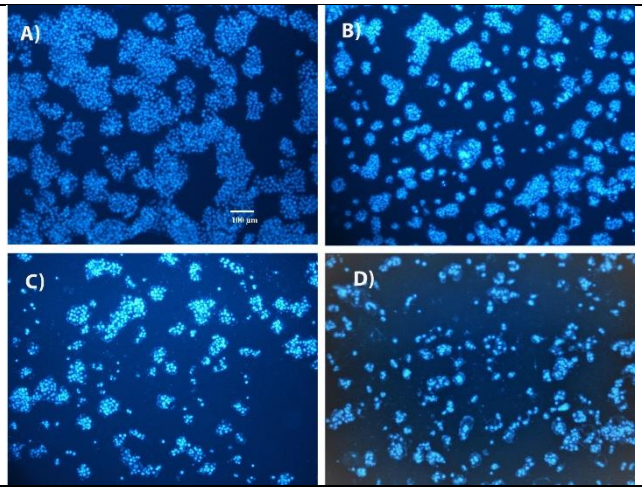

Figure 3. DAPI staining. **A)** HT-29 untreated cells and SPNs (28µg/ml) treated cells, **B)** after 24h, **C)** after 48h, and **D)** after 72h (Magnification, 10X)

the control group, the percentage of HT-29 cancer cells after treatment with SPNs indicated the significant arrest of cancer cells in the G2/M phase of the cell cycle after 24 h (Figure 4B)

4.4 SPNs effects on marker pattern in MCTSs and single cells

Figure 5 displays that hanging drop led to spheroids formation (colonospheres) during 10 days. Flow cytometry analysis revealed that 86.4%±2.75 and 77.4%± 2.12 of SPNs untreated HT29 cancer cells were $CD44^+$/ $CD133^+$ and $CD44^+$/ $CD24^+$, respectively (Figure 6). Accordingly, our result suggested that HT29 cells are cancer stem-like cells. Flow cytometry analysis identified that 70.3±4.03% and 66.7±3.18% of SPNs (28 µg/ml) treated spheroids were $CD44^+$/ $CD133^+$ and $CD44^+$/ $CD24^+$, respectively (Figure 7A), whereas these markers were much lower in SPNs treated non-spheroid HT29 cells (9.23% of $CD44^+$, 19.9% of $CD133^+$ and 7.42% of $CD24^+$) after stimulation with SPNs (28 µg/ml) (Figure 7B). It is worth noting that single cells, probably due to fast exposure to SPNs, were loosed CD markers relative to colonospheres. Nonetheless, SPNs treated MCTSs (spheroids) were completely destroyed after 72 h treatment with SPNs.

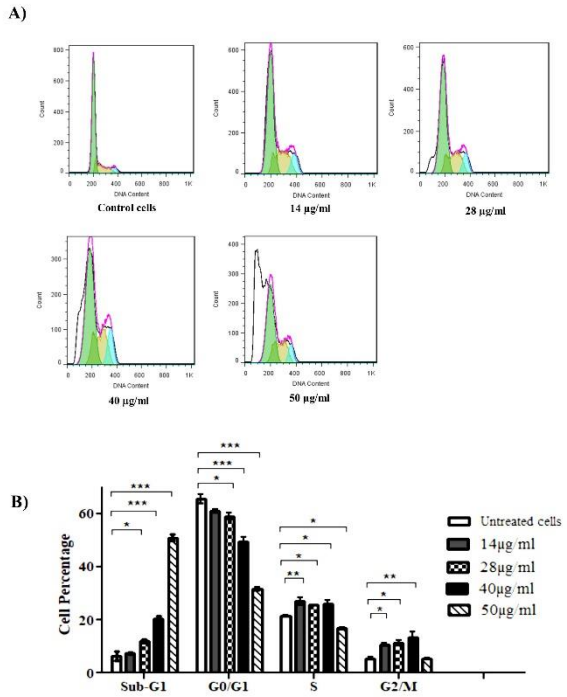

Figure 4. Cell cycle analysis. **A)** SPNs treated and untreated HT-29 cells after 24h. **B)** the cell percentage in each phase after staining with Propidium Iodide (PI) for 24 h. The results are presented as mean±SD. **P*<0.05, ***P*<0.01, ****P*<0.001

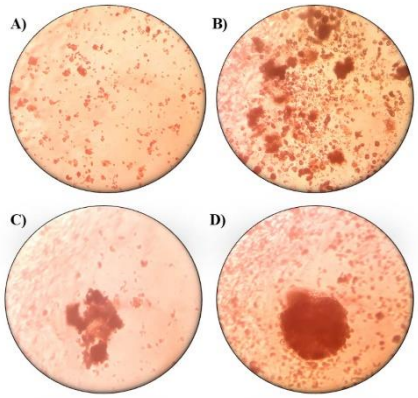

Figure 5. Hanging drop assay for HT-29 spheroid forming capacity after incubation at 37°C for **A)** 3 days, **B)** 5 days, **C)** 7 days, and **D)** 10 days. MCTSs emerged after 3 days and were completely condensed after 10 days of incubation

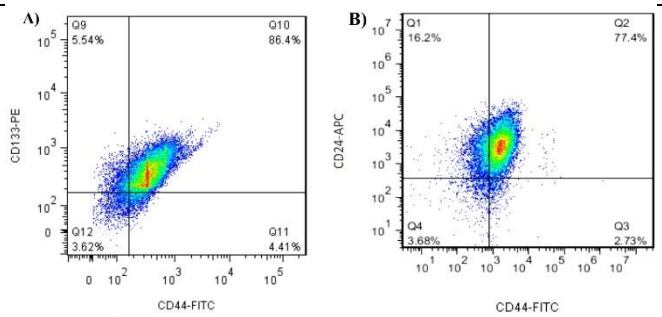

Figure 6. Evaluation of expression level of stem cell surface markers in HT-29 cancer cell line (SPNs untreated cells). Flowcytometry result of expression level of **A)** $CD44^+$/ $CD133^+$ cells and **B)** $CD44^+$/ $CD24^+$ cells were- 86.4% and- 77.4%, respectively

4.5 Deregulation of miRNAs after SPNs treatment

Q-RT-PCR analysis illustrated that in SPNs treated cells (non-spheroids), miR-34a was significantly upregulated (2.22±0.07 folds) and miR-221 and miR-

222 were significantly downregulated (-1.65±0.3 and -1.92±0.02 folds, respectively) compared to untreated cells (Figure 8A).

4.6 Potential targets of miRNAs in apoptotic pathways

In Silico analysis predicted several potential targets of desired miRNAs at intrinsic (mitochondrial) and extrinsic pathways of apoptosis, including *TP53, BAX, CASP3, CASP8, and CASP9* (Table 2).

4.7 deregulation of some potential target genes in apoptotic pathways

RT-qPCR analysis revealed that *TP53*, *BAX*, *CASP9*, *CASP3*, and *CASP8* have significantly upregulated >2

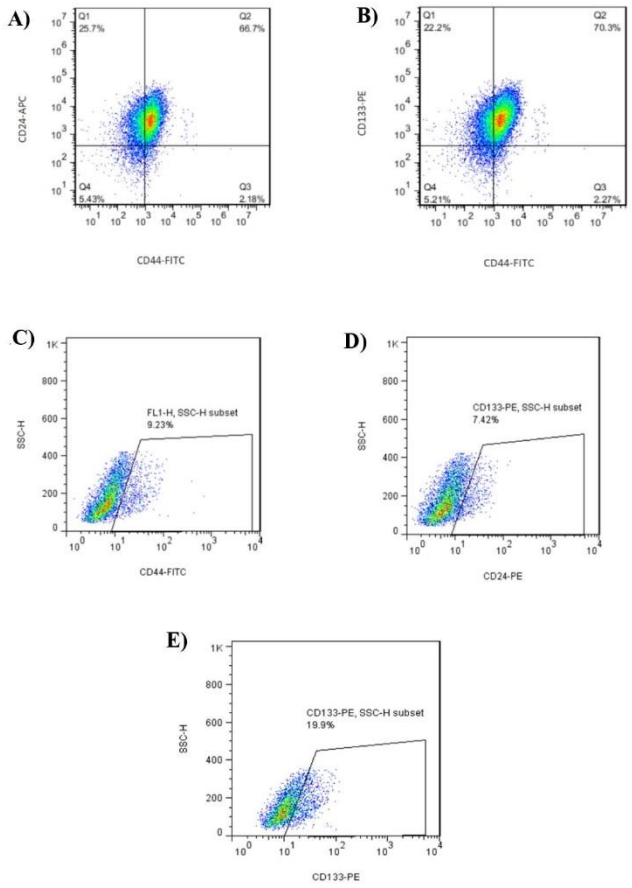

Figure 7. Assessment of HT-29 single cells (non-spheroids) and CSCs (spheroids) after treatment with SPNs. Flow cytometry analysis of **A)** HT-29 multicellular spheroids (CSCs) and **B)** HT-29 single cell suspension after treatment with SPN at IC50 dose for 24h

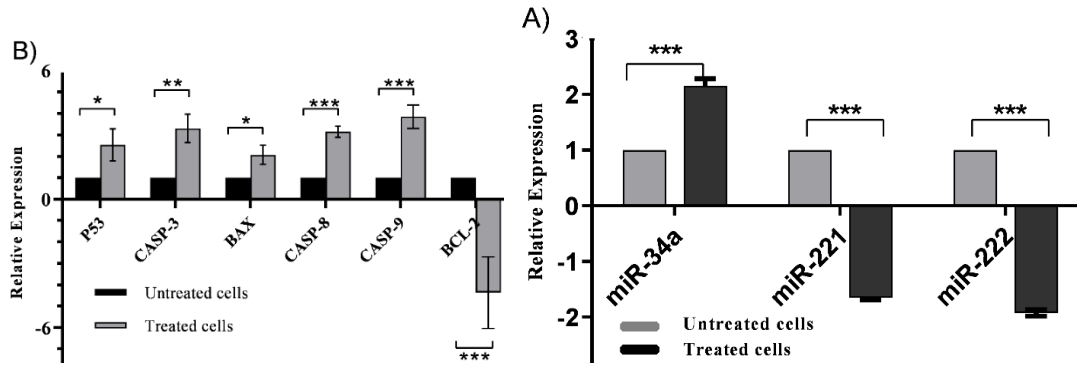

Figure 8. A) The expression level of miR-34a, miR-221, and miR-222 after treatment with SPNs (28µg/ml). B) Quantitative expression of target genes after treatment with SPNs (28µg/ml). Results was expressed as mean±SD; **P*<0.05, ***P*<0.01, ****P*<0.001

Table 2. *In silico* analysis

miRNAs	Potential Target Genes	Genes Name
miR-34a	BIK	BCL2 Interacting Killer
	BCL2	BCL2, apoptosis regulator
	PRAP1	Proline-rich acidic protein 1
	BAD	BCL2 associated agonist of cell death
	APAF1	Apoptotic peptidase activating factor 1
	AVEN	Apoptosis and caspase activation inhibitor
	BAK1	BCL2 antagonist/killer 1
	DEDD	Death effector domain containing
	BID	BH3 interacting domain death agonist
miR-221	BAX	BCL2 associated X, apoptosis regulator
	NUDT3	Nudix hydrolase 3
	PARP1	Poly (ADP-ribose) polymerase 1
	CASP9	Caspase 9, apoptosis-related cysteine peptidase
	CASP3	Caspase 3, apoptosis-related cysteine peptidase
	CASP8	Caspase 8, apoptosis-related cysteine peptidase
	PRR13	proline-rich 13
	P53	Tumor protein p53
	CCNT1	cyclin T1
miR-222	E2F2	E2F transcription factor 2
	CASP9	Caspase 9, apoptosis-related cysteine peptidase
	WNK2	WNK lysine deficient protein kinase 2
	BAX	BCL2 associated X, apoptosis regulator
	P53	Tumor protein p53
	CASP3	Caspase 3, apoptosis-related cysteine peptidase
	TGFA	transforming growth factor alpha
	PCNA	proliferating cell nuclear antigen
	CASP8	Caspase 8, apoptosis-related cysteine peptidase

folds in SPNs treated HT29 cells compared to untreated cells (Figure 8-B). Furthermore, the decrease in the expression of the anti-apoptotic *BCL2* gene was also observed in treated cells (-4.35±0.10 fold).

5. Discussion

Colorectal cancer (CRC) is the most common malignant gastrointestinal tumor cancer and the second most common cause of cancer-related death in both genders, as well as the third most common kind of cancer in oncologic pathology sampling (2). One of the most challenging issues in cancer treatment is the elimination of Cancer Stem Cells (CSCs) due to their ability to relapse cancer after treatment and drug resistance (27). The present study revealed that SPNs can HT-29 cancer stem cells after hanging drop (colonosphere formation) completely destroyed during 72 h. SPNs upregulated tumor suppressive-miRs, such as miR-34a, and down-regulated onco-miRs, such as miR-221 and miR-222. Moreover, our analysis showed that SPNs induced both intrinsic and extrinsic pathways of apoptosis.

Agarwal et al. reported that at 50-100 µg/ml of Silibinin, 8%-39%, 35%-75%, and 57%-91% of HT-29 cell growth was inhibited after 24, 48, and 72 h, respectively (28). Kauntz et al. indicated that Silibinin (40–100 µg/ml) inhibited cell growth on SW480 and SW620 colorectal cancer cell lines within eight days (29). Patel et al. showed that Silibinin inhibited cell proliferation of the HCT116-CD44^{+} subpopulation of colon cancer stem cells at ~120 µg/ml (30). In this study, Silibinin in polymersome nanoparticles (SPNs, ~220-357nm) (31) had cytotoxicity effects in lower doses (IC50=28µg/ml) during 24 h on HT29 cells relative to the abovementioned previous studies. In our previous study, SPNs can inhibit proliferation at a concentration of 45.06 µg/ml in MDA-MB-231 breast cancer cells after 24 h (31), while in this study, a lower concentration of SPNs affected proliferation inhibition. Therefore, it seems that silibinin, as a major compound of Silybum marianum, can be more effective in cancer cells derived from the gastrointestinal tract than cancer cells derived from breast tissue.

Apoptosis induction by Silibinin (~144 µg/ml) for 48-72h on SW480 and SW620 colorectal cancer cell lines was reported (21%-31% and 23%-40% late apoptosis, respectively) (29). The total percentage rates of apoptosis in colorectal cancer cell line HCT116-CD44^{+} after the induction of 120 µg/ml of Silibinin were 11.6% and 29.5% at 24 and 48h, respectively (30). Our analysis revealed that late and total apoptosis percentages increased significantly to 25.5% and 32.36% in SPNs treated cells (28µg/ml) only after 24 h. It is worth noting that, as observed in DAPI staining, the apoptotic percentage of treated cells was regularly increased in a dose-dependent manner. Thereafter, nanostructures in our study increased apoptosis induction by Silibinin on cancer cells at lower doses and time relative to previous studies.

A previous study on human colon carcinoma HT-29 cells demonstrated that Silibinin can induce cell cycle arrest in G0/G1 phases at 50 µg/ml, and also cause G2/M arrest with a higher dose (100 µg/ml) and longer treatment time (28). Further studies on colon cancer HT-29 and HCT-116 cell lines also indicated

G0/G1 and G2/M arrest in cell cycle progression with Silibinin in a dose/time-dependent manner (32). The current research pointed out that SPN can be considered an effective cell cycle blocker in two checkpoints at lower doses. Our analysis indicated that SPN could strongly induce cell cycle arrest in G2/M and proportionally in S at 28µg/ml at 24h (IC50 dose). This finding is indicative of the greater performance of encapsulated Silibinin to regulate cell mitosis and apoptosis, respectively.

In this study, we intended to evaluate our HT29 CRC cell line in terms of having CSC characteristics, including specific surface CD markers and the ability to form colonospheres. Our flow cytometry analysis revealed that HT29 CRC cell lines have 77.4% of $CD44^+/CD24^+$ and 86.4% of $CD44^+/CD133^+$, as well as forming colonospheres (33). Therefore, it seems that we can regard the HT29 cancer cell line as cancer stem-like cells.

The first assessment of the SPN effect on HT29 Cancer Stem-like Cells is to evaluate the ability of the drug to reduce the CSC CD markers. Previous studies indicated that Silibinin can significantly decrease the percentage of colorectal CSC by targeting their Specific CD markers and the colonosphere forming (12,30,34). Following this, for a more accurate evaluation of the SPN effect on CSC, the efficacy of the drug was calculated in two different cell culture models, including Multicellular Tumor Spheroids (MCTS) and single-cell suspension (non-spheroids). The result indicated that SPNs succeeded in decreasing the expression level of CSC CD markers in the two abovementioned groups compared to the control group (untreated cells). The percentage of $CD44^+/CD24^+$ and $CD44^+/CD133^+$ in MCTS after treatment with SPN decreased to 10.7% and 16.1%, respectively. In the same way, the percentage of $CD44^+$, $CD24^+$ and, $CD133^+$ in single-cell suspension with a great extent of reduction changed to 9.23%, 7.42% and, 19.9%, respectively. Nevertheless, due to the lower accessibility of SPNs to the center of mass in tumor spheroids (in MCTS models) after 24 h, the decrease of surface markers was slighter than in the single cells model. Nonetheless, after 72 h, SPNs-treated spheroid cells were completely lost. Therefore, it suggested that the use of SPNs may be an effective strategy in the removal of colorectal cancer stem cells in the future.

miR-34a is an important miRNA in various cancers, as well as Colorectal CSCs. This miRNA is a tumor suppressor and it decreases in cancer stem cells (23,35,36). miR-221 and mir-222 can activate and upregulate NFκB and STAT3 in CRC (37). Our study pointed to the upregulation of miR-34a and downregulation of miR-221 and mir-222 in SPNs treated HT29 Cancer Stem-like Cells compared to untreated cells. This data suggested that the SPNs may have the ability to inhibit the proliferation of colorectal cancer stem-like cells by mediating miRNAs, such as miR-34a, miR-221, and miR-222.

Our bioinformatics analysis pointed out that miR34a can potentially target apoptotic genes, such as *TP53, BAX, CASP9, CASP3, and CASP8.* A related study revealed that miR34a regulates gene expression in the intrinsic apoptotic pathway, such as *TP53* (38), *BAX* (39), *CASP9*, and extrinsic apoptotic pathways, such as *CASP3* and *CASP8* (40). Our quantitative analysis revealed that *P53*, *BAX*, *CASP3, CASP8,* and *CASP98* were upregulated after SPNs treatment in HT29 Cancer Stem-like Cells. Furthermore, the present study pointed out that *Bcl2* becomes downregulated after SPNs induction. It appropriated that SPNs can inhibit cancer stem cell formation through the regulation of miRNAs and their potential apoptotic targets.

6. Conclusion

In this study, silibinin loaded in SPNs increased the effects of silibinin on cancer cells compared to previous studies. SPNs (28µg/ml) can induce apoptosis in HT-29 non-spheroids and spheroids (cancer stem cells) and decrease cancer markers on the surface of spheroid and non-spheroids compared to SPNs untreated cells. Furthermore, SPNs downregulated oncomiRs, such as miR-221/miR-222, and upregulated tumor suppressive miRs, such as miR-34a (a cancer stem cell inhibitor). In addition, apoptotic genes, such as P53 and caspases, as potential targets of miR-221/miR-222 upregulated and Bcl2 as a potential target of miR-34a downregulated.

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Footnotes

Conflicts of Interest: The authors declare no conflict of interest

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