Deregulation of miR-34a, miR-221 and miR-222 after HT29 cells treatment with Silibinin encapsulated in polymersomes as an anti-cancer stem cell agent

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Abstract

Colorectal Cancer (CRC) has the most common malignant gastrointestinal cancer which representing about13% of all malignant tumor. CRC Cancer Stem Cell is the major reasons for recurrence of disease cause of solid tumor metastasis, relapse of cancer after treatment and drug resistance. Silibinin, an herbal extract from milk thistle plant, has been identified as a potential cancer medicine that can target the signaling pathway of CSCs and change their abilities. In our study, the results of CSC confirmation test such as specific surface CD markers and ability to form colonospheres was indicated the HT-29 cells as CSC-CRC. To increase the effectiveness of Silibinin, and also, to evaluate therapeutic intentions on HT-29 cancer stem-like cells, we encapsulated Silibinin in polymersome nanoparticle and validated the anti-proliferative and apoptotic activities of this new patent by MTT assay, AnnexinV/PI method, cell cycle analysis and DAPI staining. Furthermore, the efficacy of drug on Multicellular Tumor Spheroid (MCTS) and single cell suspension was showed that SPN had succeed to decrees the expression level of CSC CD markers compared with control group. Follow by using miRNAs as a novel and minus invasive expertise for prognostic, RT-qPCR confirmed that SPNs can repress oncogenic miRNAs such as miR-221 and miR-222. Silibinin encapsulated in Polymersome Nanoparticles (SPNs) can also enhance the expression of tumor suppressor miR-34a and some of its proapoptotic target genes such as P53, BAX, CASP9, CASP3, and CASP8. Our results suggested that SPNs can be recognized as a new stimulant factor to direct the HT-29 cancer cells toward apoptosis pathways thorough modify expression of some miRNAs and their apoptotic target genes directly and/or indirectly.

Introduction

Colorectal cancer (CRC) is the third common malignancy and the second cause of death related to cancer which represented in men and women aged over 65. However, due to increasing risk factors in recent decades such as obesity, bad nutritional habits, smoking and etc., frequency of disease has expanded in younger peoples [1]. In addition to physical, environmental aspects and also, genetic factors also play a significant role resulting in cancer. One of these genetic factors is miRNAs[2]. Many studies have indicated that dysregulation of small noncoding RNAs which are responsible for correct genes expression, can lead to irregular cellular behavior and bringing cancerousness [3][4]. One of these RNAs that called micro RNAs (miRNAs) have a validated role in cancer emersion. According to previous studies on relationship between miRNAs, tumorigenesis and their effect on CRC, the connection between altered miRNAs expression and caner progression was confirmed [5].These small RNAs inhibits genes expression by binding to 3'noncoding region of their targets [6]. *In silico* analysis showed that miRNAs such as miR-34a, miR-221 and miR-222, can target apoptotic genes such as *TP53, BAX, CASP9, CASP3 and CASP8.*

Due to the importance of cancer in life quality, with more studies on CRC, the heterogenous population cells were found with specific surface CD markers than common CRC cells such as CD44, CD24, epCAM, ALDH1, and CD133 [7][8][9]. These exclusive cells, called Cancer Stem Cells (CSCs), have individual features and responsible for self-renewal, produce differentiated cancer cells, initiate tumor growth, lost regulated proliferation, and etc. [1]. Another particular ability of CRC-CSC is the spontaneous

accumulation of cancer cells in floating spheroid form (known as colonospheres) in vitro[10]. These notable specifications causing chemotherapy drug resistant of CSCs as well as cancer recurrence, such a way that 15 to 30 percent of patient have has recurrence of CRC within 2 to 5 years. Therefore, finding the new drug strategy to overcome the CSC can provide a hopeful prospect to have a normal life for cancer patients[11][12].

Nowadays, one of the most current treatment is chemotherapy, that recommended before or after surgery [13][14]. due to many side effects of chemotherapies, researcher try to find new benefit and without adverse effect strategies such as native drugs (herbals)[15]. Using of natural drugs has a long history and can be suggested as a candidate for cancer therapy [16][17]. Silibinin is a polyphonic flavonoid which has widely properties such as hepatoprotective activity, antioxidant, immunomodulatory, antiviral properties and anticancer.[18][19]. In addition, over the last decades, it has been demonstrated that Silibinin has capability to arrest the cell cycle, inhibit the DNA synthesis and cell division, activate the caspase cascade and apoptotic cell death, consequently. Despite all the specifications, Silibinin had deficiency in absorption by simple diffusion to cell. This complications cause to low bioavailability and poor cellular uptake of drug [20]. To overcome this problem and release additional amount on drug into the cells, researchers have used nano-carriers as a solution

Nowadays, different nano-carrier with individual features are applied such as, liposomes [21], dendrimers [22], micelles [23], nano emulsions [24] and polymersomes [25]. Polymersomes are a group of self-assembling polymers that are highly flexible, rather stable than liposomes and have long-time blood circulation [26]. So, this is compatible to maintenance of persistent drug concentration in the blood for a long time, with no applying to further doses. Also, with the various compounds which can be loaded in polymersomes, it will have a several capabilities in many applications in nanomedicine[27]. Thus, many lipophilic anticancer drugs, amphiphilic dyes, transgenes and membrane protein could be assembling within this nano-carrier without changing their activity. These advantages of polymersome, make it as a one of the remarkable supramolecular structure for numerous applications in nanomedicine and nanobiology[28] [25].

The goal of this study was to investigation the effect of Silibinin-loaded polymersome nanoparticle (SPN), as a new synthetic agent with nano-drug delivery strategy, on HT-29 cell line. We evaluated this new fortified drug for assessment of its effectiveness on CSC characteristics including surface CD markers and colonosphere forming. Furthermore, the effect of SPN on cell cytotoxicity and apoptosis pathways thorough adjustment the expression of miR-34a, miR-221 and miR-222, and also potential apoptotic pathways genes was examined scrutiny.

Material And Methods

2.1 Materials

Silibinin(2,3-Dihydro-3-(4-hydroxy-3-methoxyphenyl)-2(hydroxymethyl)-6-(3,5,7-trihydroxy-4-oxobenzopyran-2-yl) benzo dioxin) was purchased from Sigma-Aldrich Co. (Schnelldorf, Germany) with

purity of ≥98% (MW:482.44). All materials for the preparation of nanocarrier including oleoyl chloride and polyethylene glycol (400 KD) were purchased from Sigma-Aldrich (St Louis, MO, USA) and triethyl amine and chloroform were both purchased from EMD Millipore (Billerica, MA, USA). 40,6-diamidino-2-phenylindole (DAPI) was purchase from Sigma-Aldrich Co. (Saint Quentin, France). All of the conjugated monoclonal antibody for CD133, CD24 and CD44 surface markers were purchased from (Invitrogen, Carlsbad, CA, USA).

2.2 Preparation of Silibinin encapsulated in nanoparticle

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 hours. 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. In order to use the PEG_{400}-OA as a nanocarrier in preparation of Nanoparticles, 300mg of PEG_{400}-OA and different concentration of Silibinin were dissolved in acetone solution. After that, the acetone was evaporated and the prepared product with different weight/weight ratios of Sil/ PEG_{400}-OA (1:4 to 1:30) was stored at 4°C in a light-protected condition. Encapsulation Efficiency, drug loading of nanoparticle and also appropriate verification tests such as DLS, Zeta potential, AFM, TEM, SEM, Stability test was done to determinate the physical and chemical characterization including size, shape, zeta potential, morphology, stability, etc. [29]

2.3. In vitro study

2.3.1. Cell culture study

HT-29 cell line (Human Colorectal Adenocarcinoma Cell Line) was purchased from the National Cell Bank of Iran (NCBI, Pasteur Institute, Tehran, Iran) and cultured in DMEM medium supplemented with 10% FBS and 1% L-glutamine at 37°C in a 5% CO2 humidified atmosphere. All chemicals for cell culture were purchased from GIBCO (Gaithersburg, MD, USA).

2.3.2. Determination of cell viability (MTT assay)

MTT (3-[4,5-dimethylthiazol-2-yl]2,5-iphenyltetrazolium bromide) assay was used to evaluate the effect of Silibinin encapsulated in polymersome nanoparticles (SPNs) on proliferation and viability of HT-29 cell lines. According to the manufacturer's instructions (Sigma-Aldrich, USA), After 24h of the HT-29 cells culture in a 96-well plate (1×10^4 cells/well), the cells were treated by the serial concentrations of SPNs (0 to 200µg/ml) for 16, 18, 20, 22, 24, 48, and 72 h. Afterwards, cells were washed with PBS, and 100µL of fresh medium containing 10µL of MTT (5mg/ml) was added to each well and incubated for 3h at 37ºC in a 5% CO2 humidified atmosphere. Finally, 100µL of dimethyl sulfoxide solution (DMSO) was added to each well, and Percent of cell survival was defined as the relative absorbance of treated cells versus control groups at 540nm (ELx800, BioTek, USA). As a results, the half maximal inhibitory concentration

(IC50) of SPNs on HT-29 cell lines was determined by statistical analysis. Each experiment was carried out in triplicate and repeated at least three times.

2.3.3. Apoptosis assay

For apoptosis analysis, flow cytometry assays was carried out with using Annexin V-FITC kits (Miltenyi Biotech, Germany). According to the manufacturer's manual, 14, 28, 40, and 50 µg/ml of SPNs was effected on 1×10^4 cells/well for 24h. After washing twice with binding buffer, 10µL of Annexin V-FITC was added and cells were incubated for 15 min in dark place. After washing the cells with 1 ml of binding buffer for two times, stained cells centrifuged and cell pellet was resuspended in 500µL of binding buffer. Finally, 5µL of PI solution was added immediately prior to analysis by flow cytometry (BD FACS Calibur; BD Biosciences, San Jose, CA, USA). The experiments were repeated three times.

2.3.4. Apoptosis detection by DAPI staining

40',6-diamidino-2-phenylindole (DAPI) staining was used for assessment of HT-29 apoptotic cells with/without SPNs treatment. For this purpose, the cells were treated with SPNs at IC50 concentration (28µg/ml) and incubation for 24h, 48h, and 72h. Afterward, the cells were fixed with 4% paraformaldehyde, and stained using DAPI in PBS (2.5µg/ml) for 15 sec at 20°C in a dark condition. Finally, the stained cells were washed once with PBS, and the changes in SPNs treated cells were identified at 10-fold magnification using Nikon Eclipse-TE2000-S inverted microscopy compared with untreated cells.

2.3.5. Cell cycle analysis

After 24h of induction of similar SPNs concentration with apoptosis assay, treated and control cells were washed with cold PBS and fixed in cold 70% ethanol at 4°C for 2h. Afterwards, 500 µL PBS with 50µL RNase (100µg/ml) was added to the fixed cells incubated at RT for 30min. Finally, PI (50µg/ml) was added for cellular DNA staining, and after 30min, the cell cycle was analyzed by a flow cytometry instrument (BD FACS Calibur; BD Biosciences, San Jose, CA, USA).

2.4 Cancer Stem Cell marker assay

For sample preparation, 1×10^6 cells were centrifuged at 300g for 5m. cell pellet was resuspended in 100µL of PBS containing 3% BSA (Sigma, St. Louis, MO) and incubated with an appropriate concentration of conjugated monoclonal antibody for CD133 PE (Phycoerythrin), CD24 APC (Allophycocyanin) and CD44 FITC (Fluorescein isothiocyanate) surface markers. After 1h at 4°C, the final volume of sample was adjusted to 1000µL with PBS and centrifuged at 300g for 5m at 4°C. Finally, supernatant was removed, cell pellet was fixed by adding 500µL of 1% cold paraformaldehyde (Sigma, St. Louis, MO) and the sample was analyzed by flowcytometry instrument (BD FACS Calibur; BD Biosciences, San Jose, CA, USA).

2.5 Preparation of Multicellular Tumor Spheroids

Preparation of Multicellular Tumor Spheroids (MCTSs) are based on superiority of intracellular adhesive forces toward exogenous extracellular matrix such as cell culture flask. This advantage leads to promotion of cell-cell contact and forming spherical cancer structure such as tumor materials. For this purpose, hanging drop method was used according to the authors manual [30]. Briefly, 15 to 20 drops of harvested cells up to 30µL containing 0.5×10^6 cells/ml were deposited on a 10cm dish lid. Following to inversion of the tray over the petri dish with has 10 ml of PBS and incubation at 37°C for 3 to10 days, cells were accumulated and gradually appeared in the form of single MCTS. As a result, a large amount of sphere forming cancer cells were obtained and incubated in untreated 6 well plates at 37°C for more experiments.

2.6 Qualitative model response to drug treatment

The effect of drug treatment on 3-dimentional culture was explored by comparing two difference cell culture models. The first model is single cell suspension within DMEM and the second model, a multicellular spheroid of approximately 10^6 cells. For this purpose, single cell suspension and multicellular spheroid cells were exposed to 28µg/ml of SPNs and after 24h, were analyzed by flowcytometry instrument (BD FACS Calibur; BD Biosciences, San Jose, CA, USA) for evaluation of desired CSC surface markers.

2.7 RNA isolation and RT-qPCR analysis of miRNA expression

RNA isolation and cDNA synthesis were performed using iNtRON kit (iNtRON Biotechnology, Korea) and BON-miR high sensitivity miRNA 1st-Strand cDNA synthesis Kit (Stem cell Technology Research Center, Tehran, Iran), respectively. Briefly, with using poly (A) polymerase at 37°C, poly (A) tail was added to miRNAs during 30 min. After RNA poly(A) tail was mixed with BON-RT adaptor (primer,10µM) over 5 min incubation at 75°C, RT enzyme, dNTPs and RT buffer were added and cDNA was synthesized during 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, Shiga, Japan) in Applied Biosystems StepOne^{TM} instrument (Applied Biosystems, Foster City, USA) was used to Quantitative Real Time PCR(QRT-PCR) during 95°C for 30 seconds, followed by 40 cycles at 95°C for 3seconds and 60°C for 30 seconds.

$2^{-\Delta\Delta Ct}$ method was applied to evaluation the expression level of each miRNA with SNORD 47 (U47) as the internal control. The primers were purchased from Stem cell Technology Research Center, Tehran, Iran and All reactions were performed in triplicate.

2.8 Prediction of SPNs-inducing signaling pathways through miRNAs targeting genes

The potential targets of miR-34a, miR-221 and miR-222 in two apoptotic pathways were predicted with *Insilico* analysis according to several algorithms such as TargetScan and miRWalk. These algorithms was supposed several parameters such as complementary sites of miRNAs in 3´-UTR of different mRNAs and the minimum binding energies to determine thermodynamically stability of miRNA-mRNA bindings [31] and predict potential targets of miRNAs.

2.9Quantitative RT-PCR analyses of potential target genes

The level of miR-34a, miR-221 and miR-222 targets expression in apoptotic pathways was evaluated with RT-qPCR. For this purpose, PrimeScript^{TM} 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) in Applied Biosystems StepOneTM instrument (Applied Biosystems, Foster City, USA). The applied primers of apoptotic genes including *TP53, BCL2, BAX*, *CASP9*, *CASP8* and *CASP3* were obtained from Stem Cell Technology Research Center (Tehran, Iran) (Table 1). $2^{-\Delta\Delta Ct}$ method was performed for assessment of potential targets relative expression, and the *HPRT1* gene was used as the internal control gene.

2.10 Statistical analysis

All Statistical analysis was accomplished with Prism®7 software (GraphPad Software, Inc, La Jolla, CA, USA). 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 used to identify statistical significance. All Results were expressed as the mean±SD and each experiment was repeated at least three times independently.

Results

3.1 Effect of SPNs on HT29 cells proliferation

HT-29 cells were treated with different concentrations (0, 5, 12.5, 25, 40, 45, 50, 70, 85, 100, 150 and 200 µg/ml) of SPNs during 16 to 72h. MTT assay analysis showed that SPNs inhibited the cell proliferation in a dose/time-dependent manner. The half maximal inhibitory concentration (IC50) of SPNs was 28.13±0.78µg/ml after 24h of treatment. In addition, our analysis declared that there is no significant reduction after 20 to 72h (Fig.1).

3.2 Apoptosis induction by SPNs

Apoptosis induction in HT-29 cancer cells with 14µg/ml, 28µg/ml, 40µg/ml and 50µg/ml of SPNs was evaluated by the flow cytometry at three different times. After 24h of treatments, the population of late apoptotic cells (Annexin V^+/PI^+) were increased significantly to 25.5% in cells treated with SPNs at IC50 (28µg/ml) compared to untreated cells (2.26%). Likewise, the more accurate study of apoptotic assay showed that the apoptotic percentage of treated cells were regularly increased in a dose-dependent manner comparable to control cells (Fig.2).

3.3 Apoptosis detection by DAPI staining

Our DAPI staining analysis showed the significant decrease of stained nuclei in HT29 cells after treatment with SPNs in IC50 concentration compared to control group. The results also evaluated that the treated cells with SPNs were lost their numbers during the time within 24 to 72h (Fig.3).

3.4 cell cycle analysis

The flow cytometric analysis showed that the percentage of cells in sub-G1 phase increased with the increase of SPNs concentration. Flow cytometric analysis showed that the percentage of cells in the sub-G1 phase changed from 7.71% in the control to 11.32 at 28µg/ml (IC50) of SPNs-treated cells after 24hr. 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, 28 and 40 µg/ml of SPNs-treated cells, respectively (Fig.4A).

Compare to the control group, the amount of HT-29 cancer cells after treatment with SPNs indicated the significant arrest of cancer cells in G2/M phase of the cell cycle after 24h. Analyzed data imply that increasing drug concentration in arrested phase cause to increase accumulation of cancer cells in which 10.48% at IC50 dose compare to an un-treated one in 5.07%. (Fig.4B)

3.5 Cancer stem-like cell characterization in HT29 cell line

The HT29 cancer cell line was evaluated for expression level of stem cell surface markers such as CD133, CD24 and CD44. Applied flow cytometry revealed that 86.4% ± 2.75 and 77.4%± 2.12 of HT29 cancer cells were $CD44^+$/ $CD133^+$ and $CD44^+$/ $CD24^+$ respectively (Fig.5). In addition, observations from hanging drop assay showed that HT29 spheroid forming capacity was become visible after 3 days of incubation in 37°C and MCTSs were condensed after 10 days (Fig.6).

3.6 Qualitative model response to drug treatment

The cancerous cells drug response was evaluated by comparing two different models of HT29 cancer cells including single cell suspension and multicellular spheroid forms. The results showed that treatment of single cell suspension with SPNs was much more effective than multicellular spheroid form. Flow cytometry analysis identified that 70.3±4.03% and 66.7±3.18% of multicellular spheroids were $CD44^+$/ $CD133^+$ and $CD44^+$/ $CD24^+$ respectively whereas this CSCs marker were much lower in single cell type (9.23% of $CD44^+$, 19.9% of $CD133^+$ and 7.42% of $CD24^+$). It is worth noting that in both comparing models, the surface markers were less than untreated ones (Fig.7).

3.7 Up and down regulation of miRNAs associated with cancerous pathways

Q-RT-PCR analysis showed that in SPNs treated cells, miR-34a were significantly upregulated (2.22±0.07 folds) and miR-221 and miR-222 were significantly downregulated (0.61±0.3 and 0.52±0.02 folds, respectively) (Fig.8-A).

3.8 Potential targets of miRNAs in apoptotic pathways

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

3.9 Upregulation of potential target genes in apoptotic pathways

RT-qPCR analysis revealed that some genes involved in intrinsic and extrinsic pathways of apoptosis including *TP53, BAX*, *CASP9,CASP3* and *CASP8* were significantly upregulated >2 folds in SPNs treated HT29 cells compared to untreated cells (Fig.8-B). Furthermore, the decrease in expression of anti-apoptotic *BCL2* gene was also observed in treated cells (0.23±0.10 fold).

Discussion

Colorectal cancer (CRC) has the most common malignant gastrointestinal tumor cancer, and is the second most common cause of death related to cancer in box sex, as well as the third most common kind of cancer in oncologic pathology sampling[1][32]. One of the most challenging matter in cancer treatment is the elimination of Cancer Stem Cells (CSCs) because of their ability to relapse of cancer after treatment and drug resistance[33].

Recent studies showed that Silibinin, an herbal extract from milk thistle plant, is recognized as a potential cancer protective drug and also can target the signaling pathways in CSCs and conducted these cells to differentiate and lose their properties [34][34].

Chapla Agarwal was 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 72h, respectively[35]. Also, the cell growth inhibition doses of Silibinin on SW480 and SW620 colorectal cancer cell line was evaluated by Henriette Kauntz and observed that it was 40–100 µg/ml within 8 days [36]. Shanaya Patel et.al also showed that Silibinin inhibited cell proliferation of HCT116-CD44+ subpopulation of colon cancer stem cells at 120 µg/ml [37]. In our previous study, we synthetized a polymersome nanocarrier that due to suitable properties(appropriate size, narrow size distribution, higher Encapsulation Efficiency (EE) and Drug Loading (DL)) [29] we again used in this study. In this study, Silibinin in polymersome nanoparticles had cytotoxicity effects in lower dose (IC50=28µg/ml) during 24h relative to previous studies. It is considerable that SPNs can inhibit proliferation in concentration 45.06 µg/ml in MDA-MB-231 breast cancer cells after 24h [29], while in this study, lower concentration of SPNs effected on proliferation inhibition. Results showed that Silibinin could not only be more effective in the drug delivery system with this new nano-structure, but also at lower dose in some cancer areas such as gastrointestinal tract could be more effective than 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) [36]. The total percentage of apoptosis in colorectal cancer cell line HCT116-CD44+ after induction of 120 µg/ml of Silibinin was 11.6% and 29.5% at 24 and 48h, respectively [37]. Our analysis revealed that late and total apoptosis percentage increased significantly to 25.5% and 32.36% in SPNs treated cells (28µg/ml) only after 24h. It's worth noting that as it observed in DAPI staining, the apoptotic percentage of treated cells were regularly increased in a dose-dependent manner. Then, nano structures in our study increased apoptosis induction by Silibinin on cancer cells in lower dose and time.

Previous study on human colon carcinoma HT-29 cells showed that Silibinin can be induced cell cycle arrest in G0/G1 phases at 50 µg/ml, and also caused to G2/M arrest with higher dose (100 µg/ml) and longer treatment time [38]. Further studies on colon cancer HT-29 and HCT-116 cell line also indicated on G0/G1 and G2/M arrest in cell cycle progression with Silibinin in dose/time dependent manner [39]. Our research showed that SPN can be considered as an effective cell cycle blocker in two check point in lower dose. Data analysis indicated that SPN can strongly induced cell cycle arrest in G2/M and proportionally in S in 28µg/ml at 24h (IC50 dose). This finding shows the greater performance of encapsulated Silibinin to regulate the cell mitosis and apoptosis death, respectively.

In this study, we intended to evaluation of our HT29 CRC cell line in terms of having CSC characteristics including specific surface CD markers and ability to form colonospheres. Our flowcytometry analysis showed that HT29 CRC cell line have 77.4% of $CD44^+/CD24^+$ and 86.4% of $CD44^+/CD133^+$ as well as forming colonospheres. Therefore, it seems that we can consider the HT29 cancer cell line as CSC-CRC.

The first assessment of the SPN effect on HT29 Cancer Stem-like Cells is to evaluate the ability of drug to reduce the CSC CD markers. Previous studies showed that Silibinin can significantly decrease the percentage of colorectal CSC by targeting their Specific CD markers. it also indicated that Silibinin can effect on colonosphere forming of CSCs by reducing their specific markers [40][37][41].

Follow this, for more accurate evaluation of SPN effect on CSC, the efficacy of drug was calculated in two difference cell culture models including Multicellular Tumor Spheroid (MCTS) and single cell suspension. The result was indicated that SPN had succeeded to decrees the expression level of CSC CD markers compared with control group. The percentage of $CD44^+/CD24^+$ and $CD44^+/CD133^+$ in MCTS after treatment with SPN decreased to 10.7% and 16.1%, respectively. As the same way, the percentage of $CD44^+$, $CD24^+$ and $CD133^+$ in single cell suspension with a great extent reduced were changed to 9.23%, 7.42% and 19.9%, respectively. However, due to lower accessibility of SPNs to center of mass in tumor spheroids (in MCTS models) after 24h, decrease of surface markers were less than single cells model. Nonetheless, after 72h, most cells in MCTS was lost. Therefore, using SPNs can effective on removal colorectal CSCs. Our finding suggested that the use of polymersome nanoparticles can increas anti-stem less cancerous properties of Silibinin. This higher performance of Silibinin as an anti-cancer stem cell agent is due to its new and outperform nanostructure.

Previous investigation showed that one of the influencer miRNAs in various cancers[42] as well as Colorectal CSCs is miR-34a. This miRNA is known as a tumor suppressor and so it has been made clear that miR34a may decrease in cancerous stem cells. [43][44][45]. The result of our study showed that after the treatment of HT29 Cancer Stem-like Cells with SPNs, the expression of miR34a increased compared to control cells. This data suggested that the SPNs may have the ability to inhibit proliferation of the colorectal CSCs by mediating miR-34a.

The two other miRNAs that have been extensively researched for the past nine years on their association with Colorectal cancer is miR-221 and miR-222. In 2014 scientists also showed that miR-221 and mir-222

can activate NFkB and STAT3 and increase expression of both in CRC[46].

From the point of view of the results, it seems that SPN can direct the HT-29 cancer cells toward apoptosis pathways thorough modification of mentioned miRNAs expression.

From another perspective, previous studies showed that Silibinin can accelerate the treatment of CRC-CSC by reducing specific CD markers[47][48].

According to mentioned studies and also the results of our research based on reducing CD markers in treated-HT-29 Cancer Stem-like Cells, it can be suggested that SPN can perform its therapeutic effect on the CRC-CSCs by modulate the miRNAs expression and decrease the CSC specific CD markers.

Since that miRNA's deregulation leads to dysfunction of genes, using *in silico* analysis, the predicted potential apoptotic pathways genes that can be controlled by miR-34a, miR-221 and miR-222 were selected.

The result of our study showed that after the treatment of HT29 Cancer Stem-like Cells with SPNs, the expression of miR34a increased compared to control cells. This data suggested that the SPNs may have the ability to inhibit proliferation of the colorectal CSCs by mediating miR-34a.

Our bioinformatic analysis showed that miR34a can target apoptotic genes such as *TP53, BAX, CASP9, CASP3 and CASP8.* In previous studies, our predictions validated as miR-34a targets Previous studies validated that expression of miR34a was related in regulation of intrinsic apoptosis pathway genes such as *TP53* [49], *BAX* [50], *CASP9* [51], , and extrinsic apoptosis pathway genes such as *CASP3* and *CASP8* [51]. In addition, the relationship between miR221/222 and expression of mentioned apoptosis pathway genes were predicted by *in silico* analysis.

As a consequence, quantitative analysis revealed that *P53*, *BAX*, *CASP9,CASP3* and *CASP8* were upregulated after treatment of HT29 Cancer Stem-like Cells with SPNs. On the other hand, our study also showed that *Bcl2* becomes downregulated after SPNs induction. According to validated and predicted target genes of miR34a, miR-221 and mir-222, it seems that modifying of the expression of apoptotic pathway genes may be cause of change in expression of desired miRNAs by SPNs.

Conclusion

Due to the results, HT-29 cancer cell line can be notice as CSC-CRC and the apoptotic percentage of treated cells after treatment with SPNs were regularly increased. Moreover, the results can suggest that SPNs with a new, functional and unique structure can be identified as a Stimulus to drive HT-29 cancer cells to apoptosis. Our nano- effective structure does its responsibility toward apoptosis pathways thorough modification of miR34a, miR-221 and mir-222, expression. This new nano-Silibinin can be also directly and/or indirectly adjust the expression of intrinsic/extrinsic apoptotic pathways genes.

Declarations

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Compliance with ethical standards:

The article contains no research in which animals were used. Also, This Experiment involved no biological materials obtained from people, patient cohorts, and etc.

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Tables

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

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

Note. P53-F/R: Tumor protein p53- Forward/Reverse; BAX-F/R: BCL2
Associated X- Forward/Reverse; CASP9-F/R: Caspase 9, apoptosis-related cysteine peptidase- Forward/Reverse; BCL2-F/R: BCL2, apoptosis regulator- Forward/Reverse; CASP3-F/R: Caspase 3, apoptosis-related cysteine peptidase- Forward/Reverse; CASP8-F/R: Caspase 8, apoptosis-related cysteine peptidase- Forward/Reverse; HPRT1-F/R: Hypoxanthine PhosphoRibosyl Transferase1- Forward/Reverse; SNORD47-F/R: small nucleolar RNA, C/D box 47 - Forward/Reverse; miR-34a/221/222-F/R: microRNA-34a/221/222- Forward/Reverse.

Table 2. Some potential targets of miRNAs

miRNAs	Some 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

Note. miR-34a: microRNA-34a; miR-221: microRNA-221; miR-222: microRNA-222.

Figures

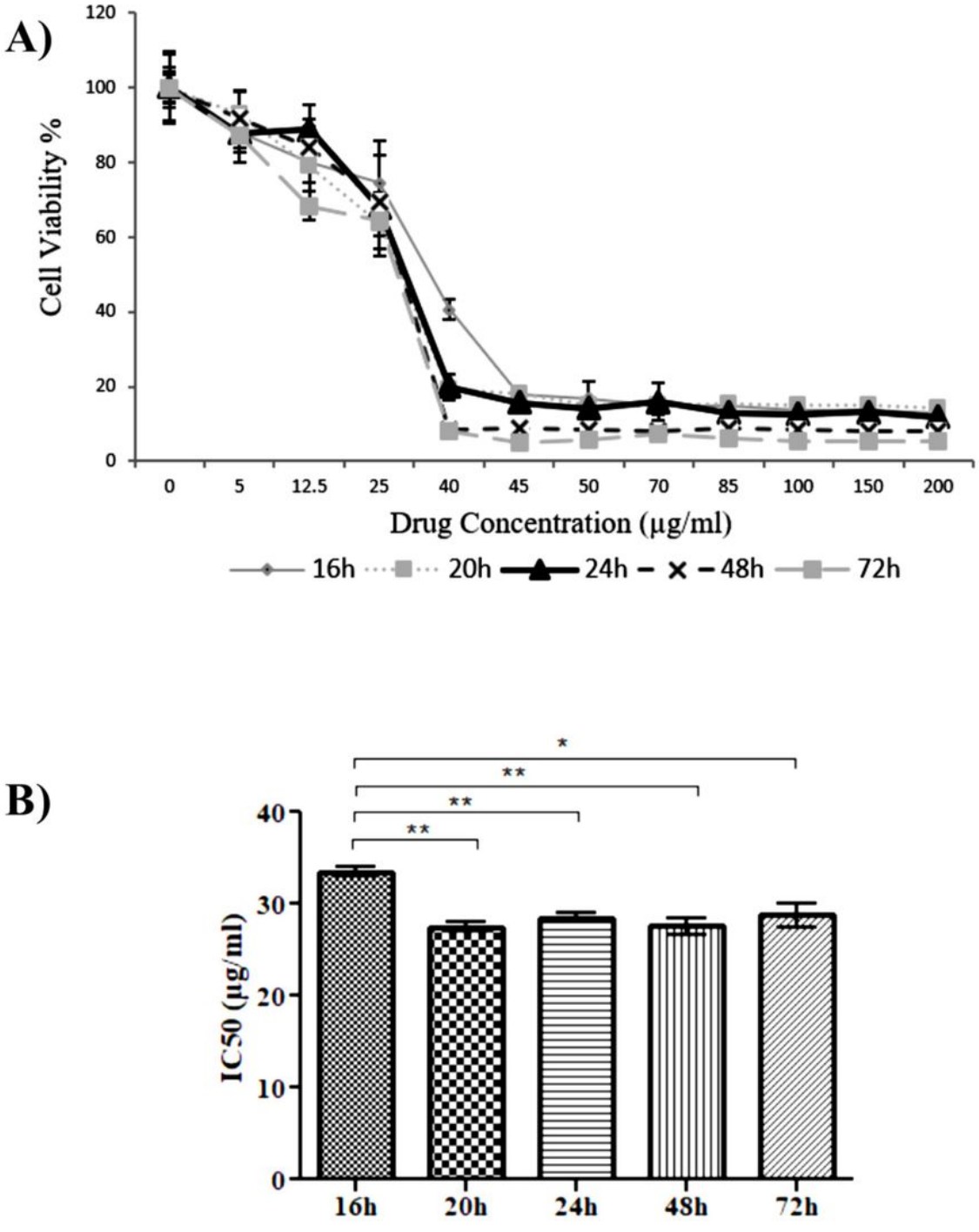

Figure 1

Effect of SPNs on HT-29 cells proliferation. A) Cell viability of HT-29 cells after treatment of SPNs with different concentrations (0–200 μg/mL) for 16 to 72h. B) Statistical analysis of the half maximal inhibitory concentration (IC50) of SPNs (μg/ml) during 16 to 72h. The results are presented as mean ± SD; *P<0.05, **P<0.01.

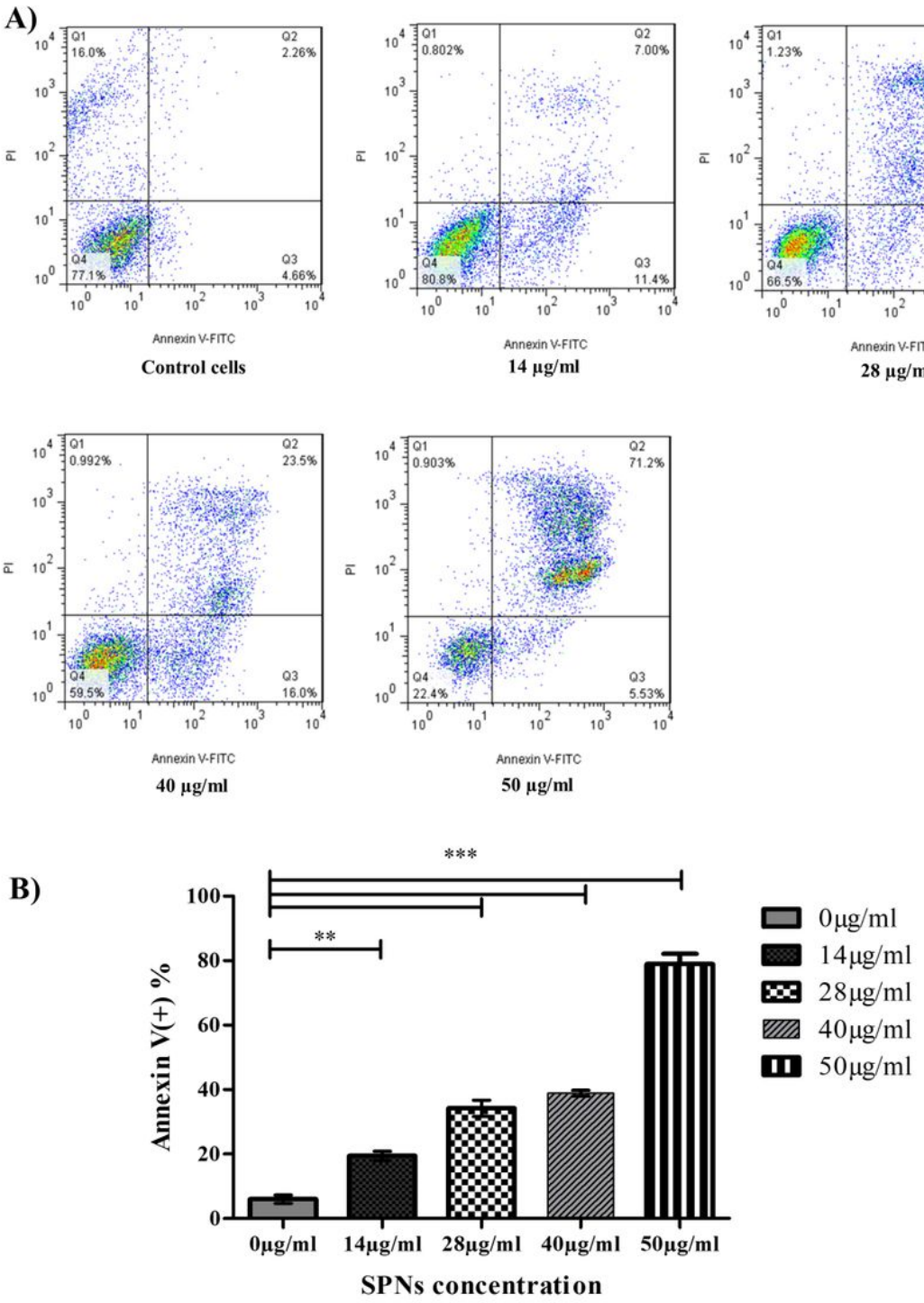

Figure 2

Flow cytometry analysis of AnnexinV/PI staining of HT-29 cells after induction of SPNs for 24h with the concentrations of 0μg/ml, 14μg/ml, and 28μg/ml, 40μg/ml, and 50μg/ml (A). B) Statistical analysis of Annexin V (+) cell percentage after treatment with SPNs for 24h (0, 14, 28, 40, and 50 μg/ml). Data was expressed as mean ± standard deviation. **P<0.01; ***P<0.001.

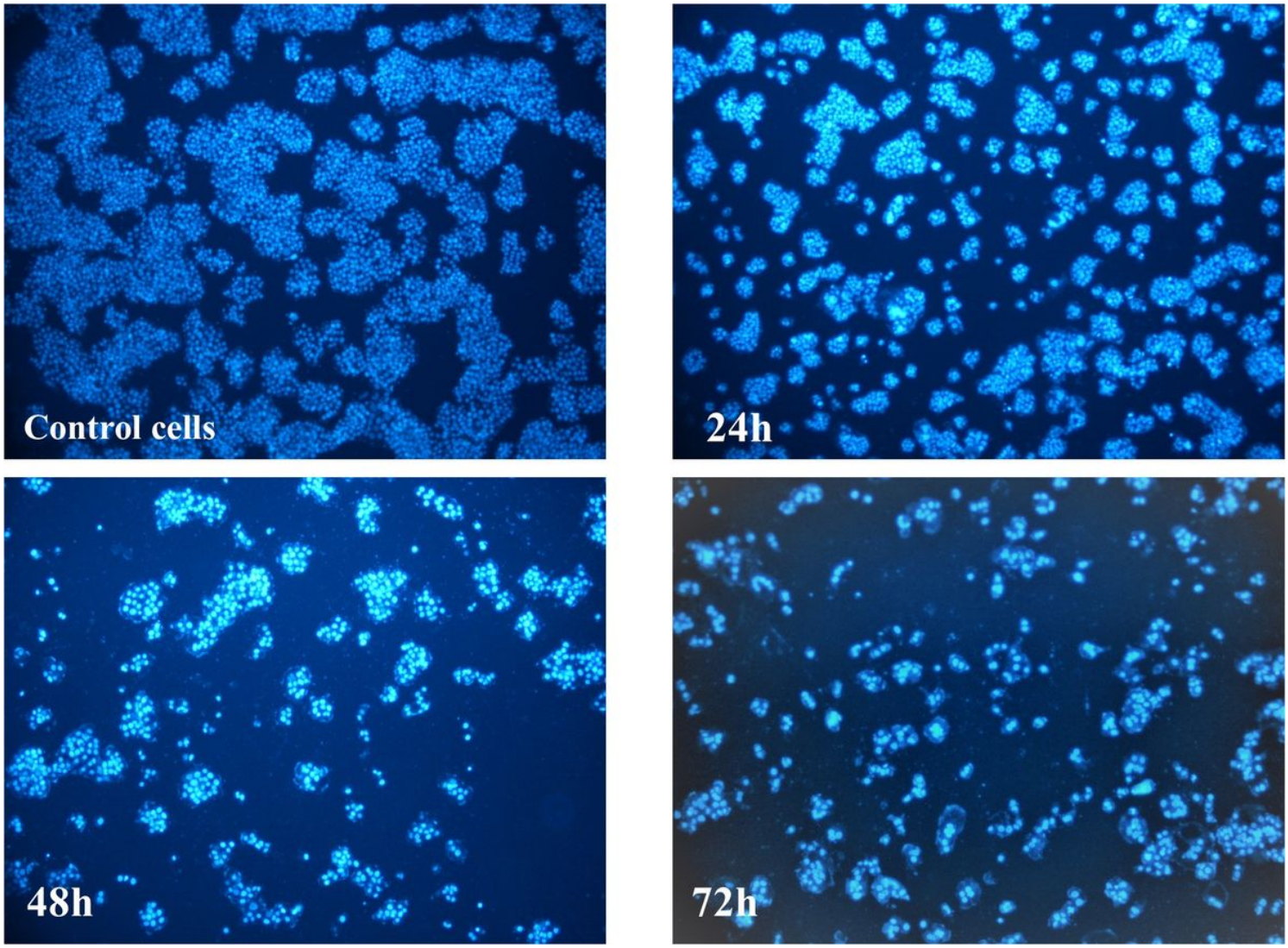

Figure 3

Apoptosis detection by DAPI staining. HT-29 cells were treated with SPNs at IC50 concentration (28µg/ml) and incubation for 24h, 48h, and 72h. The untreated cells were remained completely and shaped, were as apoptotic cells became visible in a time-dependent manner after treatment with SPNs (Magnification, 10X).

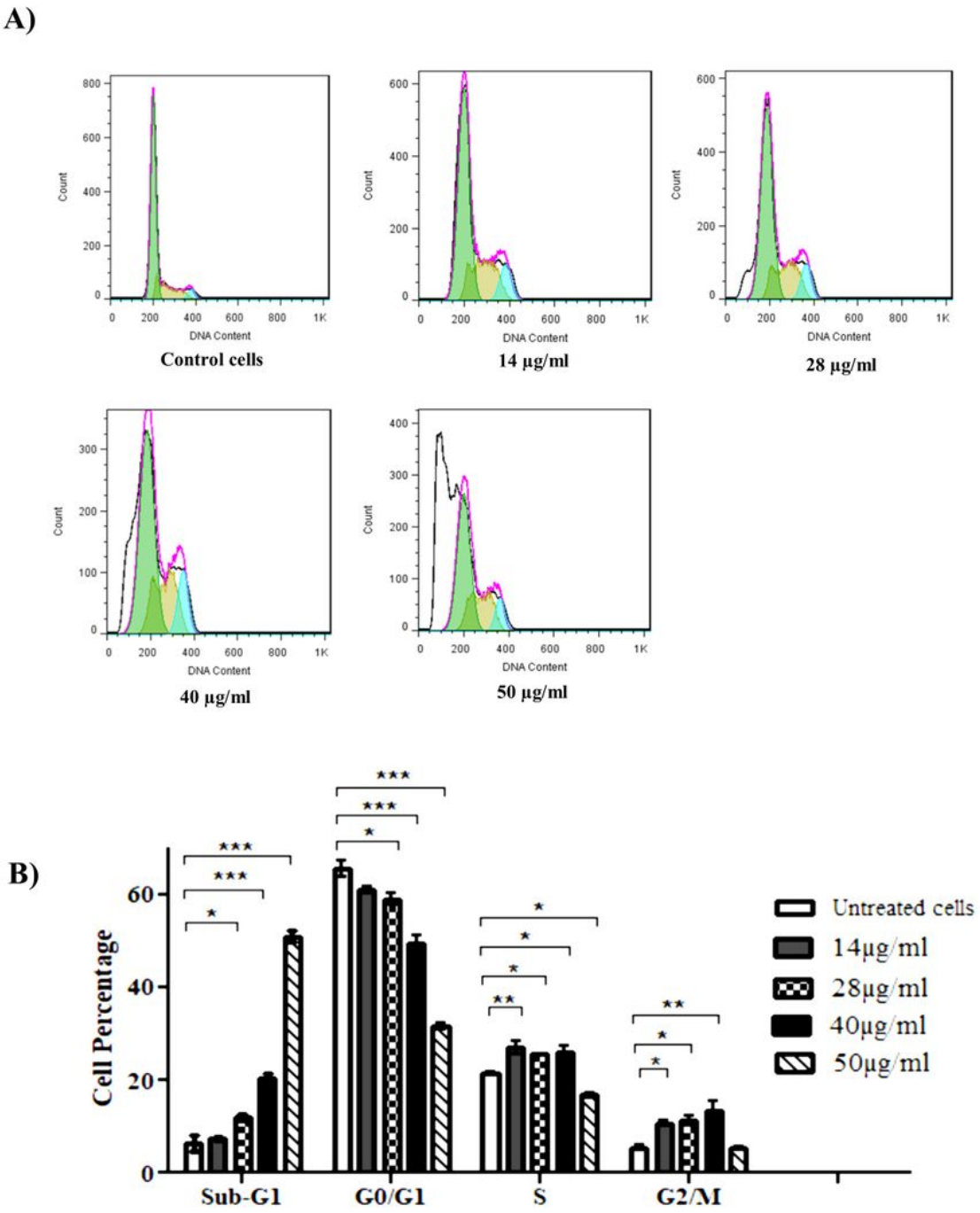

Figure 4

cell cycle analysis of HT-29 cells. A) Cell cycle distribution after 24h of dose-dependent manner with SPNs (0, 14, 28, 40, and 50µg/ml) using flow cytometry analysis. B) Statistical analysis of cell percentage in each phase after staining with Propidium Iodide (PI) during 24h compared in treatment different doses. The results are presented as mean ± SD. *P<0.05, **P<0.01, ***P<0.001.

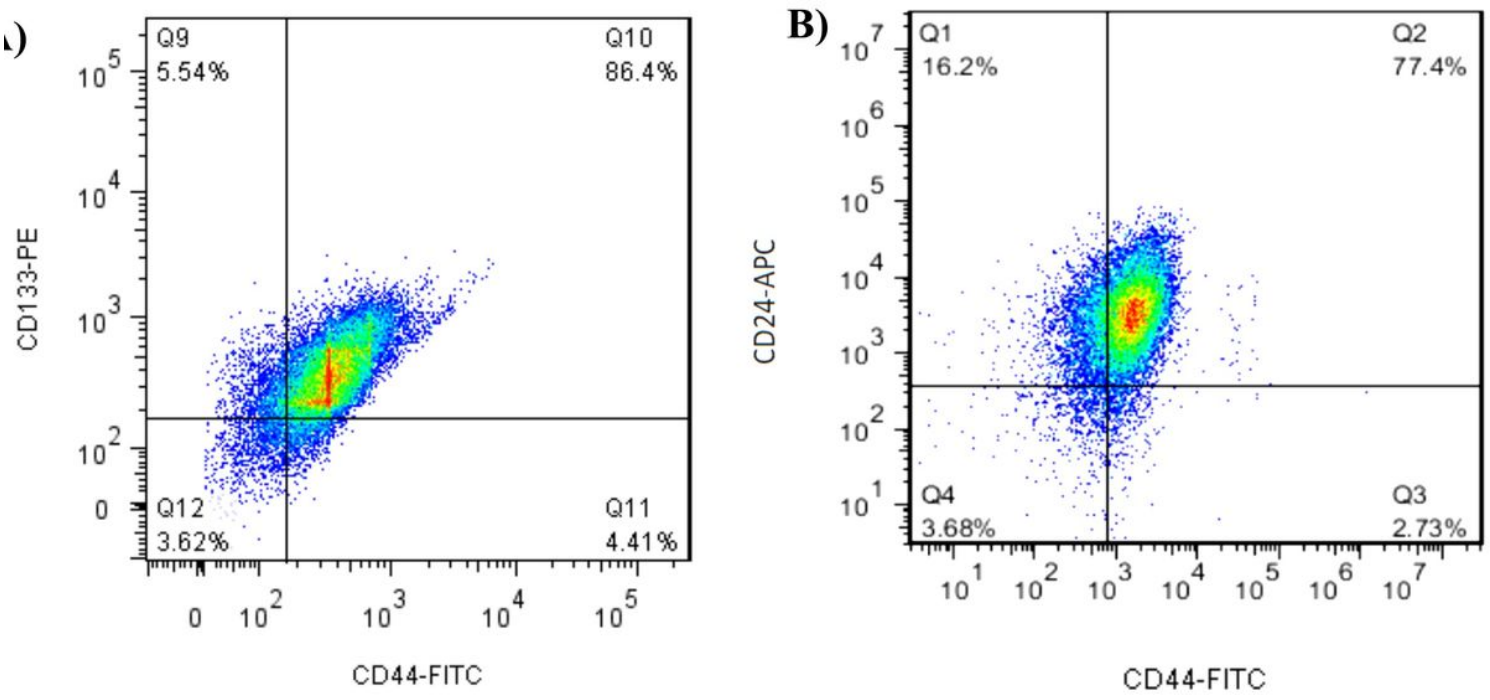

Figure 5

Evaluation of HT29 cancer cell line for expression level of stem cell surface markers. Flowcytometry result of expression level of CD44+/ CD133+ cells (A), and CD44+/ CD24+ cells (B) were 86.4% and 77.4% in untreated HT-29 CSCs, respectively.

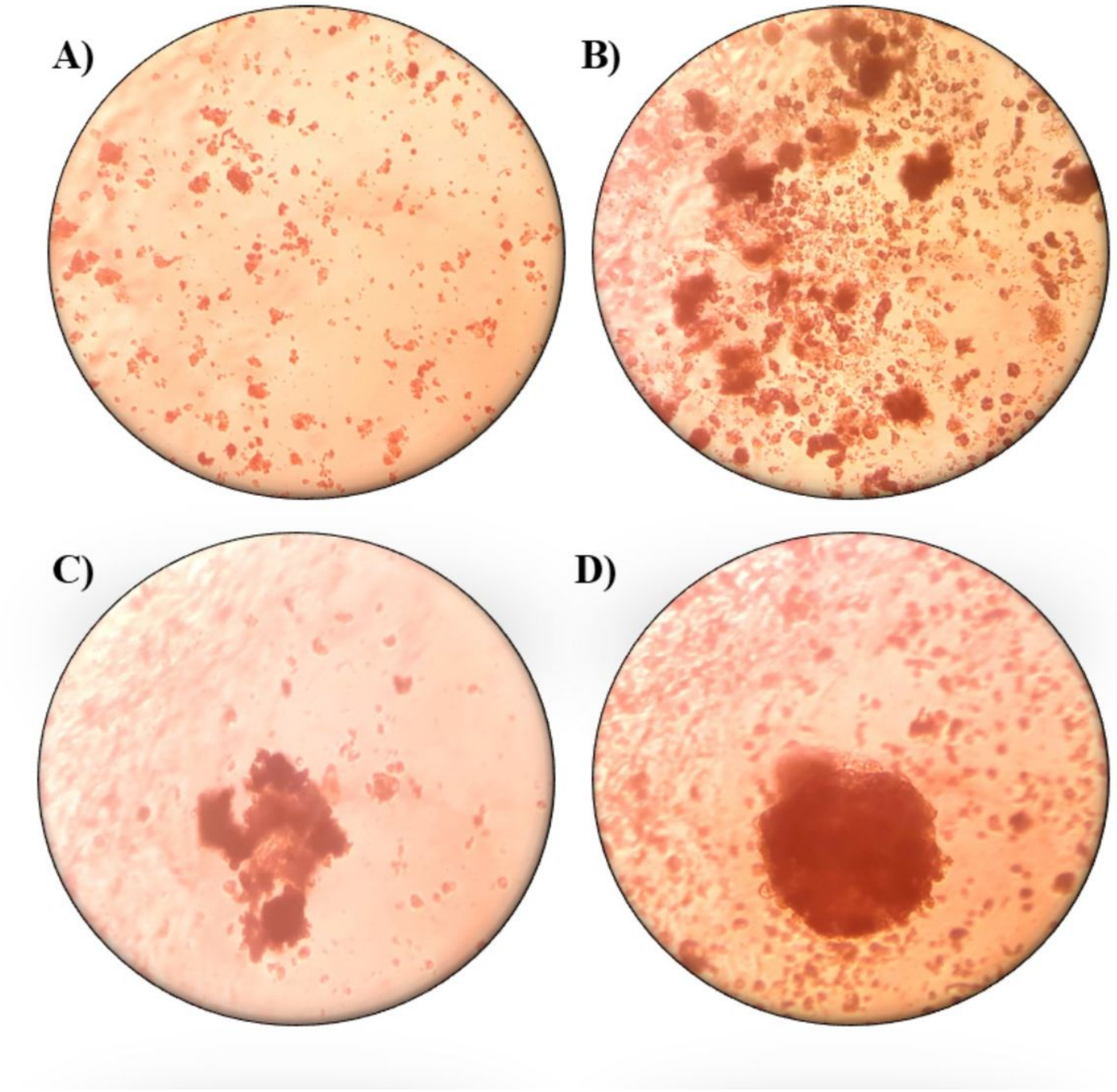

Figure 6

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

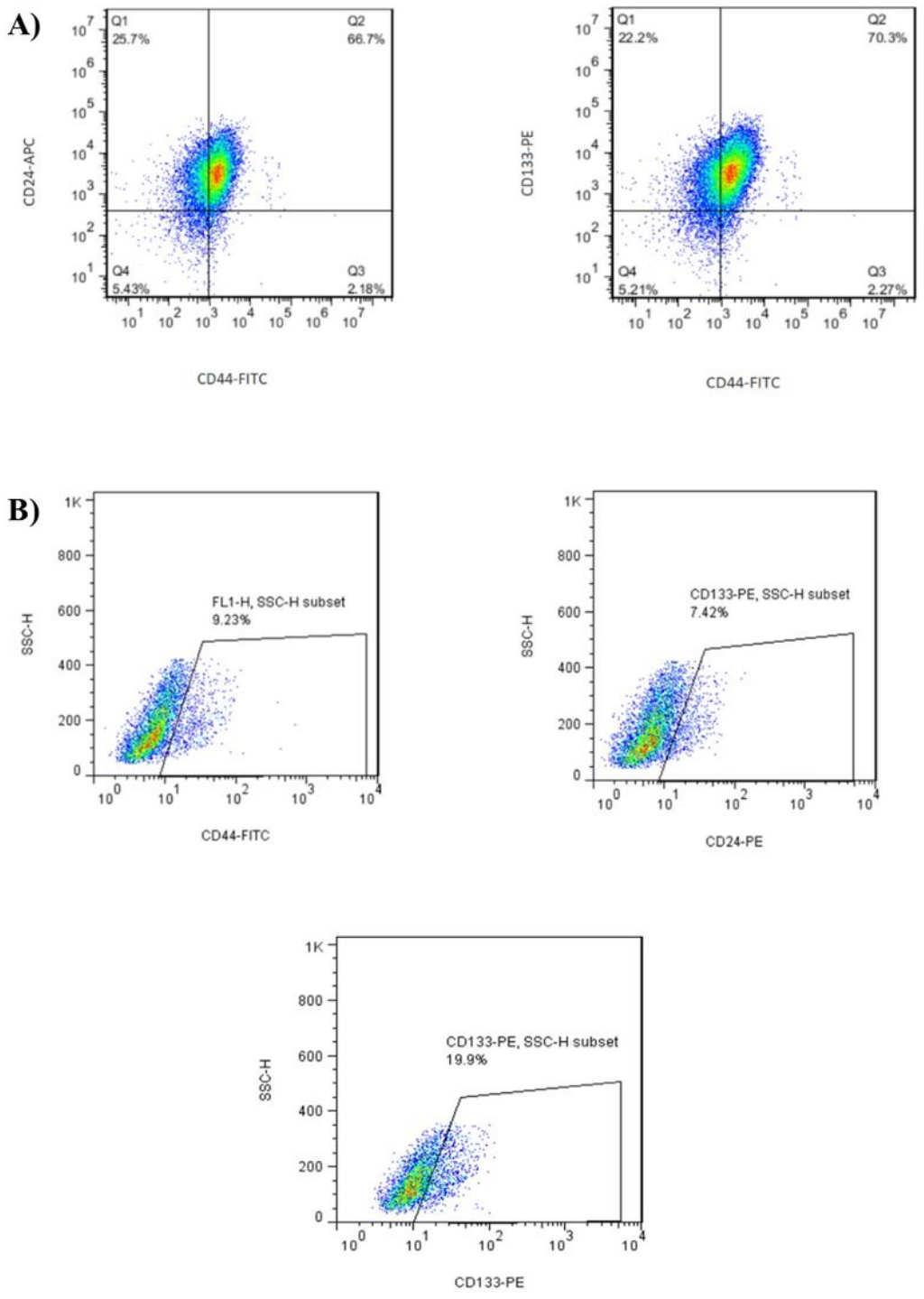

Figure 7

Assessment of HT-29 CSCs drug response in two different model of cancer cells. Flowcytometry analysis of multicellular spheroid forms(A), and single cell suspension(B) after treatment with SPN at IC50 dose for 24h. The results demonstrated that treatment of single cell suspension was more effective than multicellular spheroid form.

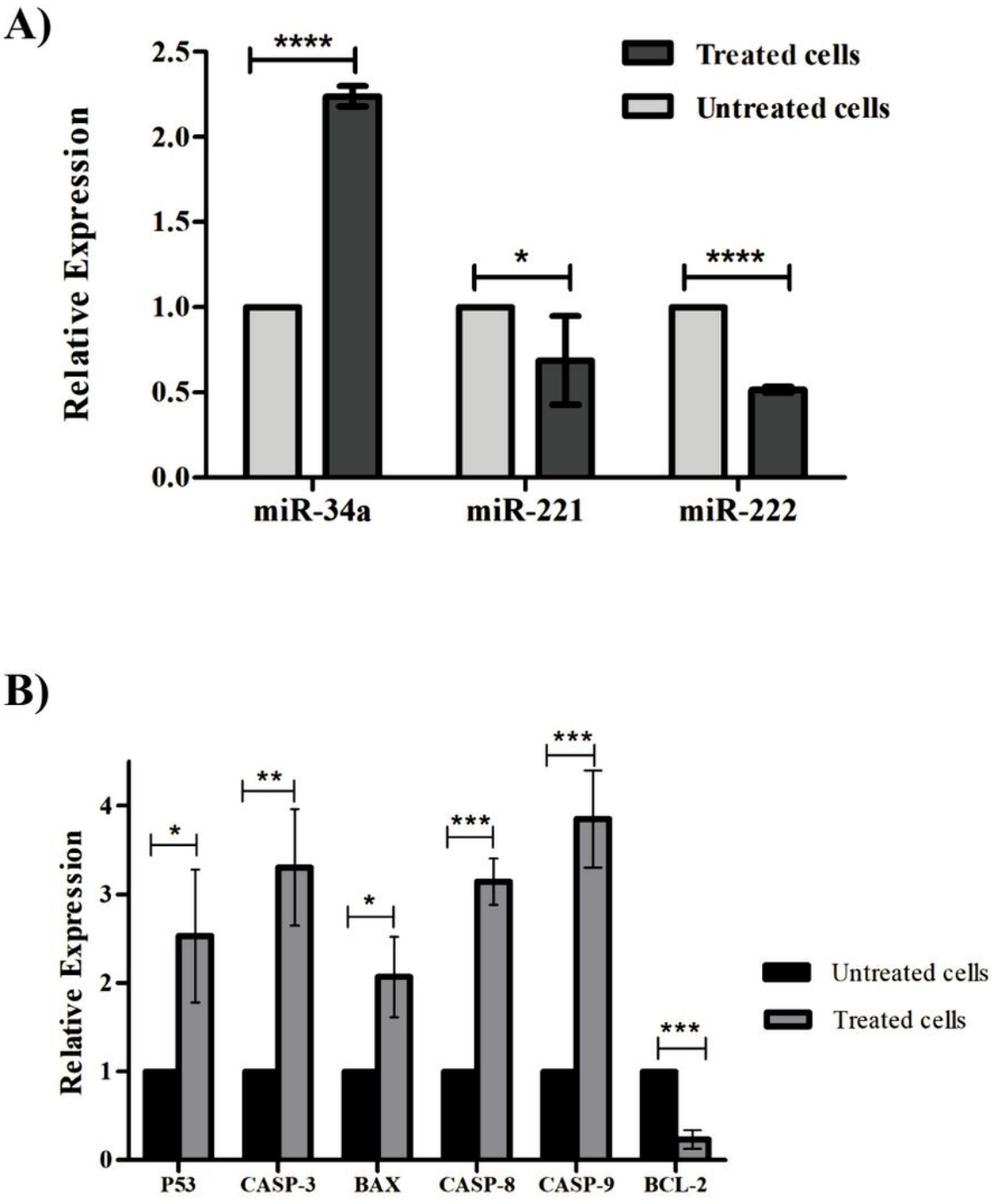

Figure 8

(A) The expression level of miR-34a, miR-221 and miR-222 after treatment with SPNs (28µg/ml). Relative expression was normalized to U47 snRNA as an endogenous control. All of the expression folds had significantly difference with untreated cells with P<0.001 (*P<0.05, ***P<0.001). (B) Quantitative expression of P53, CASP3, BAX, CASP8, CASP9, and BCL2 in treated cells with SPNs (28µg/ml) compared

to control group. Relative expression of target genes was normalized to HPRT1. Results was expressed as mean±SD; *P<0.05, **P<0.01, ***P<0.001.