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RESEARCH ARTICLE

Inhibition of miR-96 and miR-183 expression in treatment with fisetin-loaded chitosan nanoparticles in breast cancer cell lines

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ABSTRACT

Objective(s): Several efforts have been made to improve the treatment of breast cancer. Nanotechnology has been proposed as a promising strategy for the formulation of pharmaceutical compounds and increasing their efficiency. Fisetin is a natural flavonoid with anti-cancer properties. This study was conducted with the aim of improving the anti-cancer effect of fisetin with the chitosan nanoparticles delivery system and investigating some molecular pathways after treating breast cancer cell lines (MCF7 and MDA-MB231).

Methods: After the synthesis of chitosan nanoparticles, the shape, size and surface charge of nanoparticles were measured by SEM, DLS and zeta sizer. Drug loading in nanoparticles was confirmed by FTIR. The drug release rate was evaluated in acidic and neutral environments. Also, the cell cytotoxicity of fisetin and nanofisetin was investigated using MTT assay. The expression level of miR-183 and miR-96 investigated in both cell lines using real-time PCR.

Results: Our results showed that fisetin was successfully loaded into nanoparticles. The pH-dependent release of this nanosystem facilitated the release of fisetin in the acidic environment of the tumor. Chitosan nanoparticles containing fisetin with a size of 60 nm with significant efficiency exhibited higher toxicity to the viability of cells than free fisetin, and this decrease in survival was more pronounced for the triple negative MDA-MB-231. Fisetin also significantly decreased the expression of miR-96 and miR-183 in both cell lines.

Conclusions: The nanoformulation of this herbal compound and its effect can present nanofisetin as a potential candidate for the treatment of metastatic breast cancer.

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INTRODUCTION

Breast cancer is the second leading cancer among cancers and the leading cancer among women worldwide, both in developing and developed countries [1]. There are different treatment methods for cancer. Effective treatment of the disease mainly depends on early diagnosis, especially in the case of solid tumors [2]. Most cancer deaths are due to secondary tumors that occur due to metastasis. The un-targetedness of current treatment methods and their undesirable side effects have caused a lot of pain and suffering to the patients, prolonging the treatment process and revealing the necessity of studying new treatment methods [3]. To enrich the current treatment methods for cancer, much research has addressed the effect of herbal compounds in cancer. The use of natural compounds belonging to various groups such as flavonoids, alkaloids, and polyphenols in cancer studies provides promising results on treatment of this life-threatening disease [4]. Fisetin (3,7,3',4'-tetrahydroxyflavone)

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is a natural flavone [5]. This compound has a molecular weight of 286.24 daltons. Cucumbers, strawberries, apples, persimmons, grapes, and onions are among the sources of this natural compound [6]. Studies have shown that fisetin has antimicrobial, anti-inflammatory, antioxidant and anti-carcinogenic properties. To date, many in vitro and in vivo studies have been conducted on fisetin with a special focus on its anticancer role. In addition, different molecular mechanisms have been reported regarding the inhibitory effects of tumor growth in all types of cancers by fisetin. One of the main challenges facing the use of fisetin is its low solubility in aqueous solvents. Therefore, the design of a carrier system that contains a large amount of the desired compound and improves drug solubility will be very worthwhile for the use of these compounds in cancer therapy [7].

In the last decade, many nanocarriers including nanoparticles, nanocapsules, solid lipid nanoemulsions and nanopolymers have been designed to encapsulate biologically active drugs. By allowing the dispersion of insoluble drugs in aqueous solution, these nanocarriers have made it possible to use drugs that were previously left in water because of their poor solubility. Chitosan nanoparticles were used in this study to deliver fisetin to cancer cells. Chitosan is extensively used in different biomedicine and pharmacological formulations and is a type of acetylated chitin with high biodegradability and biocompatibility [8, 9]. Also, the cationic nature of this polymer, which increases adhesion to the negatively charged mucosal surface through electrostatic interactions, represents one of the most important features of chitosan that it leads to improved absorption of these nanoparticles in the desired cells [7]. In addition, these nanoparticles are able to penetrate into the environment of cancer cells in solid tumors and remain there through EPR (enhanced permeability and retention effect) due to their suitable size [10]. In EPR, the rapid growth of blood vessels in cancer causes a discontinuous epithelium with the presence of pores through which, due to defects in the tumor's lymphatic system, only molecules smaller than 4 nm are able to return to the blood via diffusion, and therefore nanoparticles with a size of around 10-100 nm can be protected from removal by the reticular endothelial system and accumulate in the tumor network [11, 12]. In the current study, chitosan nanoparticles were used to transfer fisetin to breast cancer cells. The cytotoxic effect of free fisetin and chitosan nanoparticles containing fisetin on the expression of miR-96, miR-183 in two breast cancer cell lines was also investigated. Epigenetic factors such as miRNAs play a key role in the development, progression and treatment of breast cancer. In this study, the effect of fisetin on the expression of miR-183 and miR-96 was investigated to possibly provide new therapeutic strategies based on natural drugs.

MATERIALS AND METHODS

Chitosan particles synthesis

For the synthesis of chitosan nanoparticles, the modified procedure of Raja Azalea et al. was used [13]. To this end, 50mg of chitosan was dissolved in 5ml of 5.5% acetic acid (chitosan solution 1%). Then, 50 mg of TPP was mixed with 10 ml of 5% tween and stirred until it was completely dissolved. The obtained suspension was added dropwise to the chitosan solution and stirred at 1000 rpm for 20 minutes and finally a cloudy solution was created. The suspension was sonicated for 20 minutes at 80millipulse power. Chitosan nanoparticles were dried and pulverized after centrifugation and freeze-drying for 24 hours. In this way, chitosan nanoparticles were prepared. SEM, DLS, ZETA, and FTIR were used to confirm the nature and formation of nanoparticles, determine their size, and characterize them.

Drug loading in nanoparticles

In order to load the drug into chitosan nanoparticles, 50mg of the drug (fisetin) was dissolved in 80ml of 5% Tween; and 1% chitosan was added to the solution and stirred at 1000 rpm for 20 minutes. Then, 10ml of 5.5% Tween containing 50mg of TPP is added and it is placed on the stirrer for a few minutes until it is completely dissolved. Nanoparticles were used for further studies after centrifugation and freeze-drying. In order to obtain the drug loading efficiency in the nanoparticle, 10mg of chitosan carrying the drug was dissolved in ethanol. After centrifugation, the supernatant was separated and its absorbance was calculated using a spectrophotometer. (The supernatant is used to determine the amount of fisetin un-loaded; and the deposited sediment represents the amount of fisetin loaded in the nanoparticle). To measure the amount of drug loaded in nanoparticles, the following formula was used:

$$\text{Loading efficiency} = \frac{(\text{primary drug} - \text{unloaded drug})\%}{\text{primary drug}}$$

Release assay

Twenty mg of nanoparticles containing fisetin was dissolved in 0.5 ml of PBS under acidic and neutral conditions. Nanoparticles dissolved in PBS (acidic and neutral) were inserted into the dialysis bag (cutoff: 12KD) and the bags were dialyzed in 20 ml of neutral PBS in a shaker incubator (37°C with 100 rpm shake). Then PBS outside the dialysis bag was sampled at 1, 2, 3, 4, 6, 8, 12, 24, and 72-hour intervals. To this end, at each of the intervals, 1 ml of the solution inside the falcon was removed, and 1 ml of PBS was replaced. Then, the absorbance of fisetin released in the samples was read by a spectrophotometer at 210-nm wavelength, and the curve of the release rate of fisetin from the nanoparticle was drawn.

Cell culture and investigation of cytotoxicity

In this study, two cell lines with different characteristics were used, one was MCF7 with estrogen receptor and the other was triple negative MDA-MB-231. Breast cancer cell lines were acquired from Pasteur Institute of Iran and were cultivated in RPMI-1640 culture medium with 1% of antibiotics (100µg/mL streptomycin and 100 U/mL penicillin), containing 10% (FBS) fetal bovine serum, at a temperature of 37 °C, 95% humidity and 5% CO2. After cultivation of MDA-MB-231 and MCF7 cells in 96-well plates, the cells were treated with fisetin and chitosan nanoparticle-loaded fisetin at 0-50µg/ml. 100µl of the desired suspension was added to each well in each plate. The wells containing cells, untreated with fisetin and nanoparticle carrying fisetin were considered as control. Cell treatment was performed at three intervals of 24, 48 and 72 hours. Next, cell viability was investigated using the MTT assay. Afterwards, 20µl of MTT solution was added to each well and incubated at 37°C for 3 hours. Then, the solution on the cells was removed and in order to dissolve the formed formazan crystals, 100µl of DMSO solution was added to each well. The absorbance for each well was read by a plate reader at 490-nm wavelength.

The expression of miR-183 and miR-96

The effect of fisetin on the expression of miR-96 and miR-183 in MCF7 and MDA-MB231 cell lines was investigated by real-time PCR. The U6 gene was used as internal control. Cells were treated with IC30 of fisetin chitosan nanoparticle-loaded fisetin for 48 hours. After the incubation, the cells were separated with trypsin and RNA extraction of the total cell was done using TRIzol solution. Five hundred ng of RNA extracted from each sample was used for the synthesis of miRNAs specific cDNA. Real-time PCR was performed with SYBR Green kit (YTA, Iran). Real-time PCR protocol included an initial denaturation at 96°C for 8 min, 40 amplification cycles comprising of denaturation at 95°C for 10 s, annealing at 60°C for 15 s, and extension at 72°C for 10s. The results achieved from repeating at least three independent experiments in the Rotor-Gene Q were obtained in triplicate and the 2-ΔΔCt method was used to analyzing the results.

Prediction of target genes of miRNAs and identification of gene pathways

To determine the effective gene pathways for the treatment of cancer cells treated with fisetin and fisetin-loaded nanoparticles, the target genes miR-183 and miR-96 were searched in Targetscan, miRTarbase, miRmap, and miRDB database, and the genes with high score criteria and binding in the seed position with 7 to 8 nucleotides were selected as target genes. In the next step, gene ontology and enrichment were performed by the clusterprofiler package and the pathways involved in this analysis were identified. Then the gene network was drawn by string database and the key genes were identified using Cytoscape version 3.9.1.

Data analysis

SPSS was used to conduct data analysis. To conduct statistical comparisons, t-test and ANOVA with Tukey's post-hoc (for more than two groups) were used. P-value<0.05was considered significance level.

RESULTS AND DISCUSSION

Nanoparticle synthesis and verification

To improve the medicinal properties of fisetin, for enhanced absorption, different formulation strategies have been proposed [14, 15]. Chitosan nanoparticles, which are composed of biodegradable polymers such as chitosan, are a suitable approach to improve the absorption and therapeutic effects of bioactive components [16]. Chitosan nanoparticles were synthesized using the ionotropic gelation method with desirable characteristics such as surface charge and suitable size with the interaction between positively-charged chitosan and negatively-charged TPP. Chitosan

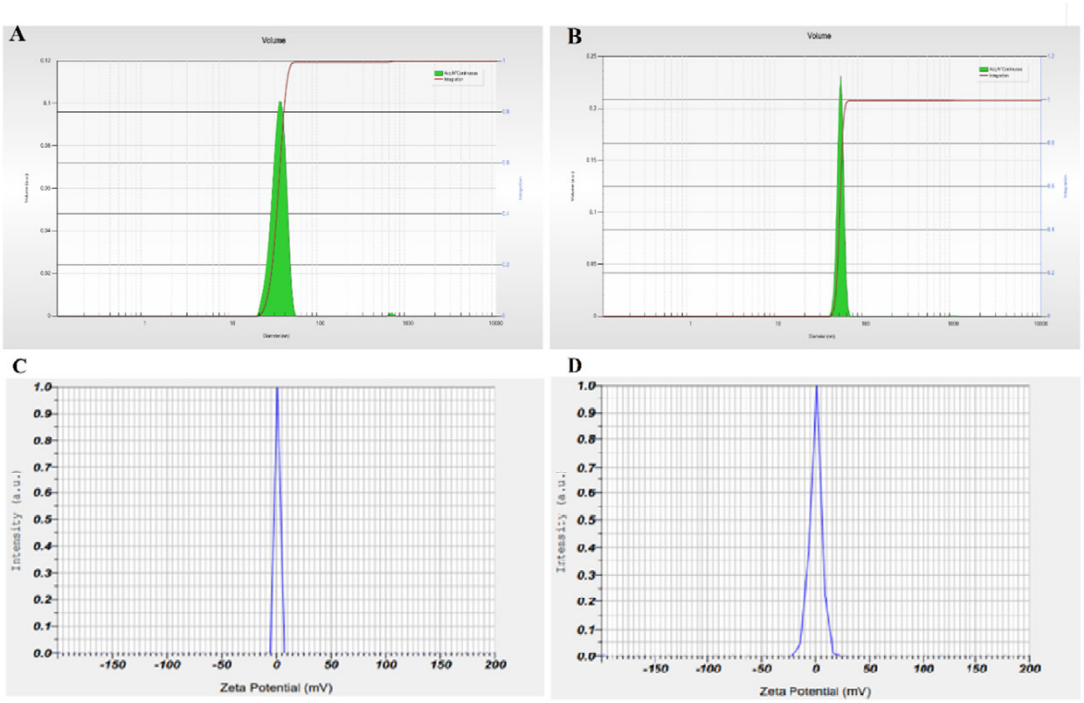

Fig. 1. The size of chitosan nanoparticles (A) and chitosan nanoparticles carrying fisetin (B.) by DLS method. The charge of chitosan nanoparticles (C) and nanoparticles carrying fisetin (D).

nanoparticles and nanoparticles containing fisetin were evaluated to confirm their size, charge, morphology and functional groups.

The chitosan nanoparticles size and fisetin carrying nanoparticles were analyzed using dynamic light scattering (DLS) method. According to Fig. 1 (A and B), the average size of chitosan particles is 31 nm, and the size of chitosan nanoparticles carrying fisetin is estimated at 60 nm, and the amount of PDI for both types of nanoparticles was calculated at 0.11. Therefore, the encapsulation of fisetin in nanoparticles caused an increase in the nanoparticles size. Also, ZETA-potential was used to determine the nanoparticles charge. The zeta potential provides information on the stability of the fisetin-nanoparticles dispersion. The greater the absolute value of this response, the higher the stability of fisetin- nanoparticles due to greater electrostatic repulsion. The value of charge for chitosan nanoparticles was measured at 1 and for chitosan carrying fisetin at 0.3. This change in charge is due to the neutrality of the charge of fisetin and its encapsulation in nanoparticles. Electron microscopy was used to investigate the surface morphology of chitosan nanoparticles (Fig. 2). The SEM results showed that chitosan nanoparticles had a spherical morphology and were uniformly distributed. The size and surface of nanoparticles are important in cancer drug delivery. Particles with an average size smaller than 10 nm are filtered by the kidneys, while particles with a size larger than 100 nm cannot spread from the liver to the bloodstream. Therefore, the synthesized chitosan nanoparticles containing drug with a size of about 60 nm, due to the appropriate size, are able to penetrate into the environment of cancer cells in solid tumors and remain there through EPR (enhanced permeability and retention effect) [17].

IR spectroscopy (FTIR) was performed to investigate the functional groups and accuracy of drug loading in nanoparticles. Fig. 3 illustrates the FTIR spectrum of chitosan nanoparticles, free fisetin and fisetin loaded chitosan nanoparticles. In the spectrum of chitosan, the peaks in the range of 3200 to 3400 indicate the peaks of intramolecular hydrogen bonds and O-H groups. The peak in the range of 1659 indicates R-CO-NH2 groups. The fisetin spectrum showed distinct peaks, the peak at 3522 cm^{-1} corresponding to the O-H group, and the peak in the range of 1615 cm^{-1} to C=O, the peak in the range of 1447 cm^{-1} to C-C, and the peak 1120 to the group C=H. Comparison of the peaks in the

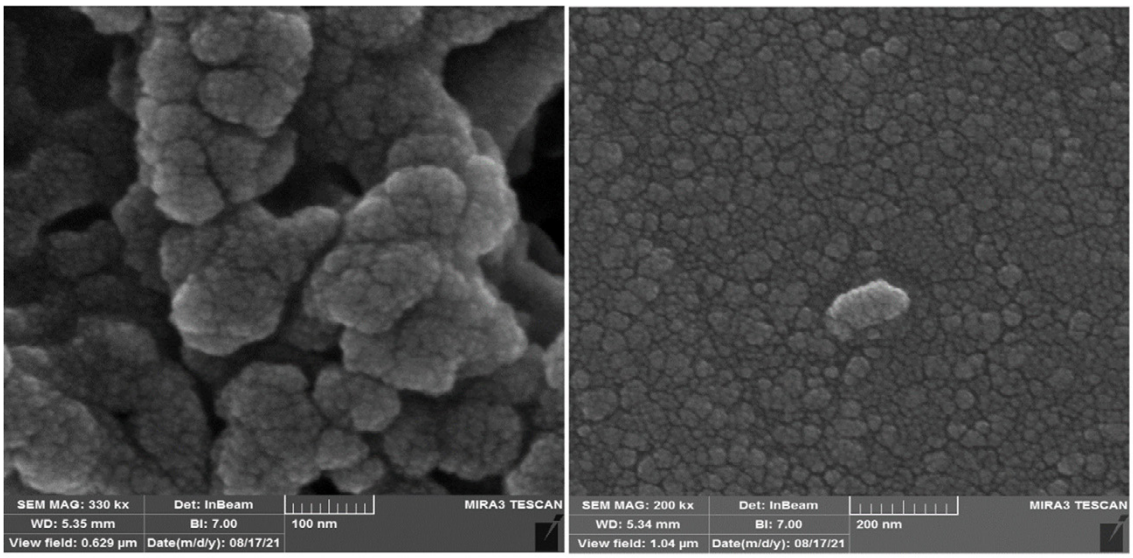

Fig. 2. Electron microscope images (SEM) at two scales of 100 and 200 nm

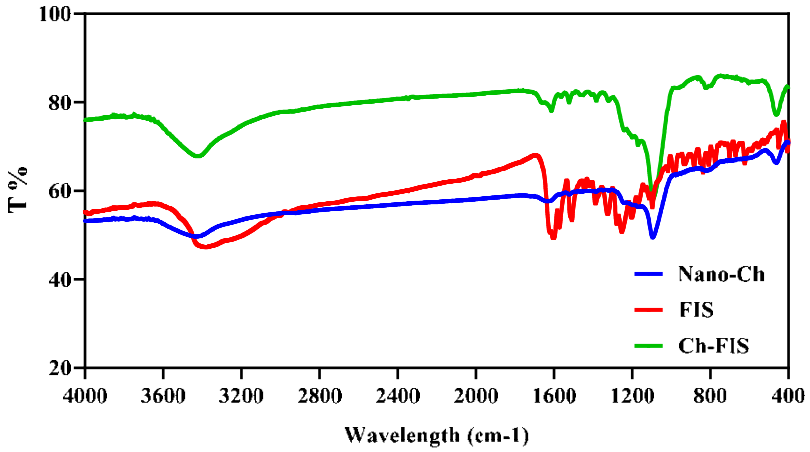

Fig. 3. FTIR spectrum of free chitosan nanoparticles, free fisetin and chitosan nanoparticles containing fisetin.

FTIR spectra of chitosan nanoparticles, free fisetin and fisetin-loaded chitosan, the presence of new absorption bands and the change of the spectrum of chitosan indicated the interaction of chitosan with fisetin, and the successful loading of the drug into the nanoparticle.

Investigating the loading efficiency of fisetin in nanoparticles and its release

The amount of fisetin encapsulation in chitosan nanoparticles was calculated at 95% after examining the unloaded drug. In the next step, the dialysis method was used to investigate the release conditions (acidic or neutral). As Fig. 4 illustrates, it was found that the amount of drug release in acidic conditions, which can be attributed to the tumor microenvironment, was slightly higher than in physiological conditions. According to our study, most of the release occurred at the interval of 8 to 12 hours for fisetin, which was the same for both acidic and neutral conditions.

The amount of surface charge of chitosan nanoparticles was 1 mV and the surface charge of nanofisetin was 0.3 mV. This change in charge of nanofisetin compared to fistin is due to the fistin loaded into chitosan. The efficiency of the drug loaded in the nanoparticle was calculated at 95%. Fisetin release from chitosan nanoparticles

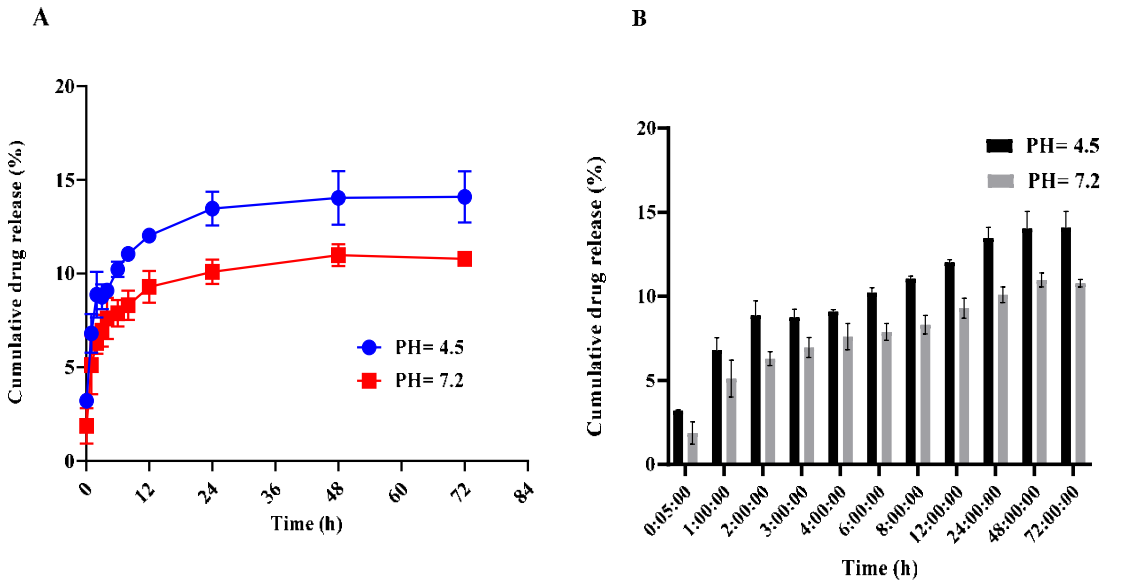

Fig. 4. The chart of fisetin release from chitosan nanoparticles in physiological (PH=7.2) and acidic (PH=4.5) conditions in A) Curve and B) bar-plot

was investigated at neutral pH. With the change of pH from neutral to acidic, the rate of drug release from chitosan increased, which is probably due to the better opening of chitosan in acidic conditions. Chitosan-based drug delivery systems are inductive systems sensitive to pH, which allows drug release in the tumor environment or the acidic environment of endosomes due to the lower pH of tumor cells[18, 19].

Cytotoxicity

Cytotoxic potential of fisetin and chitosan nanoparticles containing fisetin was evaluated against MCF7 and MDA-MB-231 strains using MTT assay (Figs. 5 and 6). According to the analysis, it was found that fisetin and chitosan nanoparticles containing fisetin could inhibit cell proliferation in both lines. Inhibition of cell proliferation in treatment with fisetin and chitosan nanoparticles containing fisetin was both dose-dependent and time-dependent. As Fig. 5 illustrates, at 24 and 48 hours at concentrations above 10µg/ml, they exhibited toxicity effects on MCF7 cell line. According to the study conducted on this cell line, compared to free fisetin, chitosan nanoparticles at 5 micrograms and above significantly inhibited cell proliferation at 72-hour interval. This difference between fisetin and chitosan nanoparticles containing fisetin was more evident at higher concentrations. Therefore, different concentrations of free fistin and nanofistin cause a reduction in proliferation and an increase in cell death.

Fig. 6 also illustrates the viability of fisetin and chitosan nanoparticles containing fisetin in MDA-MB-231 cell line. As the Figure illustrates, at 48 and 72 hours, a very significant decrease in viability is observed. The difference in effect between free fisetin and chitosan nanoparticles containing fisetin could be seen clearly that was statistically significant, so that at equal concentrations of the two compounds, the viability percentage for free fisetin was approximately 50% while this amount for nanoparticles containing fisetin was calculated at 30%. Therefore, compared to free fisetin, nanoparticles were effective for drug delivery to MDA-MB-231 cells.

Due to chitosan's proton sponge property, these nanoparticles are able to escape from endosomes, so they can enter the drug into the cell cytoplasm. And due to the positive charge of the nanoparticles, the cell uptake and the drug flow to the cells are improved. Chitosan nanoparticles containing fisetin and free fisetin have a dose- and time-dependent cytotoxic effect on both cell lines. Free chitosan nanoparticles at the concentrations used in this study had no lethal effect on cancer cells and healthy cells (data not presented). MCF7 cell line has estrogen receptor (ER) and lacks

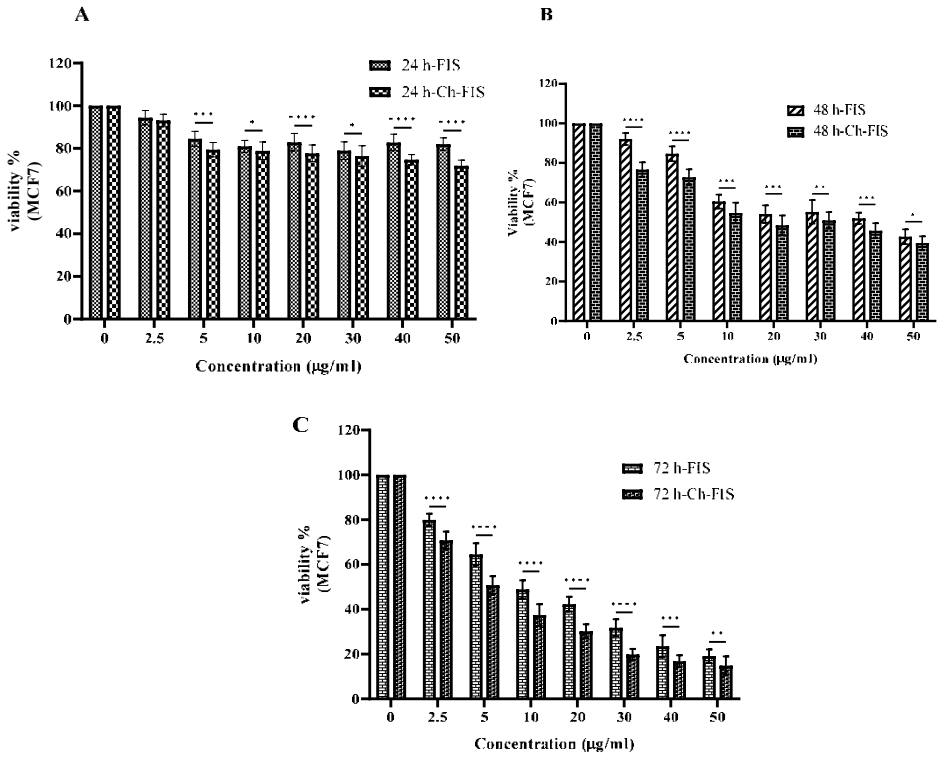

Fig. 5. MCF7 cell line viability bar-plot at intervals of 24 hours (A), 48 hours (B) and 72 hours (C).

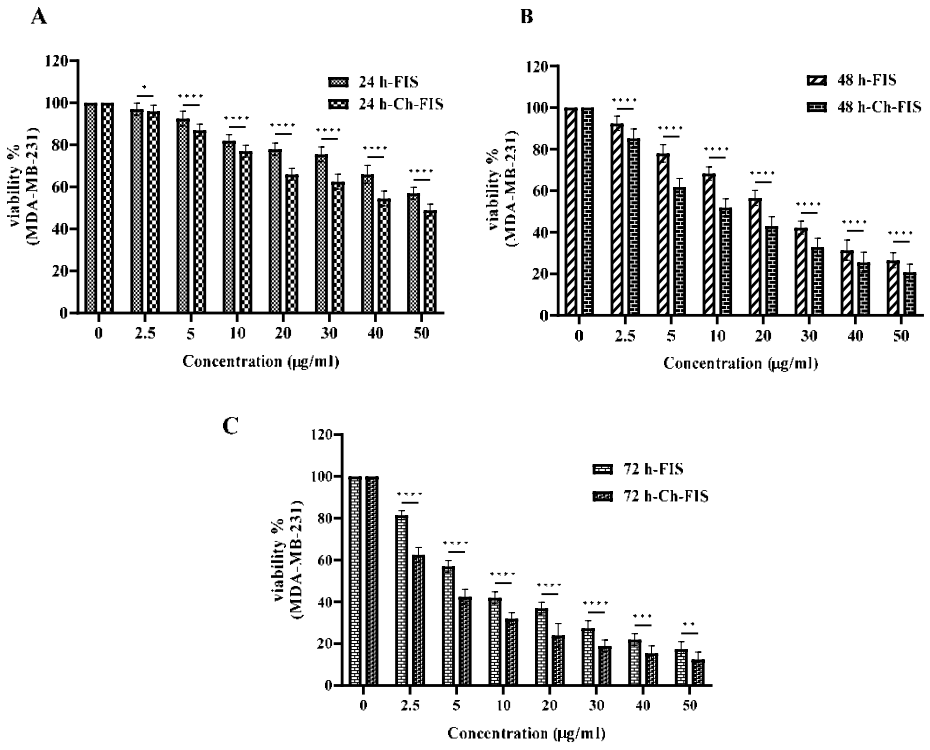

Fig. 6. Viability bar-plot of MDA-MB-231 cell line at intervals of 24 hours (A), 48 hours (B) and 72 hours (C).

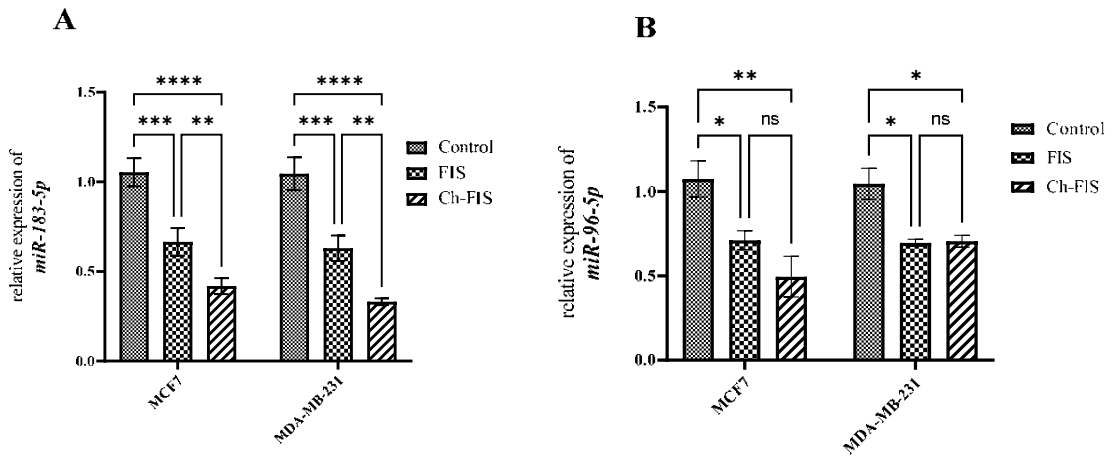

Fig. 7. The expression levels of miR-183-5p and miR-96-5p in two breast cancer cell lines MDA-MB-231 and MCF7 in the presence of fisetin and fisetin loaded chitosan nanoparticles.

progesterone receptor (PR), and MDA-MB231 cell line is commonly used as a model of triple negative breast cancer. It also lacks the expression of estrogen receptor (ER), progesterone (PR) and HER2 receptor. Fisetin is a phytoestrogen; Studies have shown that fisetin interferes with the ligand-binding domain of androgen due to its structural similarity, and reduces the development of androgen-dependent and independent cancer cells. Due to the absence of the estrogen receptor, MDA-MB231 cells show less toxicity when free fisetin is used compared to when fisetin is used using a chitosan carrier, which indicates a more effective delivery of fisetin into the cell through the chitosan carrier, and therefore it leads to cell death by preventing cell proliferation. Pawar et al. reported that the toxicity effect of fisetin loaded into nanoparticles was greater than that of free fisetin in MCF7 cell line [20]. In another study by Chunlai et al.(2019), the antitumor effect of fisetin loaded into polylactic acid nanoparticles was higher than free fisetin [21]. These studies are consistent with our findings. Treatment of A431 human epidermoid cancer cells with fisetin led to a decrease in the expression of anti-apoptotic proteins and an increase in the expression of apoptosis-inducing proteins. Basically, fisetin interacts with various cancer-associated pathways and suppresses cancer by promoting apoptosis and regulating autophagic cell death, and plays a role in cancer treatment through physical interaction with mTOR and K6S70P molecules and disruption of the Wnt/β-catenin signaling pathway and a large number of cell survival pathways [22].

miR-183 and miR-96 expression and target prediction

Fisetin inhibits the progression of cancer by modulating different gene pathways[23, 24], but there is little information about the effect of fisetin on miRNA expression. Today, the role of microRNAs in regulating the expression of genes involved in the basic processes of eukaryotic cells has been determined, and the change in their expression is associated with the occurrence of various diseases, including cancers. The role of microRNA in cancer was first shown in chronic lymphocytic leukemia [25, 26]. Breast cancer is the most common neoplastic malignancy and one of the main causes of death in women around the world, and changes in the expression pattern of several microRNAs have been reported in its development [27, 28]. qPCR method was used to investigate the effect of fisetin and fisetin loaded chitosan nanoparticles on the expression levels of miR-183-5p and miR-96-5p in two breast cancer cell lines. The expression of miR-183-5p and miR-96-5p in breast cancer compared to surrounding normal tissues, reveals increased expression that causes metastasis, cell migration and survival in cancer cells [29]. miR-96 displays a direct or indirect role as an oncogene in numerous cancers. The miR-96 is also considered to have the purpose to stimulate carcinogenesis as well as chemo-resistance in breast cancer [30]. Also, miR-183-5p, involving in cellular processes, dysregulated in a variouse of cancers. it was shown that cell proliferation, metastasis, and angiogenesis were increased by miR-183 in breast cancer [31].

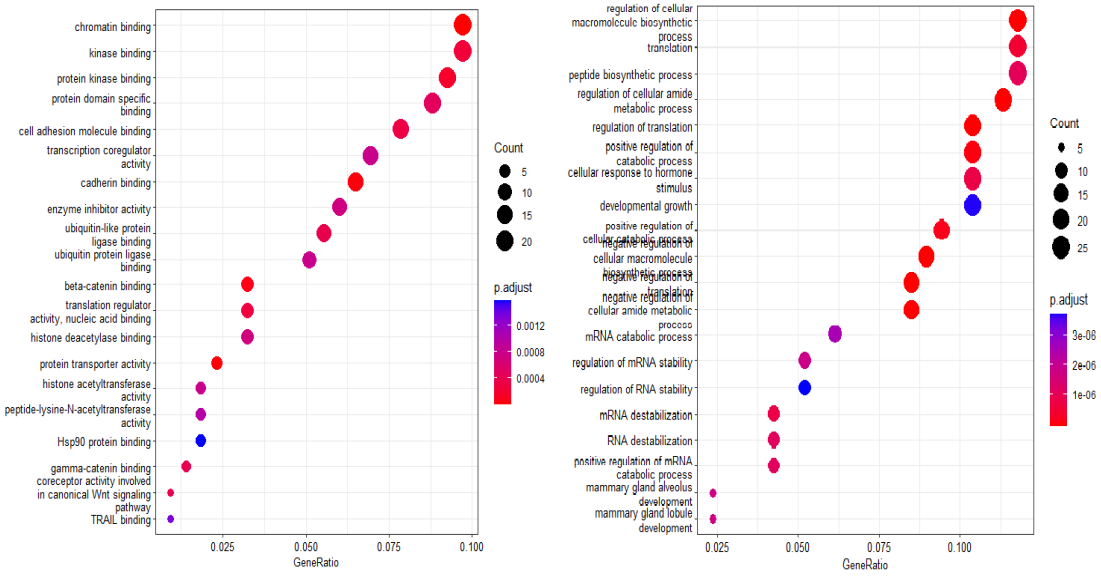

Fig. 8. GO enrichment analysis. (b) GO for overlapped target genes, Molecular function (left) and Biological process (right).

In this study, the expression of both miRNAs significantly decreased under treatment with free fisetin and chitosan nanoparticles containing fisetin. In the comparison between the effect of fisetin and fisetin loaded chitosan nanoparticles on the expression of miR-183-5p and miR-96-5p, a significant difference was observed for both cell lines for miR-183-5p ($p<0.001$). Regarding miR-96-5p, the expression difference between free fisetin and chitosan nanoparticles containing fisetin was not significant and no difference was observed.

Identification of key genes

There is limited evidence on the compounds that affect the expression of microRNAs. Due to the significant difference of the expression levels of miR-183-5p and miR-96-5p in two breast cancer cell lines compared to the untreated group, further investigations were conducted for the signaling pathways and target genes of these miRNAs. Treatment of both cell lines with fisetin caused a decrease in the expression of these two oncomiRs compared to the control sample, which confirms the anti-cancer effects of this herbal compound. The genes involved in the functional pathways of these miRNAs were identified using bioinformatics analysis. After identification of the target genes, it was found that miR-96-5p had 1155 target genes with a high score and miR-183-5p had 1256 target genes. A total of 226 genes were shared by the targets of these two miRNAs, and gene enrichment method was used to investigate the molecular functions and biological processes in which these genes are involved. As Fig. 8 illustrates, the most important molecular functions in which genes are involved include chromatin binding, cell adhesion molecule binding, and transcription coregulatory activity. Also, the most important biological processes identified included stimulation of response to hormones, regulation of biosynthesis of macromolecules, regulation of transcription, upregulation of catabolic processes and growth. According to the String database, the PPI interaction network of overlapping target genes had 502 nodes and 274 edges. Then, to identify the key genes from this network, 10 key genes were calculated among the target genes of both miRNAs using Cytoscape software (CytoHabba plugin). These genes are CCND1, HIF1A, KRAS, IGF1R, CDH1, GSK3B, FGF2, BCL2L11, ZEB1, and MCL1, respectively (Fig. 9B).Therefore, targeting these key genes for treatment or use as diagnostic markers can be useful.

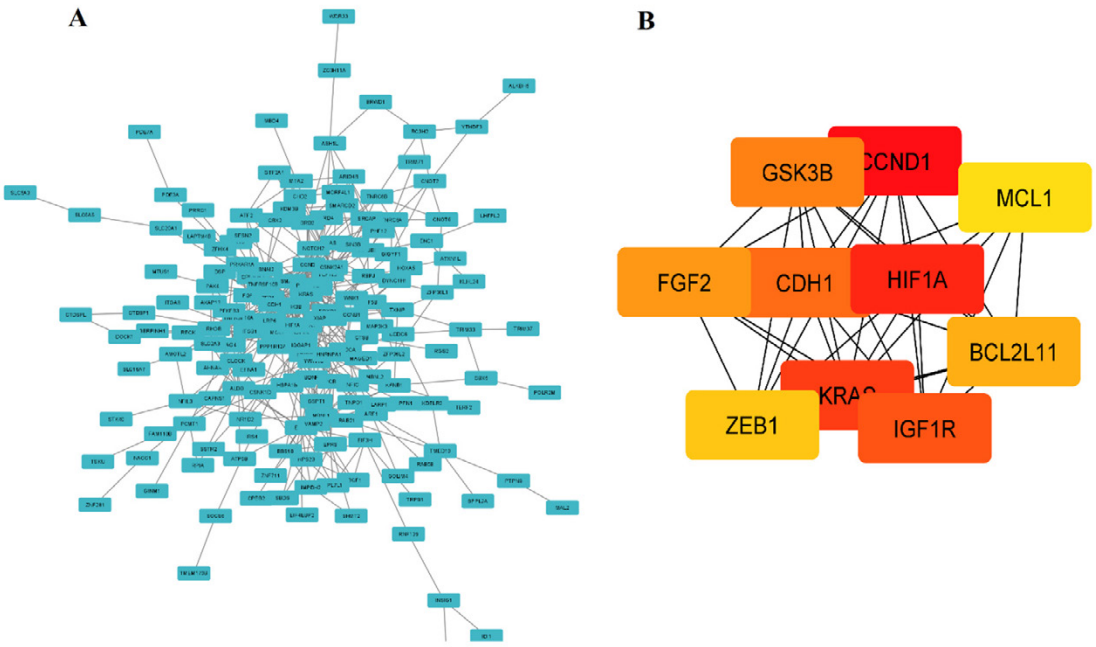

Fig. 9. Protein–protein interaction network of overlapped target genes (A). A significant hub genes selected from protein–protein interaction network (Cytoscape software) (B)

CONCLUSION

The use of natural compounds and food supplements with anti-tumor properties to control and treat cancer is expanding. In this study, two cell lines MCF7 and MDA-MB231 were treated with fisetin and chitosan containing fisetin. The formulation of chitosan nanoparticles containing fisetin was successfully synthesized with the aim of improving the delivery of fisetin to cancer cells under optimal conditions; the size and surface charge of fisetin-containing chitosan nanoparticles were in the required range for medicinal applications and cancer cell targeting. The results from cytotoxicity investigations showed that nanofisetin had a greater toxic effect on both cell lines than free fisetin. Fisetin decreased the expression of miR-183-5p and miR-96-5p that are effective in cancer progression in both MCF7 and MDA-MB231 cell lines. Therefore, it seems that it can be used in the treatment of cancer after further research.

CONFLICT OF INTEREST

There is no conflict of interest

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