Silibinin affects transcription factors of cell cycle, apoptosis, angiogenesis and metastasis in breast cancer mice model: Targeting the hallmarks of cancer

Hamid Zarei Golambahri  
University of Tehran

Nasrin Motamed (nmotamed@ut.ac.ir)  
University of Tehran

Mohammed Davoody  
University of Tehran

Article

Keywords: Silibinin, apoptosis, metastasis, Bcl-2, angiogenesis, FOXM1, HmgB1, HoxB9

Posted Date: June 10th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1695746/v1

License: © This work is licensed under a Creative Commons Attribution 4.0 International License. 
Read Full License
Silibinin affects transcription factors of cell cycle, apoptosis, angiogenesis and metastasis in breast cancer mice model: Targeting the hallmarks of cancer

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers’ bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. ("The authors declare no potential conflicts of interest.").

Hamid Zarei Golambahri¹, Nasrin Motamed²*, Mohammed Davoody³

Correspondence:

Proph Nasrin Motamed

Phone: +98-21-61112472

nmotamed@ut.ac.ir
Abbreviations used

IF: Immunofluorescence
H&E: hematoxylin and eosin
EMT: Epithelial-mesenchymal transition
Abstract
Silibinin is a phyto-flavonoid compound and has been reported to exert anticancer effects on several tumors, including breast cancer. The present study exploited Flow cytometry, H&E staining, immunofluorescence (IF), and RT-PCR to extensively observe the effect of silibinin on the expression of cell-cycle and apoptosis-related transcription factors and genes in mice breast cancer cells 4T1. Culture of 4T1 cell, MTT, flow cytometry, hematoxylin and eosin stains, immunofluorescent and Real Time PCR experiments were performed. Silibinin caused a significant cytotoxicity, induction of apoptosis and cell cycle arrest in 4T1 cells, in vitro and in vivo. CD31 and HmgB1 expression showed a considerable decline. Evaluation of gene expression showed a marked decrease in Bcl-2, FoxC2, HoxB9, and FoxM1 genes, while a significant increase in TP53, P21, Bax, FoxF1, and HmgB1. Silibinin indicates the impact on inducing apoptosis by lower expression of Bcl-2, higher expression of Bax and TP53. While increased expression of P21 is thought to cause cell cycle arrest. In the meanwhile, under-expression of HoxB9, FoxC2, and FoxM1 and over-expression of FoxF1 caused restricted EMT, metastasis, angiogenesis and invasion in cancer.
Keywords: Silibinin, apoptosis, metastasis, Bcl-2, angiogenesis, FOXM1, HmgB1, HoxB9.

Introduction
Recent anticancer chemotherapy regimen drugs have several adverse effects on the patient's body, such as; hair loss, anemia, and weaker immunity to infections (1). This has led researchers to exploit herbal drug agents as anti-cancer candidates to control the side effects of chemotherapeutic agents. Silibinin is a key constituent of antioxidant phytochemical silymarin, which is found in milk thistle (2). Silibinin has been proven to cause liver intoxication because of its unique chemical structure ((2R,3R)-3,5,7-trihydroxy-2-[(2R,3R)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydrochromen-4-one) (3, 4). Several recent studies have evaluated silibinin's effects as an apoptotic inducer, and the restrictor of the cell cycle, angiogenesis, inflammation, and metastasis in several cancers (5). The cell cycle arrest function of silibinin has been identified to occur during different phases of cell division, especially the G2/M phase (6). It also induces apoptosis in cancer cells by activating the external and internal pathways of apoptosis by increasing the expression of pro-apoptotic genes (7) and inhibit angiogenesis, metastasis, proliferation, and migration in cancer cells in vitro and in vivo (1, 8-10).
FOX (forkhead box) proteins are transcription factors that alter the expression of genes involved in the cell cycle, cell proliferation, division, migration and metastasis pathways, etc,
and hence can prove to be potential targets in cancer progression and treatment studies (11-13). FoxM1 protein positively regulates the G2/M phase of the cell cycle by activating and over-expressing PLK1, cyclinA, and cyclinB proteins (14). Restriction of aging and induction of tumorigenesis are regulated by FoxM1 through its effect on the expression of p16, p19, and β-galactosidase genes (15). FoxM1 has been observed to intensify multidrug resistance in cancer cells and its inhibition makes the tumor cells sensitive to certain anticancer drugs (16).

FoxC2 is observed to exert a significant impact on Snail, Goosecoid, and Twist genes and hence induce epithelial-mesenchymal transition (EMT) in breast cancer cells (17, 18). FoxC2 is also reported to upregulate CDK1 expression and results in a facilitated transition from G2/M phase of the cell cycle (19). FoxF1 facilitates the EMT process through underexpression of E-cadherin, while FoxC2 progresses this mechanism by transferring E-cadherin from the plasma membrane towards the cytoplasm (18).

HMG (high mobility group) proteins constitute three non-histone sub-families; HMGA, HMGB, and HMGN which are key players in the chromatin re-modeling mechanism (20). HMGB1 regulates mitochondrial and nuclear function and homeostasis (21). Keeping in mind the impact of HMGB1 on the mitochondrial structure and function, it is hypothesized that this protein may affect the apoptosis, cell-senescence, and cell-aging processes (22). HMGB1 attaches p53 and its linkage point at DNA and induces its functionality and integrity (23). HMGB1 is reported to arrest the cell cycle at G1 phase in vitro while inducing apoptosis and suppressing tumorigenesis in vivo (24). Homeobox B9 (HOXB9) upregulates EMT in cancer cells by altering the expression of Snail and other related proteins in several cancer types (25). HOXB9 is also reported to affect DNA repair mechanisms through its indirect coordination with ATM and thus causes breast cancer metastasis in the lungs (26).

Considering the vast impact of FOX transcription factors and HMG proteins on different cancers and the influence of p53, p21, Bax, and Bcl-2 on cell cycle and apoptosis, the present study was designed to observe the impact of silibinin on the expression of these genes. As it is previously reported for 4T1 cells to metastasize in lungs and considering the impact of HOXB9 gene in this mechanism, the present study focused on the expression pattern of this gene in 4T1 cells after silibinin administration as well.

Results

MTT assessment of silibinin toxicity in 4T1 cells

The MTT analysis results have shown that silibinin (50, 75, 100, 150, 200, and 250 µM/L) had a dose-dependent and time-dependent cytotoxicity in 4T1 cells. Hence, 250 µM/L
showed the highest amount of cytotoxicity in 4T1 cells during 72 h incubation period (** = P < 0.001; \textbf{Figure 1}).

\textbf{Figure 1. Dose- and time-dependent analysis of silibinin cytotoxicity in 4T1 cells by MTT assay.} 4T1 cells were exposed to different concentrations of silibinin for 24, 48, and 72 h. The results are shown as Mean ± SEM using ANOVA and the p values are as follows (P < 0.05: *, P < 0.01: **, P < 0.001: ***).

IC50 concentrations for the three incubation periods were calculated using SPSS and Excel software. The silibinin administration to 4T1 cells showed IC50 of 177.009, 104.851, and 73.677 μmol/L for 24, 48, and 72 hours, respectively. From this information, it can be inferred that 104.851 μmol/L IC50 concentration and the incubation time of 48 hours were the most suitable treatment conditions for subsequent analyses (\textbf{Figure 2}).

\textbf{Figure 2. Analysis of IC50 concentrations of silibinin, for 24, 48, and 72 hours of incubation.} The concentrations obtained were 177.009 μmol/L, 104.851 μmol/L, and 73.677 μmol/L for 24, 48, and 72 hours, respectively.
Tumor volume and weight analysis

A comparison of obtained tumors from control, placebo, and treatment groups showed that the 300 mg/kg silibinin treatment group showed a marked suppression and shrinkage of tumors, which caused lower tumor volume and weights in this group (Figures 3 A-C).

![Figure 3. Characteristics of isolated tumors.](image)

- **A)** Photographs of tumor masses, which show that silibinin treatment caused a significant shrinkage in mice tumors as compared to the control and placebo groups.
- **B)** The mean volume of tumors, shows that 300 mg/kg silibinin administration caused a marked shrinkage as compared to the control and placebo groups.
- **C)** The weight of tumors in the 300 mg/kg silibinin group showed a significant shrinkage in tumors as compared to the control and placebo groups.

H&E staining: Tissue morphology and pathology

Our H&E staining analysis showed that the tumors, excised from the control group mice, had more cell density and the frequency of dynamic cells with distinct nuclei at the mitotic stage and these nuclei are present at the correct positions in the cytosol. While, the cell nuclei of the tumors, excised from the 300 mg/kg silibinin treatment group, showed a high rate of
necrosis and a high percentage of cancer cells demonstrating the phenomenon of pycnosis being going on and the cells have dense and hyperchromatic nuclei. In these images, adipose-type connective tissue formation is also evident from the outside to the inside, with a natural angiogenesis process. These results show that silibinin caused cancer cell death through multiple phenomena while replacing them with adipocytes (Figure 4).

**Figure 4. Hematoxylin and eosin staining of treatment and control tumor tissue samples of cancerous Balb/c mice.** A) Control sample images showing normally growing and proliferating cells (left: 20X, right: 100X). B) Treatment group (300 mg/kg silibinin administration) tissues showing necrotic areas and a large number of cells with dense nuclei (left: 20X, right: 100X).

**Immunofluorescent staining assessments**

IF staining was carried out to assess the expression patterns of key genes in control and treatment groups. As shown in Figure 5A, the expression of CD31 protein (PCAM-1) showed a marked decrease in the silibinin-treated group, which indicates a decrease in the rate of angiogenesis in the treated samples, as compared to the control group. The same
reduction pattern was also seen for HMGB1 protein expression in the treated samples, and its position is also located to be intra-nuclear (Figure 5B), which may be attributed to the tumor-suppressing impact of silibinin in our study (29-32). Our DAPI staining results show that the number of stained nuclei is more in the control group as compared to the treatment group. In the meanwhile, the control nuclei have an elliptical or spherical morphology, which indicates healthy and intact nuclei. While, the treatment group nuclei seem smaller and denser with irregular shapes, which may indicate a high rate of apoptosis going on inside them.

Figure 5. Expression of CD31 and HMGB1 in the treatment and control groups by IF staining. A and B) CD31 and HMGB1 both showed decreased expression in the treatment group as compared to the control group.

Flow cytometry analysis: cell cycle and apoptosis profile

Flow cytometry was carried out to assess the exact impact of silibinin on the cell cycle and apoptotic profile of breast cancer mice tissues. According to Figure 6A, the 300 mg/kg
silibinin administration group showed 52.91% late apoptosis as compared to the control with 13.91% apoptosis, which shows a significant increase in programmed cell death in the treatment group (p = 0.002). Figure 6B shows the rate of late and early apoptotic cells in both treatment and control groups.

![Figure 6B](image)

Figure 6. Evaluation of silibinin impact on the apoptotic profile and cell cycle parameters in the 300 mg/kg silibinin-treated group and control group. A) Annexin V/PI flow cytometric analysis apoptotic induction. B) Percentage of early and late apoptosis in treatment and control groups. Data are shown as Mean ± SEM. The significance of the results was determined using t-test. Results with p-value less than 0.05 are significant, the significance is characterized as follows: (P <0.05: *, P <0.01: **, P <0.001: ***).

Figure 7 shows the impact of silibinin treatment on the cell cycle profile of treatment and control mice groups. The 300 mg/kg silibinin-treated group showed 20.57% cells caught in the subG1 phase of the cell cycle as compared to the control group sample, having 15.74% cells in the subG1 phase, which was statistically mom-significant (p = 0.153). while, the percentage of cells in the G0/G1 phase of the cell cycle in the 300 mg/kg group was 32.52%,
which showed a marked reduction as compared to the control group with 77.51% (p = 0.003). Also, 23.33% of cells from the 300 mg/kg silibinin group were tracked to be in the S phase of the cell cycle, which did not show a significant increase as compared to the control group (16.96% cells in the S phase) (p = 0.099). The 44.59% cells were present in the G2/M phase in the 300 mg/kg silibinin group as compared to the control (6.88% cells in the G2/M phase), which showed a significant increase (p = 0.01).

**Figure 7.** Cell cycle profile of silibinin-treated and control groups through PI flow cytometry. A) PI analysis of control and 300 mg/kg silibinin-treated group. B) Comparison of the percentage in control and treatment cells in various cell cycle phases. Data are shown as the Mean ± SEM. The significance of the data was determined using t-test. The results with p-value less than 0.05 are significant. The significance of the data is denoted by (P <0.05: *, P <0.01: **, P <0.001: ***).

**Real-time PCR analysis of gene expression**

RNA extraction and Real-Time PCR analysis were carried out to evaluate the expression profiles of TP53, Bax, p21, FOXF1, HMGB1, Bcl-2, HOXB9, FOXC2, and FOXM1 genes in 300 mg/kg silibinin-treated and control group sample. GAPDH served as the housekeeping gene control. According to **Figure 8**, the expression of TP53, Bax, p21, FOXF1, and
HMGB1 genes showed a marked increase as compared to the control group. While, the expression of Bcl-2, HOXB9, FOXC2, and FOXM1 genes decreased in the 300 mg/kg silibinin-treated group as compared to the control group.

![Bar graph showing comparative expression profile of various genes](image)

**Figure 8.** Comparative expression profile of the TP53, Bax, p21, FOXF1, HMGB1, Bcl-2, HOXB9, FOXC2 and FOXM1 expression in 300 mg/kg silibinin-treated group and control group.

**Discussion**

Keeping in mind the impact of the p53 gene on the expression of Bax and Bcl-2, it can be concluded that this gene induces an apoptotic process in cancer cells by up-regulating Bax and down-regulating Bcl-2 (27). P53 has also been reported to cause cell cycle arrest at the G2/M phase by up-regulating p21 and thus has the ability to control cell cycle function at several phases, such as; G1/S, S, and G2/M (28). During the present study, the FoxM1 and FoxC2 expression decreased in the silibinin-treated samples and FoxF1 expression increased. Previous studies have shown that FoxM1 and FoxC2 play a vital role in advancing the cell cycle and facilitating the cycle through several checkpoints. It has been observed that FoxM1 and FoxC2 have the highest expression in the G2/M phase and play an activating role in expressing the genes needed to cross the checkpoints (19, 29).

FoxC2 has also been reported to up-regulate CDK1 expression, thus promoting the cell cycle through G2 and M phases (19). Therefore, reduced expression of FoxM1 and FoxC2 in the treated samples reduced the number of cells in the G1 phase and thus caused their accumulation in G2 and M phases. FoxC2 expression has also been shown to be associated with increased EMT and subsequent metastasis of breast cancer cells into other organs (30,
Therefore, it may be assumed that silibinin reduces the EMT and metastasis by down-regulating FoxC2 expression.

Some specific studies have reported that FoxM1 is directly related to the cancer growth rate and tumor size among breast cancer patients (32) and has a negative effect on p21 expression, while p21 has a negative impact on FoxM1 expression as well (33), which can be justified with our present results. There are conflicting opinions about FoxF1, as it is over-expressed in breast, lung, and prostate cancers, while its under-expression has been seen in certain cancers, such as colorectal cancer (34-36). These conflicting expression patterns may be attributed to the instability of the genome and certain protein complexes in the tumor microenvironment (TME), which in turn can over-express FoxF1 in some cancer types and down-regulate it in other types of cancers. However, in the studies that have reported its antitumor effects, FoxF1 inhibits the invasion and migration of cancer cells, causing a cell cycle arrest in the G1 phase by increasing p21 expression (34). [34]. FoxF1 expression in breast cancer is inhibited epigenetically, leading to cell cycle progression, indicating that FoxF1 causes cell cycle arrest in the G1 phase [37]. FoxF1 has been reported to increase genomic stability by increasing the expression of MRE11A and BRCA1 genes, resulting in DNA repair. While FoxF1 causes cell cycle arrest in the G2 phase by increasing the expression of ANAPC2 and ANAPC4 mitotic genes (37).

Our results have shown that silibinin could down-regulate the FoxC2 gene. Previous studies have shown that FoxC2 plays a key role in the metastasis of breast cancer into the lungs (17). During EMT, FoxC2 plays an important role in causing the mesenchymal properties in cancer cells (17). Another study focuses on the significant role of FoxC2 in facilitating breast cancer stem cells to pass through the G2 phase and enter mitosis, which is controlled by its higher expression in the G2 phase (19). HoxB9 plays a vital role in cancer onset, progression, EMT, angiogenesis, metastasis, and MDR properties of breast cancer cells (38).

Our study has shown that HoxB9 expression significantly decreased in the silibinin-treated samples. In vivo studies have also shown that reduced HoxB9 expression reduces the formation of new blood vessels (38). The reduced CD31 expression in our study can be attributed to less angiogenic potential in the silibinin-treated group. These results can show that silibinin reduces angiogenesis by reducing the expression of the HoxB9 gene. While our immune-fluorescence results have shown a decreased expression of HmgB1 levels in the treated samples, the RT-PCR results have shown its increased expression. Therefore, increasing mRNA levels and decreasing protein levels may indicate increased control of HmgB1 gene expression at the translation point. Studies have shown that miR-142-3p and
miR-129-5p inhibit the translation of HmgB1 mRNA (39, 40), which can justify the conflicting results of our IF and RT-PCR studies.

HmgB1 can play various roles in the cells (23). Various studies have shown that nuclear HmgB1 plays an important role in genome stability, repair of DNA damage, and maintenance of telomere length (23), so its down-regulation in the nucleus was observed in our IF study. It can help both the progression and metastasis of cancer and the induction of intrinsic apoptosis (caused by unrepaired DNA damage).

H&E staining revealed that silibinin increased the apoptotic and necrosis rate in breast cancer cells, while this phytochemical replaced the dead cancer cells with adipose-associated connective tissue and new healthy blood vessels. This diseased-tissue replacement through healthy adipocytes and blood vessels may create a signal for the immune cell invasion into the diseased area. There are two sources of extracellular HmgB1 production in tumor tissue, which are HmgB1 released by necrotic/ apoptotic cancer cells and the one released through immune cells, which may increase inflammation in cancer tissue and increase angiogenic process and metastasis through NF-κB signaling pathway while decreasing or inhibiting the p53 pathway (23). Thus, silibinin may inhibit metastasis through mechanisms other than HmgB1.

**Conclusion**

According to the obtained results, silibinin can have a wide range of effects on breast cancer, which may be relayed through altered expression of transcription factors, or the expression of other cellular genes that can exert inhibitory or stimulatory impact on several pathways in the cell. All these mechanisms may exert an impact on cancer progression. So, silibinin can prove to be a suitable candidate for breast cancer chemotherapy.

**Methods**

**Cell cultur**

4T1 cell line purchased from the National Cell Bank of Iran (NCBI_Iran Cat# C604, RRID: CVCL_0125) and incubated for 24 hours in Roswell Park Memorial Institute (RPMI-1640) culture medium with 10% FBS(Gipco) and 1% Penicillin/streptomycin antibiotics at 37 °C in a 5% CO₂ atmosphere.
MTT assay of cell viability profile
The effect of silibinin on 4T1 cell viability was assessed by MTT assay. For this, 500 cells were transferred to each well of the 96-well plate, according to the above-stated culture conditions, and incubated for 24 h. Then, the cell culture medium was evacuated following the addition of 50, 75, 100, 150, 200, and 250 µmol/L silibinin to each well. All experiments were carried out in triplicate and the treatments were separately undergone for 24, 48, and 72 h in three separate 96-well plates. RPMI-1640 +FBS and DMSO served as negative and positive controls for this experiment. At the end of incubation, cell supernatants were removed and 30µL of MTT solution was added to each well in darkness and incubated for three hours at 37 °C. The supernatant was then removed and 30 µL of DMSO solution was added to each well, and after shaking the plate, it was kept in the environment for 30 min and its absorption was measured in a 570 nm wavelength. Cell viability was assessed through the following equation and IC50 was calculated thereafter:

\[
\text{Cell viability} = \frac{\text{Sample Absorbance}}{\text{Control Absorbance}} \times 100
\]

Ethics
All procedures related with the in vivo experiments and animal care were approved by the college of science, University of Tehran ethical Committee (1399.021) and were conducted according to the Guideline for the Care and Use of Laboratory Animals in Iran. We complied with the ARRIVE guideline for this reporting of animal experiment conducted in the present study.

Animals
Female mice (BALB/c, 6-7 weeks old, n=36) were purchased from the Pasteur Institute, Tehran, Iran. All mice were remained in sterile cages under laminar airflow hoods in a local specific pathogen-free experimental animal facility under temperature (24±2 °C) and relative humidity (approximately 60%) with a 12-h light/dark cycle. We permitted free access to the tap water and sufficient chow for mice.

Subcutaneous 4T1 tumor model
For breast tumor generation, 4T1 cells were harvested in the exponential phase of growth by trypsinization (Sigma), washed three times with cold PBS (Thermo Fisher Scientific) and
centrifugation steps at 300 x g for 5 min at 4°C, filtered through a 70 µm cell strainer (Thermo Fisher Scientific) and resuspended in cold PBS. 35 x 10⁴ cells were injected subcutaneously (s.c.) in 100 µl of PBS into the right flank of recipient mice. Tumor growth was monitored using a hand caliper and volume was calculated by measuring the longest diameter (length) and its perpendicular (width) using the formula: (length x width²)/2. On day 5-6 post tumor cell injection, when tumors were approximately 75 mm³, mice were divided into 4 groups (each group containing n=9 mice). The control group contained n=9 tumor-bearing mice without any treatment, the placebo group was administered with 45% sterilized distilled water + 45% PEG (polyethylene glycol) + 10% DMSO. While treatment groups (each one contained 9 mice) were separately injected intraperitoneally with 200 and 300 mg/kg day⁻¹ silibinin for 14 consecutive days. Tumor weight and volume were recorded every four days and the first day of treatment administration was considered Day-1 of tumor analysis.

**Histological measurements – H&E staining**

Fresh tumor tissues were collected, fixed immediately with formalin, and then embedded in paraffin, followed cut into a thickness of 5 µm sections. Section slides with tissue were deparaffinized in xylene and rehydrated via graded ethanol (dilution from 100%, 95%, 85% and 75%). Final fixation was done in formalin. Tissue sections were treated with 0.1% triton X100 and 5% BSA (bovine serum albumin), each for 20 minutes, and stained with H&E stains.

**Immunofluorescent staining**

The previously prepared tissue sections were used for immune-fluorescent (IF) staining. Tris-EDTA (pH 6.0; 95 °C) was used for antigen retrieval. Abcam anti-HMGB1 antibody (ab18256; mouse CD31/PECAM-1 AF3628-SP) was incubated with tissue sections at the ratio of 1:300 for 24 h at 4°C. The resultant product was washed thrice with PBS and re-incubated for 2 h with a secondary conjugated antibody (Rabbit IgG Alexa Fluor 488-conjugated Antibody IC1051G) with the ration of 1:1000 at room temperature in dark conditions. Finally, the sections were washed thoroughly with PBS and stained with DAPI (Invitrogen, D1306) in order to carry out a fluorescence microscopic investigation.

**Flow cytometric assessment of cell cycle and apoptosis**

The tumors were used for flow cytometric investigation. For this, tissues were sliced into fine sections, homogenized in PBS, and cells were trypsinized by using 0.25% trypsin at room
temperature. The FBS was added to the homogenate in order to block trypsin enzyme action and the resultant homogenates were filtered and centrifuged. Lysis buffer was used to remove any erythrocytes and the resultant solution was centrifuged again; re-centrifugation of resultant lysate was done twice with PBS. The cells \( n=10^6 \) cells were re-suspended in binding buffer (BD Pharmingen, Cat. No. 556454). Five microliters of annexin V (Annexin V-FITC kit, 331200) were added to 100 µl of cell lysate in dark and after passing 15 minutes, 5 µl PI (Propidium Iodide flowcytometry kit, ab139418) and 400 µl binding buffer solution was added to be observed through BD FACSLyric flow cytometry machine for apoptotic analysis. For cell cycle analysis, only PI was used, as stated above.

**RNA extraction and RT-PCR**

TRIzol™ Plus RNA Purification Kit (Invitrogen, Cat No: 12183555) was used to extract RNA samples from tumor tissues of the control group and 300 mg/kg silibinin treatment group. cDNA synthesis was carried out using a 2X Hyperscript RT Master mix (GeneAll, Cat No:601-705) kit in a 20 µl reaction mixture and 1000 ng RNA was obtained as a final product. Real-Time PCR was done using RealQ 2x Master Mix Green Dye Without ROX (Ampliqon, Cat No: 5000770-1250) kit, and the reaction temperatures were as follows: Initial Denaturation: 95 °C - 10 min, Denaturation: 95 °C - 15 s, Annealing: 59 °C - 25 s, Extension: 72 °C - 25s. The primer sequences were designed using Oligo 7.60 software as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: 5-TGTCAAGCTCATTTCTGGTGATG-3</td>
<td>R: 5- TGGAGGGAGATGCTCAGTG-3</td>
</tr>
<tr>
<td>TP53</td>
<td>R: 5- ACTTGATGGAGATATTCCACCT-3</td>
<td>R: 5- GTCTTCAGGTAGCTGGAGTGA-3</td>
</tr>
<tr>
<td>P21</td>
<td>F: 5- GTGTGCGCTGTCTTCCGT-3</td>
<td>R: 5- CACCAGAGTGCAAGACAGC-3</td>
</tr>
<tr>
<td>Bax</td>
<td>F:5-ACTAAAGTGCCCCGAGCTGAT-3</td>
<td>R: 5-AAGATGCTACTGTCCTGCA-3</td>
</tr>
<tr>
<td>Bcl2</td>
<td>F:5-CTACCGTCGTGACTTCGC-3</td>
<td>R: 5- TCCAGCCTCCCGTTATCC-3</td>
</tr>
<tr>
<td>FOXM1</td>
<td>F:5-ACACCTGGATGGAGGACCCTT-3</td>
<td>R: 5-GGTGCCTTCGCTGTAGTCC-3</td>
</tr>
<tr>
<td>FOXC2</td>
<td>F:5-GCCACCTCTTGGTATCTGAAC-3</td>
<td>R: 5-TGGAGCAGCTCTAGTATTTGCTG-3</td>
</tr>
<tr>
<td>FOXF1</td>
<td>F:5-GCACCCATACCTTACCAACAAAC-3</td>
<td>R: 5-CACCTTGGGCTGCTACATCC-3</td>
</tr>
<tr>
<td>HOXB9</td>
<td>F:5-GGGAGGCTGGCCCTGGTAAAC-3</td>
<td>R: 5-CTTTCTTAGCCTCCAGCCTG-3</td>
</tr>
<tr>
<td>HMGB1</td>
<td>F:5- GGCGAGCATCTCGGCTAT-3</td>
<td>R: 5-GGGCTGCTGTGCTACCTG-3</td>
</tr>
</tbody>
</table>

The data were analyzed with Excel 2019 and the relative mRNA expression of the target gene was calculated using the \( 2^{-\Delta\Delta Ct} \) method.

**Statistical analysis**

Excel 2019 and GraphPad Prism 8 were used for data processing and statistical analysis of the experimental results. All data were stated as the mean (± standard deviation). Each in vitro assay was conducted in triplicate and was repeated at least three times. All data were
adjusted with one-way analyses of variance with Bonferroni’s post hoc multiple comparison analysis and t-test. P-values < 0.05 (*p < 0.05, **p < 0.01, ***p < 0.001) were considered statistically significant.

References


Author contributions

Authors and Affiliations

1. Department of Cell & Molecular Biology, School of Biology, college of science, University of Tehran, Tehran, Iran

Hamid Zarei Golambahri

2. Department of Cell & Molecular Biology, School of Biology, college of science, University of Tehran, Tehran, Iran

Nasrin Motamed

3. Department of Cell & Molecular Biology, School of Biology, college of science, University of Tehran, Tehran, Iran

Mohammed Davoody

Corresponding author

Nasrin Motamed

Data availability

The datasets generated and analyzed during the current study are not publicly available due to the policies of the relevant institution, it is not possible to make the data available to the public, but are available from the corresponding author on reasonable request.

Competing interests

The authors declare no competing interests.