Nano-curcumin’s suppression of breast cancer cells (MCF7) through the inhibition of cyclinD1 expression

Background: Breast cancer is the leading cause of cancer worldwide. The high expenses associated with chemotherapy as well as its side effects make the management of breast cancer a daunting challenge. The most common overexpressed gene in breast cancer is cyclinD1, which induces cell proliferation. Recent investigations into cancer treatment have revealed that curcumin demonstrates potential anti-cancer properties through different pathways. However, the oral bioavailability of curcumin is negligible due to its high hydrophobic structure. Nanotechnology has been employed to overcome this barrier. Nano-formulated curcumin (SinaCurcumin®) has been shown to provide a significantly higher bioavailability for oral consumption. However, the efficacy of this nano-formulated drug in breast cancer has not yet been determined. In relation to the breast cancer cell line, the present study compared nano-curcumin’s anti-cancer properties with those of cyclophosphamide, adriamycin, and 5-fluorouracil (CAF).

Methods: After treating MCF7 with nano-curcumin and CAF, the present work assessed cell viability via an MTT assay. The effects of these drugs on cyclinD1 expression were measured by real-time PCR. SPSS 16.0 was used to perform ANOVA and multiple range tests.

Results: Nano-curcumin and the CAF regimen both lowered the viability of MCF7. Nano-curcumin decreased cell proliferation by 83.6%, which was more than that achieved by cyclophosphamide (63.31%), adriamycin (70.75%), and 5-fluorouracil (75.04%). In addition, curcumin was able to significantly reduce the expression of cyclinD1, whereas CAF did not alter cyclinD1 expression.

Conclusion: Nano-curcumin has a relatively high cytotoxic effect on MCF7 breast cancer cells, suppressing the expression of cyclinD1, a critical gene in the development and metastasis of breast cancer. The current study demonstrated that nano-curcumin can be an effective drug in the CAF regimen for the treatment of breast cancer. However, further in vivo research is needed for determining its efficacy and safety in clinical applications.

Keywords: nano-curcumin, cyclinD1, MCF7, patient survival, viability

Introduction
As a result of modern lifestyle-related risk factors, the incidence of breast cancer is rapidly growing worldwide.1-3 In Iran, studies have shown that the prevalence and mortality rate of breast cancer has risen as life expectancy has increased.4

One of the main etiologies of breast cancer is cyclin D overexpression, which causes cells to initiate the G1 phase of the cell cycle and activate the proliferation of signaling pathways.5,6 Studies have reported an association between cyclinD1 overexpression and tumor growth. The overexpression of cyclinD1 induces tumor formation through the ErbB2 protein and RAS oncogene.2 In addition, cyclinD1 promotes cell migration...
and metastasis by inhibiting RhoGTPase and upregulating Rock2 and TSP-1.7 CyclinD1 through the phosphorylation of paxillin also increases tumor invasion.8 Taken together, cyclinD1 is associated with patient survival and prognosis.

Breast cancer is treated by different methods, including surgery, chemotherapy, radiotherapy, and endocrine therapies.9–11 One of the most common treatments is chemotherapy.12 Various chemotherapy agents are employed to achieve therapeutic goals, such as 5-fluorouracil, cyclophosphamide, and adriamycin. However, a universal standard regimen has not yet been developed. The maximum tolerated dose of these agents may be difficult to administer and often cause intensive side effects, including anemia,13,14 neutropenia,15 leukopenia,13 vomiting,16 liver toxicity, and hemorrhagic cystitis.17 Thus, there is a need for more effective methods to improve upon the limitations of current treatments. The usage of natural compounds, in combination with common pharmaceutical agents, has proven to be less cytotoxic and more effective.17 Many studies have demonstrated the potential use of curcumin as a natural substance in combination with other routine treatments.18 Curcumin is a yellow substance that is extracted from Curcuma longa. It is widely used as herbal supplement and food coloring agent in Asia.19

The anti-microbial,20–23 anti-inflammatory,24–26 and anticancer properties of curcumin27–30 have been mentioned in recent works. The present study investigates the potential use of SinaCurcumin® for oral use which has been developed in recent works. The present study investigates the potential use of nano-curcumin for breast cancer treatment and their interaction with the cyclinD1 pathway.

Materials and methods

Drugs

Nano-micelle curcumin was obtained from Exir Nano Sina Company in Tehran, Iran (IRC:1228225765) and nano-micelle curcumin for breast cancer treatment and their interaction with the cyclinD1 pathway.

Cell line

Human breast cancer cells (MCF-7; ATCC® HTB-22™, Cat No: 85072011, NCBI NO: C135; ATCC, Manassas, VA, USA) were purchased from the cell bank at the Pasteur Institute (Tehran, Iran) due to the presence of estrogen and progesterone receptors. The present work prepared all the reagents and mediums immediately before application.

Cell culture

MCF-7 cells were cultured in DMEM (Thermo Fisher Scientific, Waltham, MA, USA) and contained 10% FBS (Thermo Fisher Scientific), penicillin (1% v/v), and streptomycin (1% v/v). The cells were incubated in 5% CO₂ at 37°C.

MTT assay

The effects of nano-micelle curcumin, cyclophosphamide, adriamycin, and 5-fluorouracil on the viability of MCF-7 cells were assessed by MTT assay (Sigma-Aldrich Co., St Louis, MO, USA). The cells were briefly seeded into a 96-well culture plate containing 100 µL of growth medium at 5,000 cells/well and then were incubated for 48 hours at 37°C in 5% CO₂. Cells were treated with different drug concentrations: nano-micelle curcumin: 0.62, 1.24, 2.52, 5.07, 10.17, 20.35, 40.71, 81.43, and 162.87 mmol/L; cyclophosphamide: 11.95, 23.93, 47.87, 95.75, 191.5, 383.01, and 766.03 mmol/L; adriamycin: 0.28, 0.57, 1.14, 2.29, 4.59, 9.19, and 18.39 mmol/L; and 5-fluorouracil: 29.98, 59.96, 81.49, 239.85, 480.48, 960.96, and 1,921.93 mmol/L. After that, the cells were incubated for a second time for 24 hours in 5% CO₂ at 37°C. The old medium was replaced with 20 µL of MTT solution (0.5 mg/mL). The plates were incubated for 3.5 hours at 37°C. Afterward, formazan precipitate was dissolved in 200 µL of dimethyl sulfoxide and then added to the wells. An ELISA reader eventually quantified the absorption of the solution (Anthos, Carlton, VIC, Australia) at 490 nm. As the percentage of cell viability, MTT assay results were expressed as mean ± SD. The half-maximal inhibitory concentrations (IC50) were measured as mg/mL.

RNA extraction

To evaluate the gene expression, we loaded samples with nano-curcumin and drugs at IC50. An RNA extraction kit (Cat No: 11828665001; Hoffman-La Roche Ltd., Basel, Switzerland) isolated the RNAs from the breast cancer cell line. Four samples were prepared according to the following instructions: MCF7 + nano-curcumin (A); MCF7 + cyclophosphamide + adriamycin + 5-fluorouracil (B); MCF7 + nano-curcumin (first 24 hours) + CAF (the second 24 hours) (C); and group D which included MCF7 as negative control. An RNase/DNase-free environment was provided to carry out all the procedures. The present study determined the quality and quantity of the extracted RNA by gel electrophoresis and NanoDrop® (Thermo Fisher Scientific), respectively.

cDNA synthesis and real-time polymerase chain reaction

A cDNA kit (Parstous, Tehran, Iran) reverse transcribed 10 ng of RNA according to the company’s instructions. Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Nano-curcumin (A)</th>
<th>Cyclophosphamide + Adriamycin + 5-Fluorouracil (B)</th>
<th>MCF7 + Nano-curcumin (First 24 Hours) + CAF (Second 24 Hours) (C)</th>
<th>MCF7 (Negative Control)</th>
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<tbody>
<tr>
<td>IC50</td>
<td>0.62 mmol/L</td>
<td>11.95 mmol/L</td>
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<td>IC1</td>
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<td>IC0.1</td>
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presents the sequence of primers employed for real-time PCR. Ten micrograms of SYBR green PCR master mix (Parstous), 1 µg of cDNA, and 10 µg of primer were mixed to prepare a reaction mixture. The $2^{-\Delta\Delta Ct}$ method quantified the relative gene expression. For a reference gene, the results were normalized to GAPDH. The following formula calculated fold change and $\Delta\Delta Ct$:

\[
\text{Fold change} = 2^{-\Delta\Delta Ct}
\]

\[
\Delta\Delta Ct = \frac{\text{Sample (Ct}_{\text{Target gene}} - \text{Ct}_{\text{GAPDH}}) - \text{Reference sample (Ct}_{\text{Target gene}} - \text{Ct}_{\text{GAPDH}})}{\text{Sample (Ct}_{\text{Target gene}} - \text{Ct}_{\text{GAPDH}}) - \text{Reference sample (Ct}_{\text{Target gene}} - \text{Ct}_{\text{GAPDH}})}
\]

**Statistical analysis**
The results were expressed as the mean ± SD. Following normal distribution, one-way ANOVA, and Tukey’s multiple range tests were applied to calculate the significant difference. $P$-values <0.05 were considered to be statistically significant.

All the tests were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**The results of the MTT test**
The MTT test showed that the highest rate of viability was observed at a concentration of 0.62 mmol/L of curcumin. At a curcumin concentration of 162.87 mmol/L, cell viability reduced to 16%. In other words, increase in the concentration of nano-micelle curcumin causes a gradual decrease in cell viability. This also occurred with other drugs. However, curcumin reduced cell proliferation by 83.6%, which was more than by cyclophosphamide (63.31%), doxorubicin (70.75%), and 5-fluorouracil (75.04%). IC50 values were measured at 615.02, 170.97, 0.91, and 59.72 mmol/L with increasing concentrations of 5-fluorouracil, cyclophosphamide, doxorubicin, and nano-curcumin, respectively. Figure 1 presents the viability of cells after treatment.

**Results of melting curves obtained from PCR products**
CyclinD1 expression was significantly ($P<0.005$) lowered following the treatment with nano-curcumin (Sample A). The gene expression ratio was calculated as 0.715 for sample A, 3.771 for CAF treatment (Sample B), and 1.431 for the combination treatment (Sample C) as shown in Figure 2. Considering Samples A and C, nano-curcumin represented a significant inhibitory potential for cyclinD1 expression.

**Discussion**
The leading cause of cancer is breast cancer, which holds fifth place in cancer-related deaths globally. The high cost of chemotherapy for treating breast cancer and its side effects

**Table 1** Primers used in the current study

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward</th>
<th>Backward</th>
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<tr>
<td>Cyclin D1</td>
<td>TGCACCACCAACTGCTTAGC</td>
<td>GGCATGACTGTGGCTAGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGATGCTGGAGGTCTGCGAGGAAC</td>
<td>GAGAGGAAGCCTGAGGCCTAGA</td>
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![Figure 1](image-url) The percentage of viability of breast cancer cells (MCF7) after treatment with four compounds.

**Note:** Cytotoxicity of 5-fluorouracil, doxorubicin, nano-curcumin, and cyclophosphamide.
make this disease one of the most challenging. The most common gene over expressed in breast cancer is cyclinD1. Recent research on cancer treatment has reported that curcumin demonstrates potential anti-cancer properties via different pathways. One of the main targets inhibited by curcumin is NF-κB, which plays a vital role in oncogenic transformation. However, the oral bioavailability of curcumin is negligible due to its strong hydrophobic structure. Nanotechnology has been employed to overcome this barrier. Nano-formulated curcumin (SinaCurcumin) has been demonstrated to provide significantly higher bioavailability by oral consumption. Following the improvement of bioavailability of curcumin, its inhibitory effects can improve.

The current study investigated the effects of nano-curcumin on the MCF7 cell line. The results indicated that nano-curcumin decreased cell proliferation by 83.6%, which was more than that achieved by cyclophosphamide (63.31%), adriamycin (70.75%), and 5-fluorouracil (75.04%). This demonstrates that nano-curcumin has more potential to inhibit MCF7 cell proliferation in comparison to CAF. CyclinD1 expression was significantly downregulated following treatment with curcumin, whereas CAF demonstrated no notable inhibition of cyclinD1 expression. Liu et al revealed that curcumin inhibits MCF7 cells by suppressing the NF-κB signaling pathway. This suppression was induced by the inhibition of ubiquitin proteasome system (UPS) via curcumin. Inactivation of UPS inhibits dissociation of ikBα from NF-κB; so NF-κB cannot enter the nucleus and cyclinD1 expression will be blocked. Altogether, NF-κB signaling pathway suppression downregulates cyclinD1 expression and decreases MCF7 proliferation.

The other mechanism describing the underlying pathway of inhibition of MCF7 proliferation is through Bcl-2. It has been reported that curcumin decreases Bcl-2 in breast cancer cells. As a result, the increase in the BAX/Bcl2 ratio leads to curcumin-induced apoptosis in cancer cells. In addition, increase in P53, a tumor suppressor protein, expression lead to BAX expression after curcumin treatment in MCF7 cells. This also causes the apoptosis in cancer cells. The anti-cancer effects of curcumin on other breast cancer cell lines (MDA-MB231 and JIMT1) have been also reported.

Confirming the present work’s results, Hajigholami et al reported that nano-packaged tamoxifen and curcumin not only increased the anti-cancer properties of MCF7, but these also lowered the toxicity of normal cells. Unfortunately, the current study did not assay the curcumin toxicity toward normal cells. Also, we could not assess the protein levels of cyclinD1 and marker of apoptosis due to the financial limitation. Altogether, nano-curcumin offers an effective and safe method to treat breast cancer cells in vitro. On the other hand, compared to CAF, curcumin weakened cyclinD1 expression to a significant extent.

**Conclusion**
Curcumin may inhibit tumor proliferation, and also increase patient survival by inhibiting metastasis and changing in the expression of cyclinD1. This has made curcumin a potential anti-cancer agent. However, further in vivo investigation is needed to assess curcumin’s efficacy and safety in clinical applications.

**Acknowledgment**
Mashhad University of Medical Sciences approved this study and had no role in the design and conduct of the study.
Disclosure

The authors report no conflicts of interest in this work.

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