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Administration of Curcumin, Betanin, and CoQ10 combined with nickel oxide, iron superoxide nanoparticles show preventive effects in breast cancer: Effect on apoptosis pathway and MiR-455 expression

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In the present study, we aimed to evaluate the cytotoxicity and antitumor effect of alone and combined treatment of curcumin, betanin, and coenzyme Q10 (CoQ10) compounds and nickel oxide (NiO) and iron superoxide (Fe2O3) nanoparticles (NPs) on breast cancer cells *in vitro* and *in vivo*. The 4T1 breast cancer cells were exposed to different concentrations of Q10, NiO, and Fe2O3 NPs and the inhibitory concentration (IC_{50}) of NPs and compounds and their effect on cell viability was evaluated by MTT assay. Apoptosis induction in BALB/c mice after treatment with the IC_{50} concentration of tested compounds was evaluated by flow cytometry. Gene expression was measured using quantitative real-time polymerase chain reaction (qRT-PCR). The IC_{50} values for Fe2O3 and NiO NPs were found to be 92.42 µg/mL and 21.49 µg/mL, respectively, and were 0.87 µg/mL, 60.14 µg/mL and 83.47 µg/mL for curcumin, betanin, and CoQ10, respectively. Curcumin was more cytotoxic, whereas Fe2O3 showed lower cytotoxicity than the other compounds in the 4T1 line. All treatments significantly exerted anticancer activity against breast tumors. qRT-PCR analysis revealed that treatment with IC_{50} concentrations of all alone and combined compounds downregulated the expression of *Bcl2* and upregulated *Bax* in breast tumor. The results revealed a significant reduction in *TFAM* and MiR-455 expression levels. The combination of aforementioned antitumor agents with Fe2O3 and NiO NPs shows a synergistic impact, as apoptosis induction is boosted by a combination of antitumor agents and NPs, and a higher regulatory impact on gene expression occurs compared with monotherapy.

Keywords: Betanin, Breast cancer, Coenzyme Q10, Curcumin, miRNA, Nanoparticles

Breast cancer is one of the leading causes of death worldwide, and according to estimates, its incidence rate in women will increase dramatically by 2030[1]. However, breast cancer treatment is an increasing challenge in advanced stages, requiring novel strategies for its effective therapy. Clinically, different approaches are used in breast cancer treatment. Surgery, chemotherapy, and radiotherapy are the most common methods of breast cancer treatment[2]. High relapse, severe side effects, and the multidrug resistance nature of breast cancer indicate the limited efficacy of current treatment approaches. Therefore, natural products are considered effective in breast cancer treatment because they have significant antiproliferative and anticancer activities and significant therapeutic values[3]. Recently, plant-derived natural products have received much attention for the treatment of various diseases, particularly cancer[4]. According to reports, natural products can mainly suppress breast cancer growth and progression by inducing apoptosis and inhibiting metastasis. For example, Li *et al*. reported that glycoprotein-56 (SPG-56) extracted from sweet potato inhibited proliferation and promoted apoptosis of MCF-7 cells in a dose- and time-dependent manner[5]. Centipeda minima (CME) significantly induces apoptosis and inhibits cancer cell migration and invasion by inhibiting the EGFR (epidermal growth factor receptor), PI3K/AKT/mTOR (Protein kinase B /phosphatidylinositol-3 kinase/mammalian target of rapamycin), NF-κB (Nuclear factor-kappa B), and STAT3 (signal transducer and activator of transcription 3) signaling pathways. Furthermore, treatment with CME downregulated matrix metalloproteinase-9 (MMP-9) activity, inhibited

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metastasis, and reduced tumor burden in MDA-MB-231 xenograft mice[6].

Curcumin is a well-known antitumor agent derived from the root and rhizome of Curcuma longa and is a regulator of molecular pathways in cancer therapy. Curcumin is extensively used for treating breast cancer, and its administration is associated with reduced metastasis by suppressing epithelial to mesenchymal transition (EMT)[7]. *In vitro* and *in vivo* experiments have shown the role of curcumin in inducing mitochondrial apoptosis and cell cycle arrest (G1 phase) in breast cancer. However, the antitumor activity of curcumin needs to be improved to provide better results in breast cancer therapy. Therefore, combination therapy with other anti-tumor agents is performed. Betanin is the predominant betacyanin in red beetroot species and is considered a potent antitumor agent. Administration of betanin induces apoptotic and autophagic cell death in breast cancer cells[8].

Furthermore, betanin can inhibit lung carcinogenesis by activating caspase cascade in triggering apoptosis. Chandrasekaran *et al*. demonstrated that betanin exerts cytotoxic effects, induces apoptosis, and inhibits cell proliferation in the A549 lung cancer cell line by blocking *Bcl2*, leading to membrane permeability and loss of mitochondrial membrane potential[9]. In addition, a recent study reported that betanin/isobetanin remarkably reduced the proliferation and survival of MCF-7-treated cells. Another antitumor agent used in cancer therapy is coenzyme Q10 (CoQ10), which reduces CD59 expression and phospholipase D activity in liver cancer therapy[10]. Reports suggest that CoQ10 has favorable effects on the regression of tumor masses and remission of distant metastasis of breast cancer[11]. Low plasma CoQ10 levels are an independent prognostic factor for melanoma and breast cancer progression[12].

Despite significant progress in drug discovery and novel antitumor compounds, cancer remains a major public health. Hence, scientists have had a special view toward using bioengineering in cancer therapy. NPs are structures with particle sizes less than 100 nm, and they have opened a new gate in cancer therapy by providing targeted delivery of therapeutics[13]. Nickel oxide NPs (NiO-NPs) are extensively used as catalysts, oxidizers, and drug carriers[14]. In addition, NiO-NPs are considered antitumor agents[15]. It has been reported that NiO-NPs can promote oxidative stress and activate the c-Jun N-terminal kinase (JNK) pathway in triggering apoptosis and autophagy in human cancers[16]. Iron oxide (Fe2O3) NPs are other promising candidates in cancer therapy[17] and can induce cell death due to the presence of Fe^{2+}, which mediates ferroptosis[18].

In this experiment, we compared the application of curcumin, betanin, and CoQ10 along with NiO and Fe2O3 NPs in breast cancer therapy. This study highlights that combination therapy is more efficient in cancer therapy than monotherapy; therefore, applying NPs and the aforementioned antitumor agents can boost cell death induction in breast cancer. Furthermore, this experiment will provide a direction for future studies focusing on other combination therapies for breast cancer.

Material and Methods

Nanoparticles properties

NP powder was purchased from Mehregan Shimi Saman Co (Tehran, Iran). The size of NPs was checked by TEM (Transmission Electron Microscope) and SEM (Scanning Electron Microscope) and investigated by XRD (Siemens X-ray diffractometer D5000, Germany). Using a (DLS) Dynamic Light Scattering analyzer (Nanotrac Wave mode, Microcrac, Inc., USA was done the hydrodynamic size and surface charges of NPs.

Cell culture and treatment

4T1 cells were purchased from the cell bank of Iran Pasteur Institute (National Cell Bank of Iran). RPMI-1640 (Roswell Park Memorial Institute) culture medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin/streptomycin, and trypsin were obtained from Gibco (Gibco-Invitrogen); Annexin V-FITC kit was purchased from BD Biosciences (San Jose, CA, USA). Curcumin, betanin, and Q10 were purchased from Sigma Chemicals (Sigma-Aldrich, USA). Thiazolyl blue tetrazolium bromide (MTT) cell viability dye was purchased from DNAbiotech (KalaZist, IRAN). RNA was extracted using the RiboEX kit (GeneAll Biotechnology, Seoul, South Korea). The cDNA Synthesis Kit was purchased from BioFACT (Daejeon, South Korea). The MiR-Amp kit was purchased from Parsgenome (Iran). All other materials were obtained from domestic providers at analytical grade.

In vitro cytotoxicity assay

The pre-synthesized Fe2O3 and NiO NPs and purified curcumin, betanin, and CoQ10 compounds were used in this assay and were purchased from Sigma delegates in Iran. The cytotoxicity of NPs

(Fe2O3 and NiO) and compounds (curcumin, betanin, and CoQ10) was assessed by MTT (3, -4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide) assay using a mouse mammary carcinoma cell line (4T1) according to the manufacturer's instructions (DNAbiotech, KalaZist, IRAN).

Study design and MTT assay

4T1 cells were purchased from the cell bank of Iran Pasteur Institute (National Cell Bank of Iran). Cells were cultured in RPMI-1640 culture medium (Gibco-Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco-Invitrogen) and 1% penicillin-streptomycin (P/S). Briefly, 4T1 cells in the log phase of growth were trypsinized and seeded at a density of 8×10^4 cells/well in a 96-well plate and incubated for 24 h. Then, the wells were replaced with 100 µL of the fresh medium containing different concentrations of Fe2O3 NPs (50, 100, 150 and 200 µg/mL), NiO NPs (10, 20, 30 and 40 µg/mL), curcumin (0.5, 1, 1.5 and 2 µg/mL), betanin (10, 50, 100 and 200 µg/mL) and CoQ10 (20, 60, 80 and 100 µg/mL) and was further incubated for 48 h at 37°C with 5% CO_2. Subsequently, 20 µL of MTT solution (5 mg/mL) was replaced with the culture medium, and the cells were incubated for an additional 4 h at 37°C. Then, the supernatant liquid in each well was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to solubilize the resulting formazan crystals. Finally, the optical densities were read using a microplate reader (BioTek-ELx800, USA) at 570 nm, and the percentage of cell viability was expressed as: A treatment/A control × 100% (where, A = absorbance). Untreated cells incubated in the culture medium alone served as controls. The mean of three absorbance values was calculated for each concentration. The IC_{50} value (µg/mL) was determined as the concentration of the tested compound, that inhibited cellular growth by 50%[19]. All experiments performed in triplicates.

***In vivo* toxicity and anticancer efficacy**

NPs breast cancer mic model and treatment All experiments involving animals were approved by the Ethical Committee of Islamic Azad University, Tehran Medical Sciences branch (IR.IAU.TNB.REC.1399.024) and carried out following the relevant guidelines and regulations. *In vivo* cytotoxicity study was performed using 6-week BALB/c female mice obtained from the Pasteur Institute, Karaj, Iran. The animals were maintained in isolation under controlled temperature (22±0.5°C), humidity (50%-60%), and light (12:12 h light/ dark cycle) in polypropylene cages. The animals received filtered water and food ad libitum. The mice were randomly divided into two main healthy and cancerous groups. The tumor implantation model was established as previously described by Shahid Beheshti Research Center[20]. In brief, 1×10^6 4T1 cells suspended in 0.1 mL PBS were injected into the mammary fat pad of each mouse[21]. After inoculation of 4T1 cells, tumor growth was monitored using caliper measurement. Then, healthy and cancerous groups were divided into twelve subgroups (n = 10 per subgroup) and treated with IC_{50} concentrations of NPs and compounds. Subgroup 1 received only normal saline solution as a control. Experimental subgroups 2 and 3 were treated with Fe2O3 and NiO NPs, respectively. Subgroups 4, 5, and 6 received curcumin, betanin, and Q10 compounds, respectively. The experimental subgroups 7, 8, and 9 received Fe2O3 NPs containing curcumin, betanin, and CoQ10 compounds. Finally, subgroups 10, 11, and 12 received NiO NPs containing curcumin, betanin, and CoQ10 compounds, respectively. All mice were anesthetized after the treatment at the end of 14 days; tumors and breast tissues from healthy groups were dissected and subjected to subsequent analyses. For measuring cell viability, 20 mg of tissue was homogenized in ice-cold PBS and centrifuged at 12000×g for 15 min at 4°C. The obtained pellet was resuspended in 20 µL of MTT solution, and the rest of the protocol was performed as described previously.

Apoptosis assay (Flow cytometric analysis of cells)

Flow cytometry to quantitatively determine the extent of cell apoptosis following treatment with IC_{50} concentration of NPs and compounds performed using annexin-V/PI Kit according to the manufacturer's instructions as described in Khakrizi *et al.* with slight modifications[22]. In brief, single cells prepared from tissues (3×10^5 cells/ml) were stained with 3 µL of Annexin V- fluorescein isothiocyanate (FITC) and 5 µL of propidium iodide for 15 min at room temperature in the dark. Analysis was performed using a Becton Dickinson flow cytometer (Franklin Lakes, NJ, USA) equipped with FlowJo software (Tree Star Inc., version 9.6.3).

Gene Expression Assay

cDNA synthesis and qRT-PCR

Bax, *Bcl2*, *TFAM*, and MiR-455 gene expressions were assessed by qRT-PCR. RNA was extracted from

tissues using a RiboEX kit (Gene All Biotechnology, Seoul, South Korea) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using BioFACT cDNA Synthesis kit (Daejeon, South Korea), and qRT-PCR analysis was conducted in triplicate using SYBR Green Master Mix (TAKARA, Japan) on a LightCycler TM 96 (Roche) in 20 µL reactions containing 1×SYBR Green PCR Master Mix, 0.5µM of each forward and reverse primer and 1.5 µL cDNA template. The thermocycling conditions were as follows: an initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 20 sec. ACTB (β-actin) was used to normalize the expression of *Bax*, *Bcl2*, and *TFAM*, whereas U6 was used as an internal normalized reference for MiR-455. Expression levels were measured using the $2^{-\Delta\Delta Ct}$ method[23]. All primers were designed using Primer3plus software, and the sequences are listed in (Table 1).

Statistical analysis

Statistical comparisons were performed using Student's t-test. Results are expressed as means ± standard errors (SE). P-values of less than 0.05 were considered significant.

Results

***In vitro* cytotoxicity assay**

The *in vitro* cytotoxicity effects of Fe2O3 and NiO NPs, curcumin, betanin, and CoQ10 compounds were evaluated in breast cancer cell line (4T1) to adjust the optimal doses (IC_{50}) administered to animals (Fig. 1A-E). The results showed that all compounds reduced cell viability in a dose-dependent manner. As shown in Figure HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/". HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-

Table 1 — Primer sequences used for qRT-PCR

Target transcript	Primer type	Sequence (5′→3′)
Bax	Forward	CTACCGTGAATCTTGGCTGTAAAC
	Reverse	AATCAACGCAGTTGTCCGTGGC
Bcl2	Forward	CCTGTGGATGACTGAGTACCTG
	Reverse	AGCCAGGAGAAATCAAACAGAGG
TFAM	Forward	GATACACCAGATAGAGATAG
	Reverse	CGAATCCTATCATCTTTAGCAAGC
MiR-455	Forward	GCAGTCCATGGGCATATACAC
	Reverse	GCTGTCAACGATACGCTACCTA
ACTB	Forward	GATCAAGATCATTGCTCCTCCTG
	Reverse	CTAGAAGCATTTGCGGTGGAC
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AGAGCAGGGTCCGAGGT

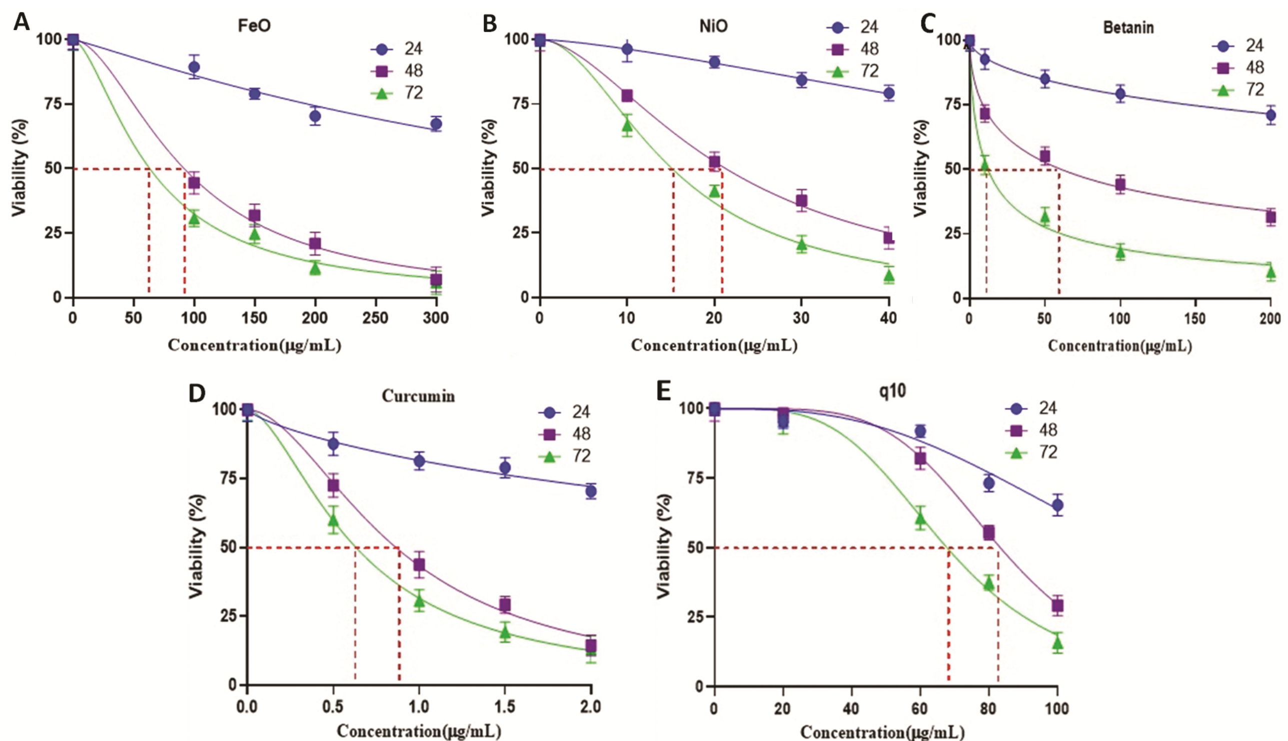

Fig. 1 — Cytotoxicity of Fe2O3 (A), NiO (B), curcumin (C), betanin (D), and CoQ10 (E) after 24, 48 , 72 h on 4T1 cell line by MTT assay. Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 4T1, mouse mammary carcinoma cell line

4545/"HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/"1 HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/"A and B, the IC_{50} values for Fe2O3 and NiO NPs were 92.42 µg/mL and 21.49 µg/mL, respectively. IC_{50} values obtained for curcumin, betanin, and CoQ10 were 0.87 µg/mL, 60.14 µg/mL and 83.47 µg/mL, respectively (Fig HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/". HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/" HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/"1 HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/"C HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/"- HYPERLINK "https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5019472/figure/f2-ijn-11-4545/"E). Curcumin exhibited higher cytotoxicity in the tested concentration range against cancer cells, which was found to be more than approximately 69 and 95 times lower in concentration than betanin and CoQ10 compounds. Fe2O3 showed lower cytotoxicity than other compounds with an IC_{50} of 92.42 µg/mL. The IC_{50} value of NiO NPs was approximately four times lower than that of Fe2O3.

In vivo toxicity and anticancer efficacy

To determine the cytotoxic effect of NPs (Fe2O3 and NiO) and compounds (Cur, B, and Q10) *in vivo*, we treated healthy and carrying breast tumor groups of BALB/c mice with IC_{50} concentrations and examined cell viability using the MTT assay. As depicted in (Fig. 2A), unlike the combined treatment of compounds and NPs, no significant decrease in cell viability was observed in the healthy group after treatment with compounds and NiO NPs alone compared with the untreated control. The results showed that cell viability significantly decreased in all breast tumor groups treated with the tested compounds and NPs either alone or in combination compared with the untreated control and healthy groups ($P< 0.05$, Fig. 2B). As depicted in Figure 2B, an effective inhibition in cell viability was observed in the treatment with NPs than compounds in breast tumors groups. In addition, the results showed a significantly higher cell viability reduction in the combined treatments than in the treatment with compounds and NPs alone ($P< 0.001$). Curcumin treatment induced a higher decrease in cell viability (58.36%±2.32) than other compounds in the breast tumor groups. Importantly, among all tested compounds in breast tumor groups, Cur-Fe2O3 performed the most inhibition potency in cell viability by 43.26%±1.42 with the respective IC_{50} values of 0.87 µg/mL and 92.42 µg/mL, respectively (Fig. 2B).

Determination of cell apoptosis by flow cytometry

To determine whether cell viability inhibition following treatment with IC_{50} concentrations of NPs and compounds was associated with apoptosis, healthy and breast tumor groups treated with the tested compounds were analyzed by flow cytometry. As depicted in Fig. 3(A), the healthygroups treated with NPs alone did not display any significant apoptosis. In contrast, the percentage of apoptotic

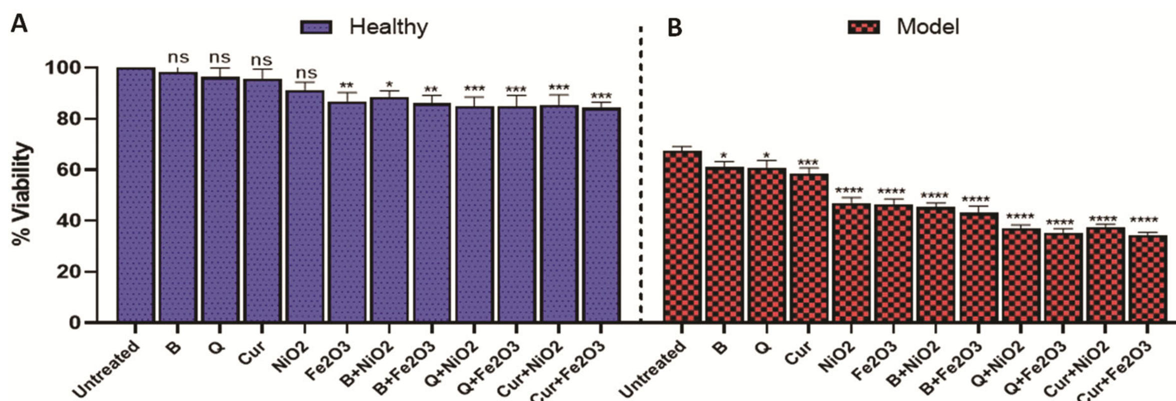

Fig. 2 — *In vivo* cytotoxicity studies on healthy (A) and breast tumor groups (Model) (B) studied by the MTT method. Data are presented as mean ± SD. *$P< 0.05$, **$P< 0.01$, ****$P< 0.001$. Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Untreated, no treatment with nanoparticles and or compounds; Fe2O3, iron superoxide nanoparticle; NiO, nickel oxide nanoparticle; Cur, curcumin; B, betanin; Q10, coenzyme Q10; B+NiO, Cur+NiO, Q+NiO, B+ Fe2O3, Cur+ Fe2O3, and Q+ Fe2O3, combined treatment of nanoparticles and compounds

cells in the other treated groups was significantly higher than that in the control group ($P< 0.05$). The results demonstrated that the percentage of apoptotic cells significantly increased in all tested compounds in the breast tumor groups compared with the control and healthy groups. In addition, the percentage of apoptosis increased more obviously in the combined treatment group than in those treated alone in breast tumor groups ($P< 0.01$, Fig. 3B). The potential to induce apoptosis in cells exposed to NPs and compounds either alone or in combination was further confirmed and quantified through annexin V-FITC/PI staining assays using flow cytometry (Fig. 4A-C). The representative dot plot in (Fig. 4D, G, and J) shows that treatment with compounds increased the population of early apoptosis (17.0%, 17.7%, and 18.8% for betanin, CoQ10, and curcumin, respectively) and late apoptosis cells (14.3%, 14.5%, and 17.6% for betanin, CoQ10, and curcumin, respectively) in breast tumor groups as compared to NPs treatment and control. Figure 4F and I show that the combination of NiO NPs with betanin (B+NiO) did not display any significant increase in apoptosis, whereas its combined treatment with other compounds (Q+NiO and Cur+NiO compounds) caused an increase in early apoptotic cell populations (17. 5% and 19.9%) and an apoptotic population (33.7% and 35.4%). Based on the Annexin V assay shown in (Fig. 4E, H, and K) combination treatment of Fe2O3 NPs and CoQ10 (Q-Fe2O3) could induce 40.2% cell death, in which 20.9% were early apoptotic (Annexin (+) PI (−)), 19.3% were late apoptotic (Annexin (+) PI (+)), 18.1% were necrotic (Annexin (−) PI (+)) in breast tumor groups.

Gene expression profile

The possible alterations in the expression of *Bax* (pro-apoptotic) and *Bcl2* (anti-apoptotic) as apoptotic genes were further measured using qRT-PCR (Fig. 5 and Fig. 6). As depicted in Figure 5, the mRNA expression level of the pro-apoptotic gene *Bax* was significantly upregulated in breast tumors after treatment with IC_{50} concentrations of all alone and combined compounds of NPs and compounds compared with control ($P< 0.001$). The highest mRNA expression level of the *Bax* gene was found after the combined treatment of NiO and curcumin (1.51fold) in healthy and CoQ10 alone (5.61fold) in breast tumor groups, respectively ($P< 0.05$ compared to control). Furthermore, the relative expression levels of *Bax* in the breast tumor groups were significantly elevated to 1.93 ($P< 0.001$), 2.6 ($P< 0.001$), and 1.99 ($P< 0.001$) fold, after treatment of Fe2O3 NPs with curcumin, betanin, and CoQ10 compounds, respectively, compared with Fe2O3 NPs. Similarly, the expression of *Bax* also increased to 2.07 ($P< 0.001$), 2.04 ($P< 0.001$), and 2.13 ($P< 0.001$) fold, following the treatment of NiO NPs with curcumin, betanin, and CoQ10 compounds, respectively, compared to NiO NPs. Our results suggest that combined treatments with NPs and compounds induce apoptosis via increased gene expression levels.

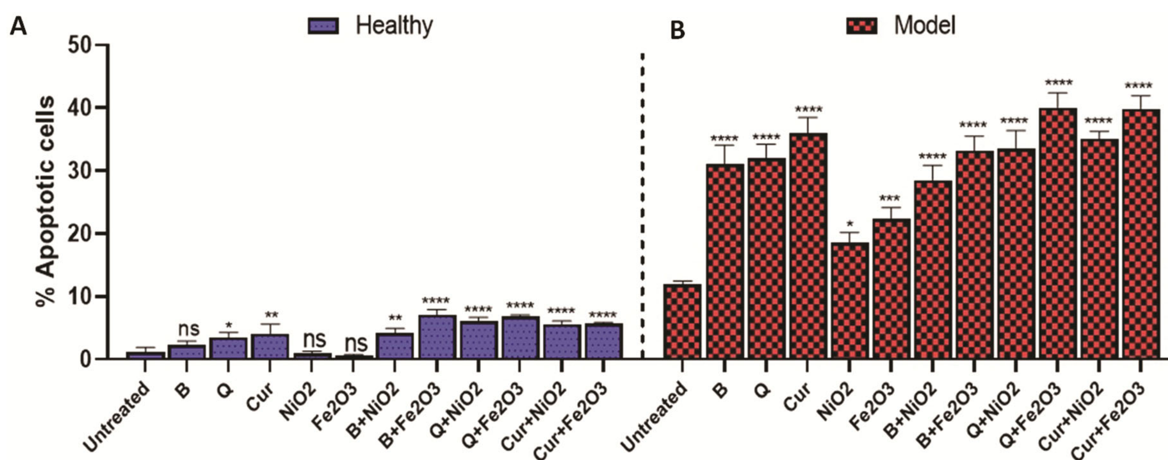

Fig. 3 — *In vivo* apoptosis studies on healthy (A) and breast tumor groups (Model); and (B) studied by flow cytometry. Data are presented as means ± SD. *$P< 0.05$, **$P< 0.01$, ****$P< 0.001$. Abbreviations: Untreated, no treatment with nanoparticles and or compounds; Fe2O3, iron superoxide nanoparticle; NiO, nickel oxide nanoparticle; Cur, curcumin; B, betanin; Q10, coenzyme Q10; B+NiO, Cur+NiO, Q+NiO, B+ Fe2O3, Cur+ Fe2O3, and Q+ Fe2O3, combined treatment of nanoparticles and compounds

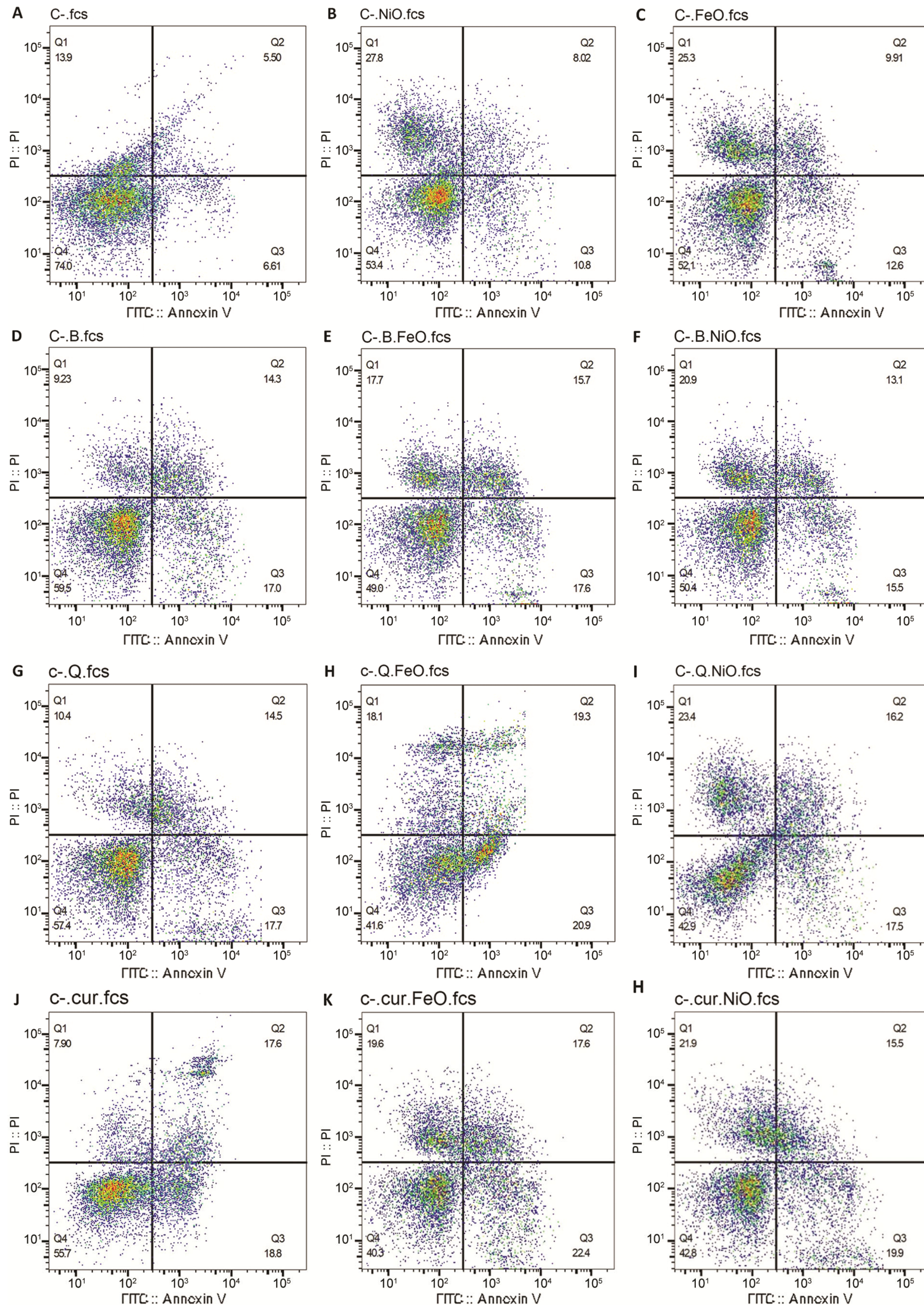

Fig. 4 — Flow cytometric analysis of apoptosis in breast tumor groups after staining with FITC-conjugated annexin V and PI. Abbreviations: C-, breast tumor group without treatment with nanoparticles and compounds; Fe2O3, iron superoxide nanoparticle; NiO, nickel oxide nanoparticle; Cur, curcumin; B, betanin; Q10, coenzyme Q10; B.NiO, Cur.NiO, Q.NiO, B.Fe2O3, Cur.Fe2O3, and Q. Fe2O3, combined treatment of nanoparticles and compounds

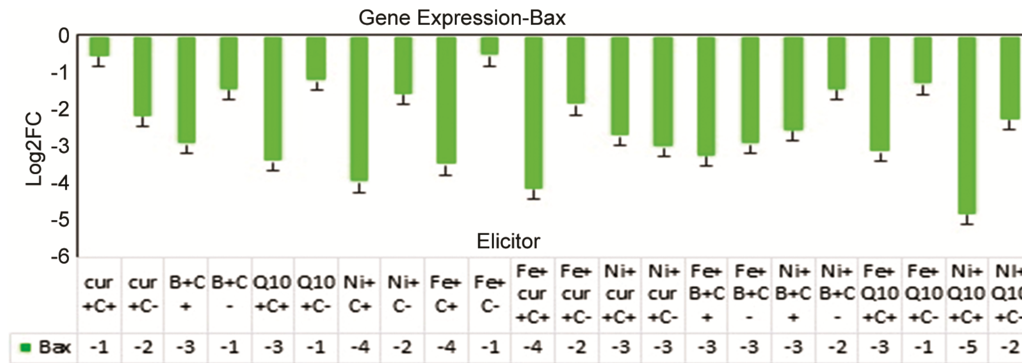

Fig. 5 — Expression level of *Bax* gene following the treatment with IC_{50} concentrations of alone and combined nanoparticles and compounds. Data are presented as means ± SD. Abbreviations: C+, Healthy group without treatment with nanoparticles and compounds C-, breast tumor group without treatment with nanoparticles and compounds; FeO, iron superoxide nanoparticle; NiO, nickel oxide nanoparticle; Cur, curcumin; B, betanin; Q10, coenzyme Q10; B.NiO, Cur.NiO, Q.NiO, B. Fe2O3, Cur. Fe2O3, and Q. Fe2O3, combined treatment of nanoparticles and compounds

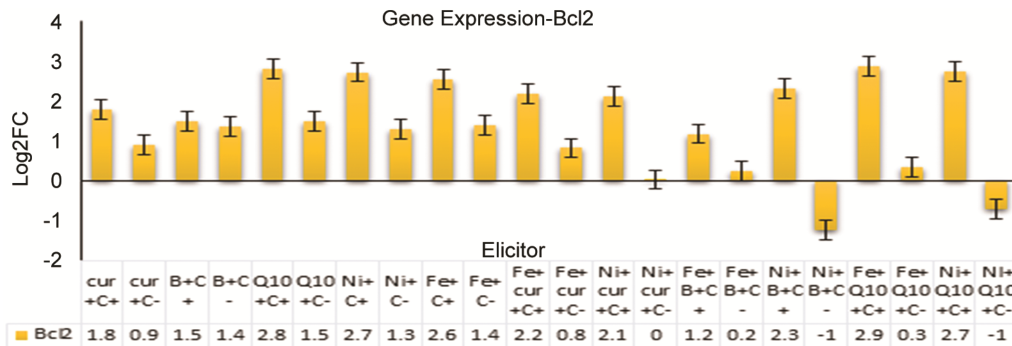

Fig. 6 — Expression level of *Bcl2* gene following the treatment with IC_{50} concentrations of alone and combined nanoparticles and compounds. Data are presented as means ± SD. Abbreviations: C+, Healthy group without treatment with nanoparticles and compounds C-, breast tumor group without treatment with nanoparticles and compounds; Fe, iron superoxide nanoparticle; Ni, nickel oxide nanoparticle; Cur, curcumin; B, betanin; Q10, coenzyme Q10; B.NiO, Cur.NiO, Q.NiO, B. Fe2O3, Cur. Fe2O3, and Q. Fe2O3, combined treatment of nanoparticles and compounds

In addition, the relative expression levels of *Bcl2* decreased after treatment with all compounds in both healthy and breast tumor groups compared with the control group ($P > 0.05$ for healthy groups) (shows in Fig. 6). The combined treatment with Fe2O3 and curcumin in the healthy group and curcumin alone in the breast tumor groups had the lowest *Bcl2* expression to 0.81 ($P > 0.05$) and 0.198 ($P < 0.001$) fold, respectively, compared to control. The relative expression levels of *Bcl2* were markedly downregulated after combined treatment with Fe2O3 NPs and compounds, which were 2.06, 2.86, and 2.34 folds for curcumin, betanin, and CoQ10 alone groups, respectively ($P < 0.001$). Combined treatment with NiO NPs also showed a similar expression pattern to Fe2O3 NPs; the relative expression levels of *Bcl2* markedly downregulated after combined treatment with NiO NPs and compounds, which was 1.18-fold that of the curcumin alone group, 2.14-fold that of the B-alone and 1.61-fold that of the CoQ10 alone ($P < 0.001$) (Fig. 6). The results showed that the combination treatment of NPs and compounds was more efficient than using NPs and compounds alone.

Our results showed that the treatment of NPs and the compounds in both alone and or combination treatment significantly reduced the expression level of the *TFAM* gene in breast tumor groups compared to control ($P < 0.001$). As shown in (Fig. 7), *TFAM* gene expression increased in all breast tumor groups treated either alone or in combination with NPs and compounds compared with the healthy group, which was statistically significant ($P = 0.0000$). The highest expression of the *TFAM* gene was related to the treatment of CoQ10 alone in both healthy (0.93-fold, not significant compared to control) and breast tumor groups (3.57-fold, $P < 0.01$ compared to

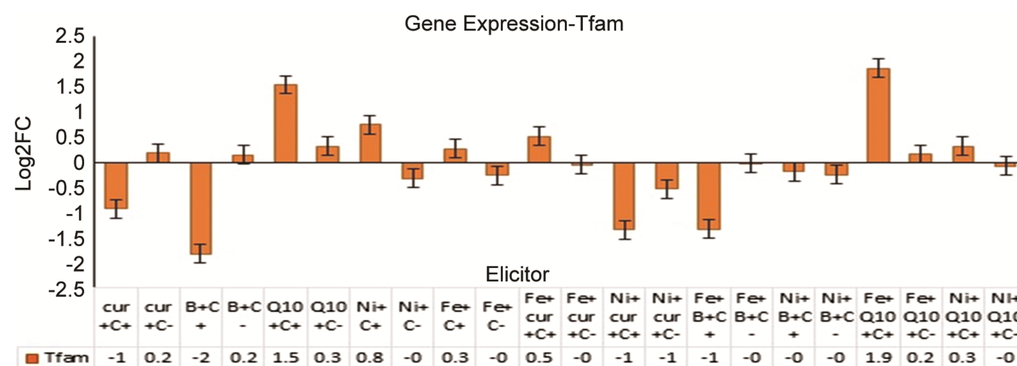

Fig. 7 — Expression level of *TFAM* gene following the treatment with IC_{50} concentrations of alone and combined nanoparticles and compounds. Data are presented as means ± SD. Abbreviations: C+, Healthy group without treatment with nanoparticles and compounds C-, breast tumor group without treatment with nanoparticles and compounds; Fe, iron superoxide nanoparticle; Ni, nickel oxide nanoparticle; Cur, curcumin; B, betanin; Q10, coenzyme Q10; B.NiO, Cur.NiO, Q.NiO, B.Fe2O3 Cur. Fe2O3, and Q. Fe2O3, combined treatment of nanoparticles and compounds

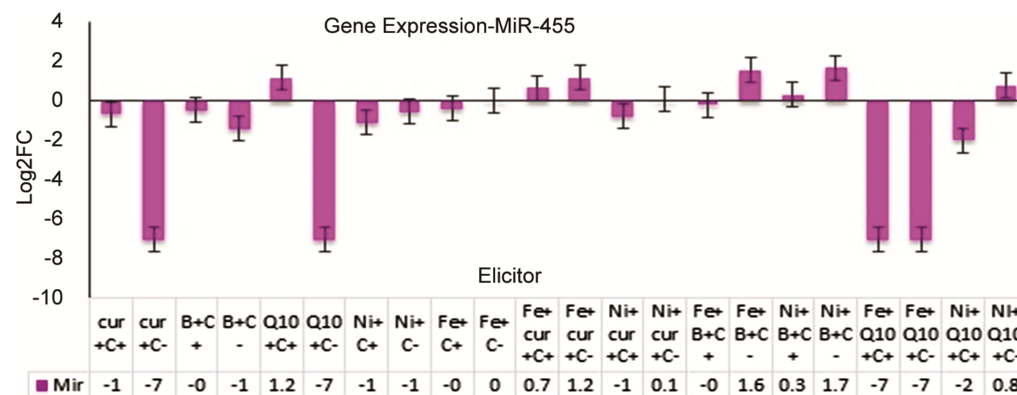

Fig. 8 — Expression level of MiR-455 following the treatment with IC_{50} concentrations of alone and combined nanoparticles and compounds. Data are presented as means ± SD. Abbreviations: C+, Healthy group without treatment with nanoparticles and compounds C-, breast tumor group without treatment with nanoparticles and compounds; Fe, iron superoxide nanoparticle; Ni, nickel oxide nanoparticle; Cur, curcumin; B, betanin; Q10, coenzyme Q10; B.NiO, Cur.NiO, Q.NiO, B.Fe2O3, Cur.Fe2O3, and Q.Fe2O3, combined treatment of nanoparticles and compounds

control). The relative expression levels of *TFAM* significantly decreased after combined treatment with Fe2O3 NPs and compounds, which were 2.77, 2.41, and 1.97 folds for curcumin, betanin, and CoQ10 alone groups, respectively (P=0.001) (Fig. 7). Similar to the expression pattern for Fe2O3 NPs, the relative expression levels of *TFAM* were markedly downregulated after combined treatment with NiO NPs and compounds, which was 3.72-fold that of the curcumin alone group, 2.23-fold that of the B-alone group, and 1.68-fold that of the Q10-alone (P=0.001) (Fig. 7).

As shown in Figure 8 HYPERLINK "https://www.dovepress.com/silver-nanoparticles-enhance-the-apoptotic-potential-of-gemcitabine-in-peer-reviewed-fulltext-article-IJN". HYPERLINK "https://www.dovepress.com/silver-nanoparticles-enhance-the-apoptotic-potential-of-gemcitabine-in-peer-reviewed-fulltext-article-IJN" HYPERLINK "https://www.dovepress.com/silver-nanoparticles-enhance-the-apoptotic-potential-of-gemcitabine-in-peer-reviewed-fulltext-article-IJN"8, treatments with NPs alone and combined treatment with NiO and betanin reduced the expression of MiR-455 in the breast cancer group compared with that in the control group, whereas other treatments resulted in increased expression (*P*> 0.05). The relative expression levels of MiR-455 in the breast tumor groups were significantly elevated to 1.67 (*P*< 0.05), 1.21 (*P*> 0.05), and 1.82 (*P*< 0.001) fold after combination treatment of Fe2O3 NPs with curcumin, betanin, and CoQ10 compounds, respectively, compared to Fe2O3 NPs. In this study, the combined treatment of NPs and compounds was associated with reduced expression of MiR-455

compared to independent compound treatments. In addition, the expression of MiR-455 was significantly lower in curcumin and Q10 than in betanin ($P < 0.05$) (Figure HYPERLINK "https://www.dovepress.com/silver-nanoparticles-enhance-the-apoptotic-potential-of-gemcitabine-in-peer-reviewed-fulltext-article-IJN". HYPERLINK "https://www.dovepress.com/silver-nanoparticles-enhance-the-apoptotic-potential-of-gemcitabine-in-peer-reviewed-fulltext-article-IJN"HYPERLINK "https://www.dovepress.com/silver-nanoparticles-enhance-the-apoptotic-potential-of-gemcitabine-in-peer-reviewed-fulltext-article-IJN"8). Our results showed that changes in tumor size /day the treatment combined groups were reduced more than compared to the single group.

Discussion

Applications of NPs and compounds as promising alternative therapies for cancer diagnosis and treatment have been drawing significant attention. However, most traditional chemically derived anticancer agents are limited because of cancer cell resistance to drugs and side effects on non-targeted tissues[24]. It has been demonstrated that naturally occurring compounds could potentially be used as therapeutic strategies in breast cancer. However, the efficacy of curcumin, betanin, and CoQ10 compounds in decreasing chemotherapy-induced toxicity and plausible adjuvants in radiotherapy or chemotherapy has not been thoroughly evaluated, and further investigation and randomized controlled clinical trials are needed to determine the optimal dose. Therefore, we evaluated the cytotoxicity of Fe2O3 and NiO NPs and curcumin, betanin, and CoQ10 compounds against 4T1 cells using the MTT assay. Our results showed a dose-dependent effect on 4T1 cells where cell viability progressively reduced at higher concentrations of NPs and compounds. Multiple lines of evidence suggest notable cytotoxicity and antitumor efficacy of curcumin against various types of cancers such as breast cancer[24,25], human skin melanoma (A385)[26], colon carcinoma (C-26) cells[27], and human prostate cancer (LNCa, C4-2B)[28]. This supports our observation that curcumin showed higher cytotoxicity; therefore, the viability of 4T1 cell lines decreased by 50% following 48 h of incubation at a concentration of 0.87 μg/mL. Moreover, our results also demonstrated that the cytotoxic potential of NiO was higher than Fe2O3 NPs. This finding was in line with that Pandey *et al*[29] which demonstrated lower cytotoxic activity Fe2O3 NPs than NiO NPs on the lung cancer cell line A549. To study the anti-tumor and apoptosis effects of NPs and compounds *in vivo* and whether the combined treatment increased the cytotoxicity of tested compounds, we further treated healthy and tumor-carrying mice with IC_{50} concentrations of alone and combined compounds. In the present study, the combination of NPs and compounds significantly inhibited cell viability in breast tumor groups, which may be associated with the synergistic effect of NPs in increasing ROS production ($P < 0.01$). Similarly, the results of flow cytometric analysis clearly showed a greater extent of apoptotic cell death in the breast tumor groups with combined compounds. Successful induction of apoptosis using novel therapeutic agents is suggested to be an efficient strategy for cancer treatment. Alteration in the regulation of pro-apoptotic *Bax* and anti-apoptotic *Bcl2* family genes result in increased apoptosis and increased efficiency of therapeutics[30].

Our results indicated that treatment with all of the studied NPs and compounds stimulates cell death *via* intrinsic apoptosis as there is an upregulation in the expression of *Bax* and downregulation in the expression of *Bcl2*. In the present study, CoQ10 had the most significant additive effect on *Bax* gene expression compared with other independent and combined compounds, which was consistent with the CoQ10 antitumor activity through apoptotic properties and induction of oxidative stress in cancer cells reported by others[31,32]. Reduction in the expression of *Bcl2* in the curcumin alone group was the greatest compared with the other compounds, which contributed to the cytotoxicity, induction of oxidative stress, and anticancer effect of curcumin (2.87-fold, $P < 0.001$ compared to control). Numerous studies have proposed that curcumin prevents the carcinogenesis process in several types of cancer through alteration in the expression of apoptosis-associated genes such as survivin (a member of the inhibitor of apoptosis (IAP)), vascular cell adhesion molecule 1 (VCAM-1), cyclin D1 (CCND1), *Bax*, *Bcl2*, p53 inflammatory molecules NF-κB, vascular endothelial growth factor (VGEF) or cyclooxygenase-2 (COX-2), matrix metalloproteinase-2 (MMP-2) and MMP-9, inflammatory cytokines chemokine ligand 1 (CXCL1) and chemokine ligand 2 (CXCL2)31,32. Tabatabaei Mirakabad*et al*[33] showed that curcumin-loaded PLGA-PEG (polylactide-co-glycolide-polyethylene glycol) has more cytotoxic effects than

free curcumin on the MCF-7 breast cancer cell line. Yallapu*et al*[34] showed that curcumin encapsulated in poly(lactic-co-glycolide) (PLGA) (biodegradable polymer) NPs exhibited six-fold increase in the cellular uptake performed in metastatic MDA-MB-231 breast cancer cells compared with free curcumin. In another study, curcumin was encapsulated into protamine and dextran and then functionalized by folic acid-conjugated chitosan (CUR-LbL NP). The percentage of apoptotic cells significantly increased in MCF-7 cells treated with CUR-LbL NP (70 ± 1.2%) compared with untreated cells. In addition, encapsulated curcumin in carboxymethyl cellulose (CMC-CUR) coated with bovine serum albumin enhances the stability of curcumin, leading to marked increases in total cell death in Huh-7 (Human Liver carcinoma cell line) and HepG2 (human liver cancer cell line) compared with control untreated cells[35].

Mitochondrial transcription factor A (mtTFA, mtTF1, *TFAM*) promotes the transcription and replication of mitochondrial DNA (mtDNA) and is crucial in the functional integration of the mitochondrial respiratory chain and in maintaining a balance between compounds and oxidation[36]. *TFAM* mRNA and protein expression has been proposed to be positive in patients with breast cancer compared with adjacent normal tissue in patients and cell lines (MCF-7, MDA-MB-231, and MDA-MB-453[37,38]. However, the pattern of *TFAM* expression in breast cancer and adjacent normal tissues, and their clinical significance, has not yet been well explored. For example, the results of Gao *et al*.'s[39,9] study showed that Nuclear Respiratory Factor 1 (Nrf1) and *TFAM* protein expression increased in breast cancer patients. In another study, Balliet *et al*. used an *in vivo* mouse xenograft model to evaluate the tumor-promoting properties of fibroblasts without *TFAM*. According to their findings, *TFAM*-free fibroblasts increased breast tumor growth *in vivo* without increasing tumor angiogenesis[40].

Therefore, evaluation of *TFAM* expression in breast cancer and adjacent normal tissues can be effective in identifying new targets for cancer treatment, preventing invasive cancer formation, and improving the therapeutic efficacy and prognosis of breast cancer patients. Therefore, in the present study, for the first time, *TFAM* gene expression was investigated before and after treatment with NPs alone and combined with NPs and compounds in healthy and breast tumor mice. Our results indicated that alone and combined treatments with NPs (Fe2O3 and NiO) and compounds (curcumin, betanin, and CoQ10) reduced *TFAM* gene expression in cancer cells, which can be attributed to ROS overproduction and enhanced oxidative stress. Downregulation of *TFAM* after treatment in cancer cells can be attributed to the overproduction of ROS and mitochondrial dysfunction, which lead to increased oxidative stress and cell apoptosis. Therefore, the results of this study were in line with reports of increased apoptosis by reducing or suppressing *TFAM* expression. In this study, the combination of curcumin and NiO NPs had the most significant effect on *TFAM* down-expression compared with other compounds in breast tumor groups. Thus, the enhanced apoptotic properties of curcumin in combination with NiO NPs may increase oxidative stress and apoptosis by reducing *TFAM* gene expression. However, further studies on protein levels are required to confirm these results. Consistent with our results, decreased expression of the *TFAM* gene after curcumin treatment was observed by Feizolahi *et al*[41].

Epigenetic factors play a significant role in cancer initiation and development. MicroRNAs (miRNAs) are endogenous, short non-coding RNAs that have 19-22 nucleotides and can affect vital biological mechanisms in cells, such as proliferation, migration, and differentiation. MiRNAs can bind to 3′-UTR for gene expression reduction. Cancer development occurs as a result of aberrant expression of miRNAs and regulating their expression level is important for effective cancer therapy[42,43]. MiR-455 is a tumor-associated miRNA gene located in the fragile region of chromosome 9q32 and is abnormally expressed in several tumors, including breast cancer, non-small cell lung cancer, and gastric cancer[37]. Aili *et al*. showed that MiR-455-5p expression significantly increased in breast cancer tissues and is considered an independent prognostic factor for poor survival in breast cancer patients[44]. *In vitro* experiments of MiR-455-5p overexpression revealed accelerated breast cancer cell invasion and rapid migration. In addition, Zeng *et al*[45] found that MiR-455-3p plays an important role in inhibiting EMT and transforming growth factor-beta (TGF-β) signaling pathway and maintaining cell differentiation and could be a promising therapeutic intervention for breast cancer. Our results showed that the expression level of MiR-455 decreased in all control and treated breast cancer groups compared with that in the healthy group. In

addition, treatment with NPs alone and combined treatment with NiO NPs and betanin decreased the expression of MiR-455 in breast tumors compared with the control. In contrast, the expression of MiR-455 increased in the other treatments.

Conclusion

This study highlighted that monotherapy for breast cancer is not sufficient for its treatment. Therefore, it is vital to utilize combination therapies for this malignant condition. The combination of curcumin, betanin and CoQ10 with Fe2O3 and NiO NPs shows a synergistic impact, as apoptosis induction is boosted by a combination of antitumor agents and NPs, and a higher regulatory impact on gene expression occurs compared to monotherapy. Thus, this experiment revealed that natural anti-tumor agents and NPs could be promising candidates for breast cancer therapy. Apoptosis induced by the tested compounds through the upregulation of *Bax* and downregulation of *Bcl2*. Our study confirmed that combined treatment with NPs and compounds has more cytotoxic and apoptotic effects on breast cancer and could be exploited as a potential source for developing novel drugs against breast cancer. We demonstrated that treatment with NPs alone and the combined treatment of NiO NPs with betanin led to apoptosis induction and metastasis prevention by reducing the expression of MiR-455. This study suggests that the tested compounds could be considered as new therapeutic targets for inducing apoptosis in breast cancer. However more studies are required to determine more precise molecular mechanisms.

Conflicts of interest

All authors declare no conflict of interest.

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