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Original Article

The Synergistic Combination of Cisplatin and Piperine Induces Apoptosis in MCF-7 Cell Line

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Abstract

Background: Piperine is a natural compound obtained from the *Piper nigrum* that exhibits anti-proliferative and anti-cancer activity in cancer cell lines. We analyzed the cytotoxic effect of piperine combined with cisplatin compound in the human MCF-7 breast cancer cell line and the underlying mechanism.

Methods: The present in vitro study was performed on MCF-7 cell line in Jahrom University of Medical Sciences between, Jahrom, Iran from 2016 to 2017. Cultured MCF-7 cells were seeded into four groups: a control group (untreated group), a group treated with cisplatin, a group treated with piperine and a group treated with cisplatin and piperine. Cell viability was analyzed using the MTT assay method. Flow c-ytometric analysis was investigated for apoptosis. The mRNA and protein expression of the apoptotic regulators p53, Bcl-2, Bax, caspase 3 and caspase 9 were detected by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting analysis.

Results: Piperine (20 and 30 μM) in combination with cisplatin (5, 10 and 15 μM) for 24 h synergistically inhibited cell viability of MCF-7 breast cancer cells more than piperine and cisplatin used alone. Synergistic anti-breast cancer activities cisplatin (5 μM) and piperine (20 μM) were via inducing apoptosis. Piperine (20 μM) and cisplatin (5 μM) for 24 h induce apoptosis strongly through reduction of Bcl-2 and increase of caspase 3, p53, caspase 9, and Bax.

Conclusion: Piperine in combination with cisplatin could trigger p53-mediated apoptosis more effective than cisplatin alone in MCF-7 breast cancer cells, reducing the toxic dose of cisplatin used in cancer chemotherapy.

Keywords: Apoptosis; Breast cancer; Caspase; Cisplatin; Piperine

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Introduction

Breast cancer is one of the most prevalent malignancy reasons for death among women in all parts of the world. This malignancy is a global health problem due to its poor response to the treatment and increasing metastasis rate to other tissues (1). Conventional systems for cancer therapy such as chemotherapy have been currently employed for treating malignant breast cancer. Platinum drugs such as cisplatin (also called **cis**-diammine-dichloro-platinum (II)) and its analogs are the most effective anti-tumor compounds which routinely used in clinics for the different types of human solid tumors remedy via multiple mechanisms (2, 3). The anti-tumor activity of cisplatin is mediated by its ability to DNA damage in tumor cells and interfering with its repair mechanism (4). Cisplatin-induced DNA damage triggers various signal transduction pathways that can induce the apoptosis mechanism in cancer cells (4, 5). Despite cisplatin's initial therapeutic success, its use can typically limit due to its toxicity in normal cells and resistance of the tumor cells to cisplatin-based chemotherapy, which leads to therapeutic failure (6). The major toxicities associated with cisplatin overdose include kidney damage (nephrotoxicity), neuropathy, bone marrow suppression and hearing problems (ototoxicity) (7). The ability to manage these side effects is a major challenge to the clinical use of cisplatin in cancer medicine. Therefore, there is a highly urgent need for searching and discovering a new effective and less cytotoxic treatment system to inhibit and treatment of patients with breast cancer (8).

Today, many types of plant-derived compounds have been discovered as important resources of new drugs with the anti-tumor activity's potential, which may decrease adverse side effects of chemotherapeutic drugs (9-11). Piperine, the major phenolic product isolated from *Piper nigrum* and *Piper longum*, has been used as a food additive and traditional medicine for many centuries in Asian countries (12-14). Recently, piperine's potential cytotoxic and anti-tumorigenic properties in against several types of cancer cells have been reported (15). In recent years, numerous experiments in vitro and vivo have reported that the anti-tumor activity of piperine may partly be due to its effects on signaling pathways involved in apoptosis of cancer cells (15-18).

Apoptosis can occur by the intrinsic apoptosis pathway (also called the mitochondrial pathway) and the extrinsic apoptosis pathway. Anti-cancer drugs predominately initiate apoptosis via the mitochondrial pathway. The mitochondrial pathway is triggered by numerous intracellular events, regulated by a balance of anti-apoptotic (Bcl-2) and pro-apoptotic (Bax) molecules (19). Besides, the combination of piperine with chemotherapeutic drugs enhances their effects against various cancers in vitro and in animal models Han et al. provided some evidence that piperine in combination with mitomycin-C significantly induced apoptosis in drug-resistant cervical cancer cells by Bcl-2/Bax and STAT3/NF-κB signaling pathways (20, 21). However, no reports show the potential cytotoxic effects of piperine in combination with cisplatin in the MCF-7 cell line of breast cancer.

In this paper, we analyzed the synergistic effect of cisplatin combined with piperine on cell viability and induction of apoptosis in MCF-7 cell line of breast cancer.

Materials and Methods

Materials

Piperine (>95% purity), cisplatin, dimethyl sulfoxide (DMSO), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), and Horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Sigma (St. Louis, MO, USA). Antibiotics (penicillin and streptomycin), fetal bovine serum (FBS) and Dulbecco Modified Eagle Medium (DMEM) were purchased from Invitrogen (Grand Island, NY, U.S.A.). Primary antibodies for anti-Bcl-2, anti-p53, and anti-Bax were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A).

Cell culture

The present in vitro study was performed on human breast cancer cells, MCF-7 cell line, in Jahrom University of Medical Sciences between, Jahrom, Iran from 2016 to 2017. MCF-7 cells were purchased from the Pasteur Institute (Tehran, Iran). Cells were maintained in Dulbecco's Modified Eagle's Medium enriched with 10% heat-inactivated fetal bovine serum and penicillin (100 units/ml)/streptomycin (100 µg/ml). The cultured cells were maintained in a humidified 37˚C incubator containing 5% CO2. Before each experimentation, cells starved with DMEM containing 0.5% FBS for 24 h.

Cell treatment

For this experimental manuscript, First, MCF-7 cells were incubated with different concentrations of piperine (0-100 µM), cisplatin (0-50 µM), the combination of cisplatin (5, 10 and 15 µM) with piperine (20 and 30 µM) for 24 h and then the viability of MCF-7 cells was detected by MTT assay. For next experimentation, MCF-7 cells were seeded into four groups: 1) a control group (untreated group), 2) a group treated with cisplatin (5 µM), 3) a groups treated with piperine (20 µM) and 4) a group treated with cisplatin (5 µM) plus piperine (20 µM) for 24 h and then cell apoptosis, Bcl-2, caspase 3, p53, caspase 9, Bax mRNA and protein expression was investigated.

Cell viability by MTT assay

The effects of piperine, cisplatin, and their combination on MCF-7 cells' viability were estimated using MTT assay. In brief, MCF-7 cells were seeded into 96 well plates at a density of 10^4 cells/well and maintain to the confluence at 37º. After 24 h of treatment with various concentrations of piperine (1-100 µM), cisplatin (1-50 µM) and their combination to induce cytotoxicity, MTT solution (5 mg/ml in PBS) was added and incubated for 4 h at 37 ºC. Then, the MTT solution was eliminated and the formazan crystals were dissolved with DMSO (100 µl), added to each well. A microplate reader (BioTek® ELx800, USA) was applied to measure the walls' optical density (OD) at 540 nm.

The combination index (CI)

The combined agents' synergistic effect was calculated using Compusyn software program based on the Chou–Talalay method. The types of combinations between piperine and cisplatin concentration were defined by the combination index (CI). CI>1, CI=1 and CI<1 indicated antagonistic effects, additive effect and synergistic effects (22). A synergistic dose of 20 µm of piperine and 5 µM cisplatin was used for all experiments.

Flow cytometry assay and apoptosis analysis

Apoptosis was evaluated by flow cytometric assay using propidium iodide (PI) staining and Annexin V-FITC according to Apoptosis Detection kit (Invitrogen, U.S.A) based on producer's protocol. Briefly, treated MCF-7 cells with piperine (20 µM), cisplatin (5 µM) and their combination for 24 h were collected, centrifuged, washed, and then were incubated with Annexin V-FITC and PI dye for 10 min in the dark at room temperature. Finally, the difference between viable cells and apoptotic cells was analyzed by flow cytometry.

Quantitative RT-PCR (qRT-PCR)

To distinguish the mRNA expression of apoptotic and anti-apoptotic markers, total RNA was extracted from the MCF-7 cells after treatment with piperine (20 µM), cisplatin (5 µM) and their combination for 24 h, using Trizol reagent (Invitrogen) based on the producer's protocol and total RNA (1 µg) was used for cDNA synthesis applying superscript II reverse transcriptase kit (Takara, Japan) according to the standard producer's protocol. Gene expression was amplified via the quantitative real-time PCR using SYBR Green PCR Master Mix (Takara, Japan) and specific forward (F) and reverse (R) primers using Applied Biosystem 7500 Fast Real-Time PCR System (USA). The specific primers used for detection of mRNA levels of p53, Bax, Bcl-2, caspase-3, caspase-9, and β-actin were as follows: P53: forward 5'-GGACCTCTTAACCTGTGGCT-3'; re-

verse 5'-AAAGCTGTTCCGTCCCAGTA-3', Bax: forward 5'-ACAGAGGGCATGGAGAGAGA-3'; reverse 5'-CTGAGAGCAGGGATGTAGCC-3', Bcl-2: forward 5'-CGGCTGAAGTCTCCATTAGC-3'; reverse 5'-CCAGGGAAGTTCTGGTGTGT-3', Caspase-3: forward 5'-CTGTGTGCAGGCTTTTGTGT-3'; reverse 5'-CTCAATCACTGCTCGTGGAA-3', Caspase-9: forward 5'-CGCCACTCTCTCATTCACAA-3', and reverse 5'-TCAAGGCAGCCTGTTCTTTT-3', β-actin: forward 5'-AGCCATGTACGTAGCCATCC-3'; and reverse 5'-CTCTCAGCTGTGGTGGTGAA-3'. The mRNA level was normalized using the housekeeping gene (β-actin) and fold-changes were derived using the comparative CT method (23).

Western blot analysis

Briefly, MCF-7 cells treated with cisplatin (5 μM), piperine (20 μM) and their combination for 24 h, cells were collected and then lysed in lysis buffer. The protein concentrations of the lysates were quantified by the Bradford assay method (24). Proteins (50 μg/well) were separated by 10% SDS-polyacrylamide gel electrophoresis, then (electroblotted) transferred onto a polyvinylidene difluoride (PVDF) membrane. The immunoblot was blocked with 5% nonfat milk as a blocking buffer at room temperature for 1 hour. After blocking, the membrane was incubated with a 1:1000 dilution of specific primary antibodies against p-p53, Bax, Bcl-2 and β-actin at 4 °C for overnight, followed by horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 dilution) at room temperature for 1 h and then enhanced chemiluminescence (ECL) detection kit. Densitometry analysis of the blots was performed by the image J software (Bio-Rad).

Statistical analysis

All experimental results were presented as the mean±standard error of the mean (SEM) of triplicate experiments. Statistical significances between groups were evaluated by one-way analysis of variance (one-way ANOVA). Probability values ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$) were considered to indicate a statistically significant difference.

Results

Co-treatment of piperine and cisplatin exerts cytotoxicity effect in MCF-7 breast cancer cells by a synergistic effect

The MTT results in Fig. 1A and Fig. 1B showed that treatment with piperine and cisplatin resulted in decreased cell viability compared to the untreated control ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$) in MCF-7 cell line in a dose-dependent manner. As shown in Fig. 1C, the combination treatment of piperine (20 and 30 μM) with cisplatin (5, 10 and 15 μM) had a higher inhibitory effect on the viability of cultured breast cancer cells of MCF-7 than the individual drugs treatment. Therefore, the piperine concentrations (20 μM) and cisplatin (5 μM) were used for further experiments. The combination index was calculated as 0.45 for MCF-7 cells (Fig. 2).

Co-treatment of piperine and cisplatin prompts apoptosis in breast cancer cells

Results from flow cytometric assay indicated a significant increase in the numbers of early and late apoptotic cells in treatment groups compared with the control (Fig. 2). The early apoptosis rate was 13.9% in the MCF-7 cells exposed with piperine, 14.1% in the MCF-7 cells exposed with cisplatin and 16.2% in the MCF-7 cells exposed with cisplatin and piperine. The late apoptosis rate was 11.2% in the MCF-7 cells exposed with piperine, 19.8% in the MCF-7 cells exposed with cisplatin and 39.8% in the MCF-7 cells exposed with cisplatin and piperine.

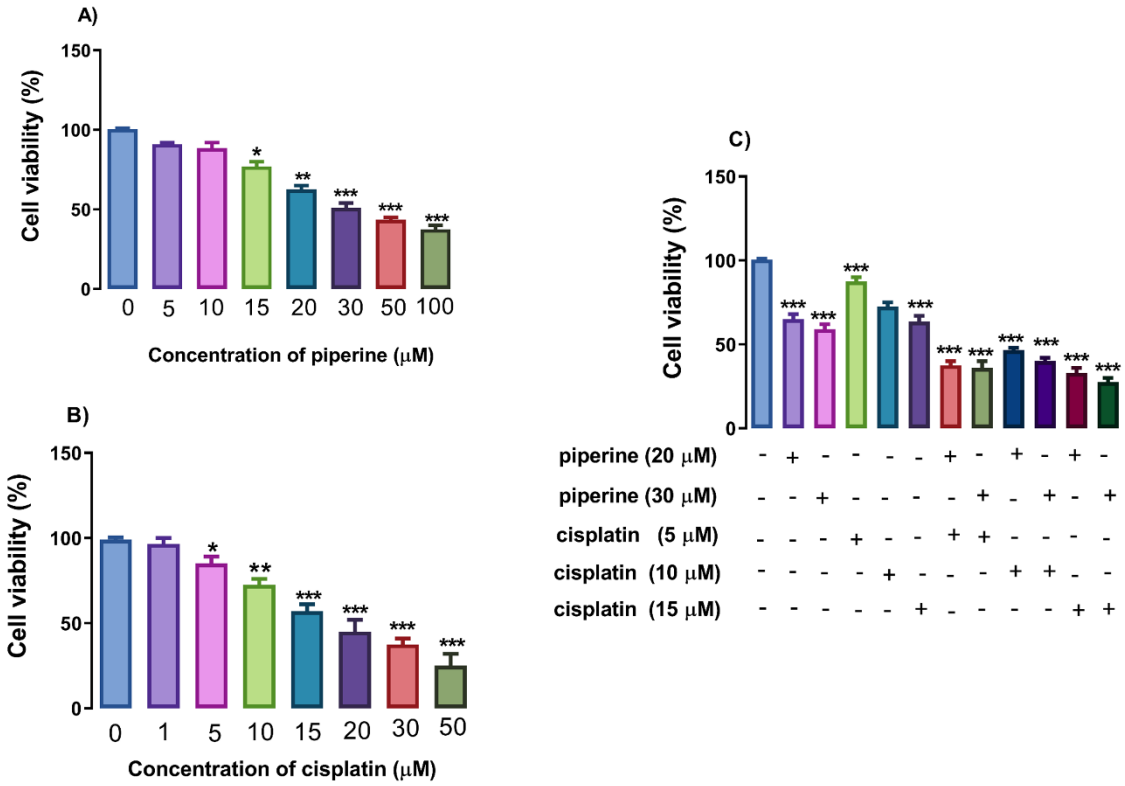

Fig. 1: Inhibition effect of (A) piperine, (B) cisplatin, and (C) their combination on cell viability of human Breast cancer MCF-7 cells. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with the untreated group

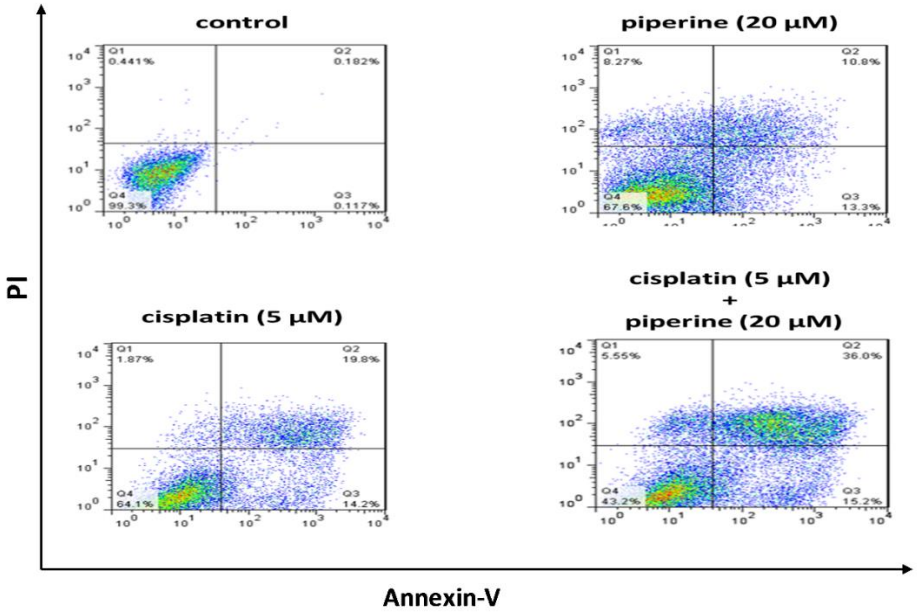

Fig. 2: The effect of piperine, cisplatin and their combination on apoptosis of human breast cancer MCF-7 cells**.** The image of flow cytometric analysis indicating necrotic cells (Q1), late apoptotic cells (Q2), early apoptotic cells (Q3) and viable cells (Q4)

Effect of piperine and cisplatin combination on p53, Bax and Bcl-2 protein expression in MCF-7 cells

Our study's western blot results demonstrated that the following combination treatment of piperine and cisplatin for 24 h, p53 protein expression was significantly augmented compared with single groups ($P<0.01$; Fig. 3A). Piperine (20 μM) and cisplatin (5 μM) increased the expression levels of Bax compared to the group of control ($P<0.05$; Fig. 3B). However, piperine in combined with cisplatin elevated the protein expression of Bax more than the two compounds alone (Fig. 3B). The Bcl-2 expression was decreased in the cells treated with piperine and cisplatin compared with these levels in the cells treated with cisplatin (5 μM) or piperine (20 μM) alone (Fig. 3C).

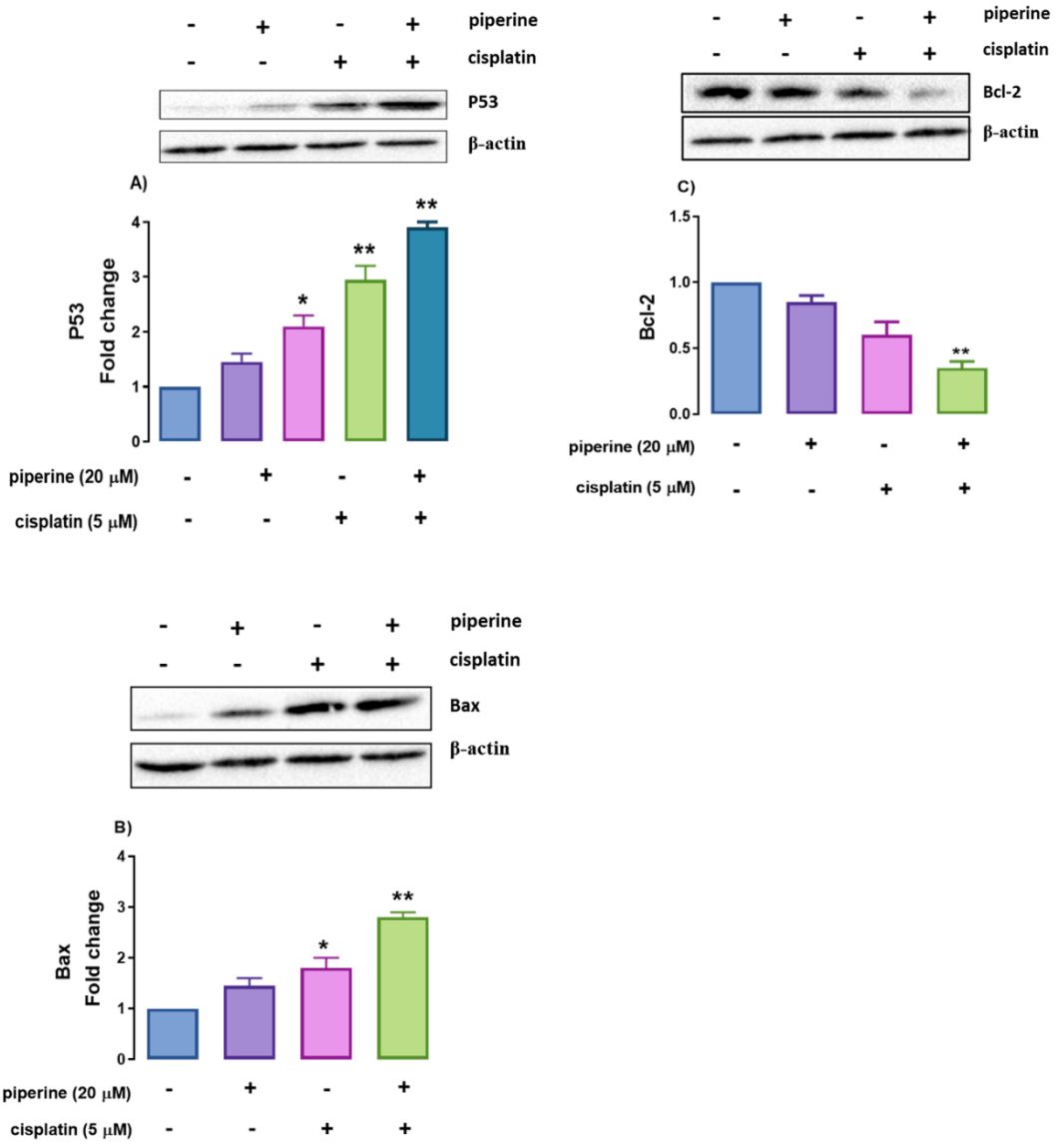

Fig. 3: The effect of piperine, cisplatin and their combined treatment on (A) P53, (B) Bax, and (C) Bcl-2 protein expression. $^{*}P<0.05$ and $^{**}P<0.01$ compared with the control group

Effect of piperine and cisplatin on the caspase-9 and caspase-3 expression

The expression levels of caspase-9 and caspase-3 in cells of MCF-7 were analyzed by qRT-PCR analysis. Piperine and cisplatin increased the caspase-9 and caspase-3 levels compared to the control group (Fig. 4A and Fig. 4B). Piperine, combined with cisplatin, increased the caspase-9 and caspase-3 expression more than these compounds alone (P<0.01).

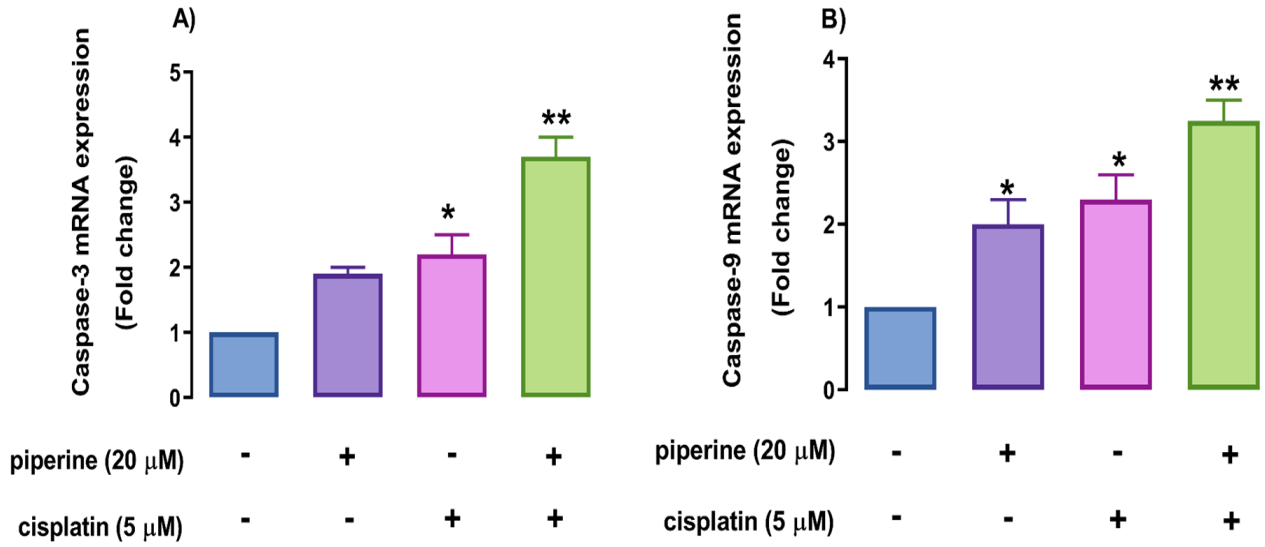

Fig. 4: The mRNA expression of **A)** Caspase-3 and **B)** Caspase-9 after treatment of piperine, cisplatin, and their combination were analyzed with qRT-PCR. $^{*}P$<0.05 and $^{**}P$<0.01 compared to the control group

Discussion

In the current research, the synergistic cytotoxic effects of piperine and cisplatin in breast cancer cells were shown in comparison to these compounds alone and analyzed the underlining mechanisms involved in the viability inhibition of breast cancer cells of MCF-7.

As a potent chemotherapeutic compound, cisplatin is widely used for the treatment of numerous human malignancies, such as breast cancer (25-28). Cisplatin, depending on cell type and concentration, kills tumor cells by DNA damage and interference with DNA repair mechanisms (7, 25). Despite the positive effects of cisplatin against breast cancer therapy, a major problem in cisplatin chemotherapy is cancer cells' resistance to cisplatin and its toxic effects on normal cells such as renal toxicity that limit the dose used (2, 29).

Thus, finding a new strategy to minimize the toxic effects of cisplatin and other anti-cancer drugs is necessary against breast cancer. Various natural products in combinational with synthetic anti-cancer drugs such as cisplatin are a good candidate for cancer therapy, due to enhance the efficacy and decrease side effects of chemotherapeutic drugs (30, 31).

Piperine, a bioactive compound of black pepper, *is* traditional medicine and has been reported to have anti-proliferative activity and anti-neoplastic properties against human cancer cells including ovarian cancer with fewer side effects (32, 33). In vitro and animal models demonstrated that piperine induces toxicity, apoptosis and cell cycle arrest in various tumor cells (34). Piperine combined with anti-cancer drugs induced more cytotoxicity in human cancer cells (20, 35, 36). Here, we explored the cytotoxic effects of piperine combined with cisplatin and the mechanisms of their effects

in breast cancer cells of MCF-7. Our study's MTT results showed that piperine and cisplatin inhibited cell viability in breast cancer cells of MCF-7. The combination of piperine with cisplatin more decreased MCF-7 cell viability in comparison to piperine or cisplatin alone. Thus, we investigate the biochemical mechanisms triggered by piperine and cisplatin in tumor cells. Piperine damages cancer cells via induction of apoptosis (32, 37). In flow cytometry analysis, the apoptosis rate of cancer cells was detected in cells treated with cisplatin, piperine and their combination. Cell apoptosis was observed in less dosage of piperine and cisplatin combination compared with piperine or cisplatin alone.

Dysregulation in the mitochondrial apoptotic pathways is strongly associated with developing tumors and diverse cancer cells' resistance to cytotoxic medications (38, 39). P53, as a central tumor suppressor protein, plays an important regulatory role in cell death through apoptosis induction. Piperine induced apoptosis in A549 cells, correlated with the elevated expression of p53 (16, 40). In our study, western blotting analysis revealed that p53 protein was increased after treatment with piperine or cisplatin, however, p53 expression was significantly more increased after co-treatment piperine with cisplatin in compared with piperine or cisplatin. Induction of apoptosis in cells involves numerous effectors that act through intrinsic or extrinsic apoptotic pathways. The genes encoding protein family of Bcl-2, which contains anti-apoptotic and pro-apoptotic proteins, are central regulators of the p53-dependent apoptotic pathway. In response to the activation of p53, the increasing Bax (pro-apoptotic effector)/ Bcl-2 (anti-apoptotic protein) ratio is an important key factor to enhance the sensitivity of cells to the triggering of process of apoptosis, associated with the permeabilization of the outer mitochondrial membrane, release of cytochrome **c** from mitochondrial into the cytoplasm and activation of caspases to induce cell death (38). In response to the treatment of cancer cells with anti-cancer drugs, there is an up-regulation in the ratio of Bax/Bcl-2 due to the decrease in expression of Bcl-2 or increase in expression of Bax (41, 42). Our findings of western blot analysis and qRT-PCR demonstrated that after MCF-7 treatment with piperine or cisplatin, the expression levels of Bax increased, but the Bcl-2 expression was significantly reduced, that leads to a high Bax/Bcl-2 ratio. Our results are in agreement with the previous study in other cancer cells (35, 41).

Bax protein expression is correlated with caspases activity that are the essential effectors of apoptosis signaling (43). In the current research, piperine and cisplatin co-treatment significantly increased the levels of caspase-3 and caspase-9 more than piperine or cisplatin alone in MCF-7 breast cancer cells, indicating that the apoptotic signal in MCF-7 cells induced by piperine and cisplatin can be due to the activation of the mitochondrial apoptotic pathways.

Conclusion

The combination of cisplatin with naturally extracted piperine has synergistic effects and more effective in inhibiting cell viability in the MCF7 breast cancer cell line than cisplatin or piperine alone. The synergistic effects of two compounds on inhibiting of cell viability have exerted through the apoptosis induction in cancer cells. Piperine combined with cisplatin may be used as a more effective, powerful and less dosage of anticancer agent for cancer therapy.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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