J Human Gen Genom. 2023 May; 7(1): A-10-371-1 doi: 10.61186/jhgg.7.1.97
Published online 2024 February 30 **Research Article**

Piperine has anti-tumor effects in breast cancer by inducing mitochondrial apoptosis pathway

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Received 2023 October 26; **Accepted** 2024 February 29

Abstract

Background: Breast cancer (BC) is the second leading cause of death due to cancer among women worldwide. Therefore, the present study investigates the cytotoxic effects of piperine on the breast cancer cell line (MCF7) and the genes of the apoptotic pathway.
Objectives: This research was performed to assess the effect of piperine on BC cells and the change in the expression level of *bax* gene through the induction of apoptosis.
Methods: MCF-7 cells were prepared by the Pasteur Institute, Iran. Cytotoxicity of piperine at concentrations of (5, 10, 15, 20, and 25 µM) during 24, 48, and 72 hours was evaluated by MTT assay. The cell apoptosis and *bax* gene expression were evaluated by Flow Cytometry and qReal-time PCR, respectively. Finally, the statistical analysis of MTT and RT- PCR data was done by SPSS software version 22.
Results: The piperine showed concentration-dependent cytotoxic effects on the MCF-7 cell line in MTT assay. The *bax* gene expression level has a significant increase in piperine-treated cells compared to the untreated ones. The MCF-7 cell apoptosis at IC50 concentration of piperine was measured at 58.3% during 48-h treatment.
Conclusions: In general, it can be concluded that piperine has cytotoxic effects against breast cancer by inducing apoptosis via overexpressing of *bax*.

Keywords: Apoptosis, Breast cancer, Flow Cytometry, MTT assay, Piperine

1. Background

Breast cancer (BC) is the most common cancer in females worldwide and causes 25% of all female malignancies with higher prevalence in developed countries [1,2]. Also, BC needs special attention because of its high prevalence and low response against conventional therapies [2,3]. Over 90% of BCs are not metastatic at diagnosis. For those with no metastatic symptoms, the treatment goals include tumor eradication and prevention of disease recurrence [4]. Current treatments for BC, including chemotherapy, surgery, and radiotherapy are not effective because of poor patient response, a high risk of relapse, and drug resistance [5,6,7]. Thus, there is a need for novel, readily available drugs with fewer side effects to treat different cancers [8,9]. In recent years, plants have been considered as a strong potential for cancer treatment. Plants are rich in bioactive natural compounds [10], and their polyphenolic compounds have many therapeutic effects [10,11]. Piperine is a hydrophobic polyphenol derived from the black pepper plant. (Figure 1) [10,12].

Piperine has been recognized as an effective anticancer [13] agent that can regulate signaling pathways and molecular targets leading to the prevention of cancer cell proliferation, angiogenesis, and metastasis and consequently, the apoptotic pathways [14,15]. The anti-tumor activity of piperine has been shown in cancers of the colon, breast, forestomach, etc. [16]. One of the essential functions of piperine is induction apoptosis through changing the expression of the gene's apoptotic pathway. (Figure 2) [17]. Apoptosis or programmed cell death is crucial to regulate homeostasis by eliminating abnormal and redundant cells and plays a role in many diseases, especially cancer. It is deregulated in carcinoma and, hence, is one of the most evaluated processes in cancer treatment [18]. The down-regulation of *bcl-2* and up-regulation of *bax* expression have been reported during apoptosis [19,20]. Studies have shown that the cross-talk between these Bax (pro-apoptotic) and Bcl-2(anti-apoptotic) proteins can indicate the fate of a cell [21].

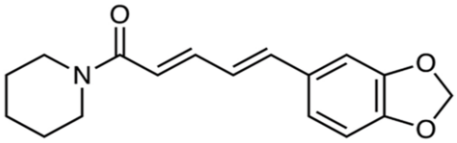
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[DOI: 10.61186/jhgg.7.1.5]

2. Objectives

This research was performed to investigate the effect of piperine on MCF-7 cells at different times and concentrations and the changes in the expression levels of *bax* and *bcl-2* genes through the induction of apoptosis in MCF -7 cells.



C17H19NO3

Figure 1. Chemical Structure of Piperine[34]

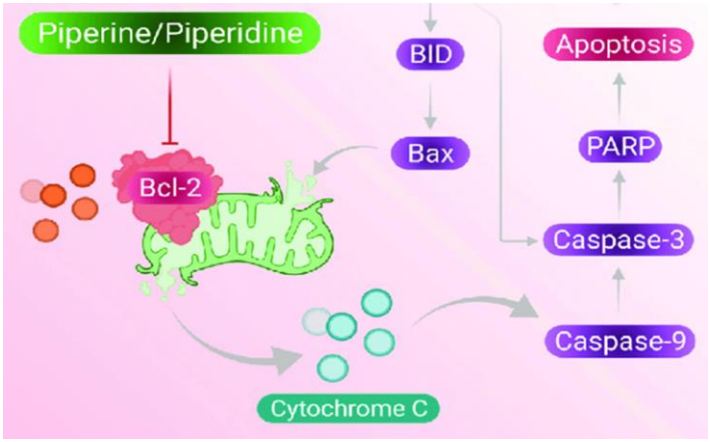


Figure 2. . Induction of apoptosis by Piperine[17]

3. Methods

3.1. Reagents

In this study, we used RPMI1640 medium (Bio IDEA), Penstrap 1% (Merck Germany), FBS (Gipco, Invitrogen), and sodium bicarbonate (Serva co, Germany). DSMO (Sigma Aldrich). EDTA (Bio IDEA), Trypsin, MTT (Solar Bio). Also, all materials required for the Real-time PCR test, including primers, Trizol, Taq polymerase, and SYBER Green fluorescence master mix, were prepared by Pishgam Company (Iran).

3.2. Cell Culture

In this study, MCF-7 BC cells were provided by the Pasteur Institute, Iran. They were cultured in RPMI1640 medium treated with Penstrap 1%, 20% heat-inactivated fetal bovine serum, and 3 mg/ml sodium bicarbonate followed by incubation at 37 °C with humidified air containing 5% CO2.

3.3. Treatment method

According to the studies, the concentrations used for piperine (5-25 μM) were considered and IC50 was calculated between the concentrations. Also, one concentration was considered as a control

3.4. MTT assey and Cell Viability

After cell culture, the piperine toxicity on MCF-7 cells was assessed using MTT assay at 24, 48, and 72 hours. Briefly, 10,000 cells underwent culturing in plates of 96 wells (NEST) and then exposed to 37 °C and CO2 5% for 24 hours. They received piperine at various concentrations (5-25 μM) at intervals of 24, 48, and 72 hours with ternary replications. A triplicate with zero μM drug dosage was regarded as a control. Following treatments for 24, 48, and 72 hours, the removal of the surface area of the cells was done, and the wells were added with 10 μl of MTT (Solar Bio) solution and 90 μl of fresh medium, followed by incubation for 4 hours. Then, the perimeter of the wells was removed, and the wells were filled with DMSO (100 μl; Sigma Co, Germany) solution. In the end, using an ELISA reader (Elx808, BioTeck, USA), the absorbance was read at 550 to 670 nm. The microscopic images of MCF-7 cells after receiving piperine compared to the control group show that the cell density decreased and the cell morphology changed, indicating the toxicity of the drug to the cell.

3.5. Apoptosis

After the MTT assay, the cells were treated for 48 hours with IC50 concentration, and finally, the study of cell apoptosis and necrosis

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was performed by the tissue culture laboratory. Figure 4(a) shows MCF-7 cells that are growing under normal conditions, but after receiving piperine, the cells lost their normal shape and underwent apoptosis Figure 4 (b).

3.6. Flow Cytometry

Flow cytometry analysis was performed by BD Biosciences (USA) device. Two groups were considered for this test: the first group, 10000 MCF-7 cells with media (controls), and the second group cells treated with piperine at IC50 concentration for 48 h. After incubation, PBS (%10) was used to wash the cells, and they were trypsinized and underwent suspension using PBS. Annexin-V (Biolegend) was mixed with the suspension followed by incubation for 10 min. Finally, data were restored using Partec Flomax software.

3.7. Total RNA extraction and cDNA synthesis

For RNA extraction, the MCF-7 BC cells were seeded in plates with two wells at 10000 cells per well. Then, 24 hours later, the cells received piperine and underwent incubation for 48 hours. Afterward, the extraction of the total RNA of the untreated and treated cells was done using Trizol (EasyBLUE total RNA extraction solution, Cat#: 17061) based on the kit guideline (Pishgam, Biotechnology, Iran). The extracted RNA was kept in water treated with DEPC at –80 °C. Then the complementary strand (cDNA) was synthesized from the RAN strand. Primer design was done by Gen runner software, and the specificity of designed primers was checked and confirmed by NCBI software and through Blast with the genome. The sequence and Amplicon size of *bax* and *βactin* (control) genes are shown in Table 1".

Table 1. Characteristics designed primers for bax and βactin (control) genes.

NO.	Gene	Seq(5-3)	TM	Amplicon Size
1	βactin	F CTT CCT TCC TGG GCA TG	60Ċ	85bp
		R GTC TTT GCG GAT GTC CAC	60Ċ	
2	bax	F AAA CTG GTG CTC AAG GC	60Ċ	187bp
		R CAC AAA GAT GGT CAC GG	60Ċ	

3.8. Real-time PCR

Real-time PCR SYBR performed by Rotor-Gene 6000 (Corbett Research, Australia). The reactions were performed with 1 µl of each primer, SYBR Green PCR Master Mix (10 µl), 1 µl cDNA (2 ng), and 7 µl of double distilled water (total volume: 20 µl). The thermal cycling conditions were 95 °C for 15 s, 60 °C for 20 s, and 30 s for 72 °C for 40 continuous cycles.

3.9. Statistical Analysis

Statistical calculations were performed by SPSS software version 22. To analyze RT- PCR data, first, the relative expression of data was done using REST software (Germany). Analysis of gene expression and flow cytometry data was done based on the unpaired t-test.

4. Results

4.1. MTT Assay

The results of the MTT assay are shown in Figure 3. As can be seen, piperine decreased cell viability in a concentration-dependent manner. After 72 h of cell treatment, the viability of cells was strongly reduced in 25µM piperine, indicating the anti-cancer effects of this natural product. Also, IC50 values for MCF-7 cells receiving piperine were 15 µM after 48 h ($p<0.05$) (Fig.3).

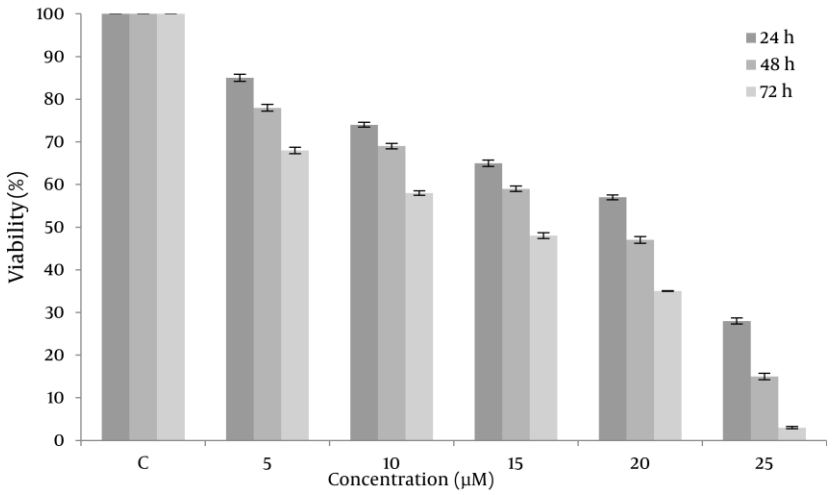


Figure 3. The viability percentage of MCF-7 cells exposed with piperine (5 to 25 µM) after 24, 48, and 72 hours treatment (*$p<0.05$).

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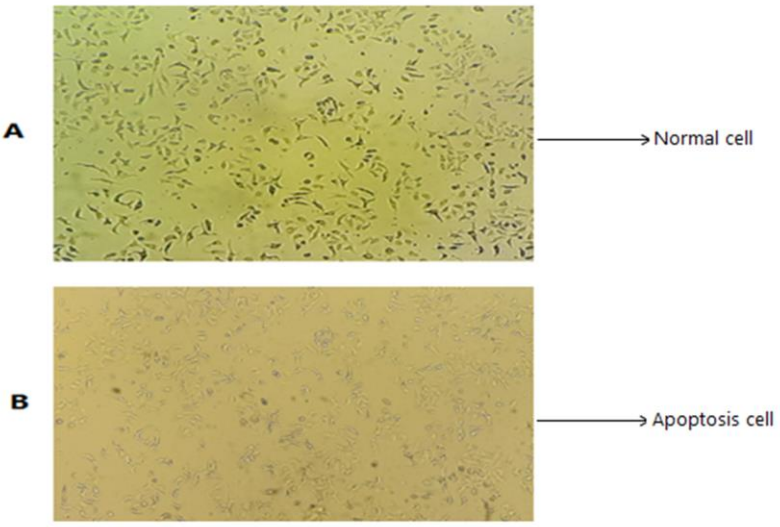


Figure 4. Microscopic images of MCF-7 cells. A: control group of MCF-7 cells, B: MCF-7 cells after receiving

4.2. Flow Cytometry

The results of the Flow Cytometry test showed the apoptosis percentage of MCF-7 cells treated with piperine IC50 concentration (15 μ M) for 48 h, 58.3%, which is 57% more than the control group (Figure 4).

Table2. The Flow Cytometry of results

Test (%)	Sample	
	Untreated cells	Cells treated with piperine at IC_{50} concentration and 48 hours
Necrosis	3.44	13.8
Late apoptosis	0.328	32.3
Early apoptosis	0.929	26.0
Live cells	95.3	27.8
Unpaired T-test	Mean = 0.628	Mean = 29.15
Significant	P value < 0.001	

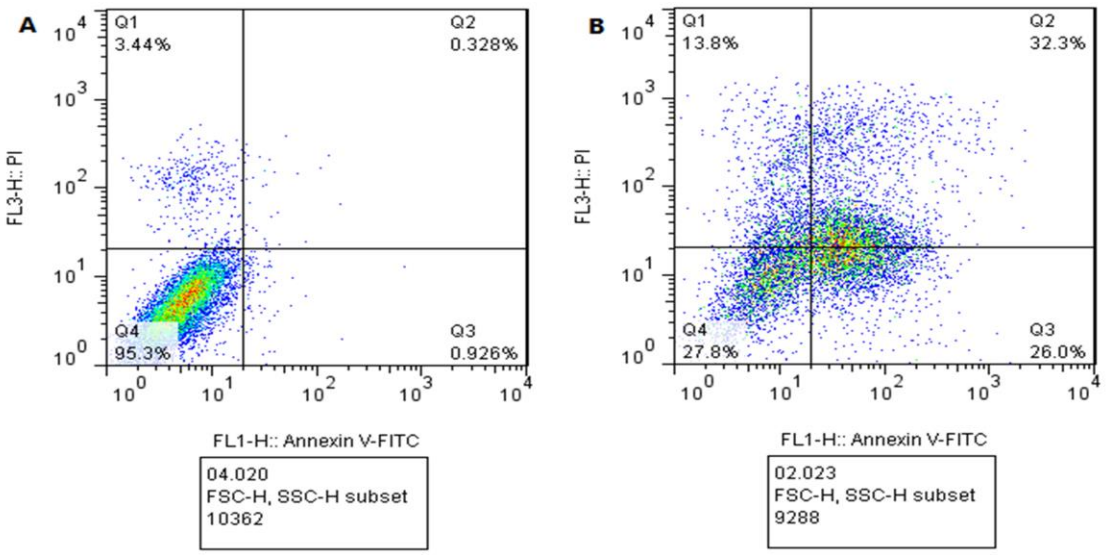


Figure 5. Flow cytometry results of untreated (A) and treated (B) MCF-7 cells with IC50 concentrations (15 μM) and 48 hours, treatment with piperine

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4.3. Real-time PCR

Piperine overexpressed bax gene in MCF-7 cell line at IC50 concentration (15μM). The comparison of the means bax expressions in control and piperine-treated cells showed almost 2.5 times increases in the expression of this gene in the treated cells (Figure 6), indicating the activation of mitochondrial apoptosis pathway induction by this natural product.

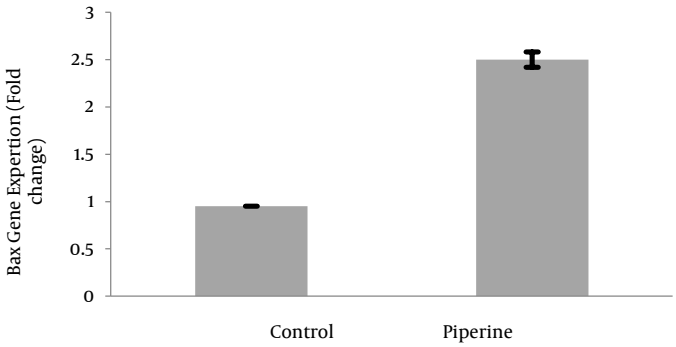


Figure 6. The effects of piperine on the *bax* expression of the MCF cell line. The cell treated with 15μM piperine for 48 hours and then the expression of *bax* gene was measured by RT-PCR technique (**p≤0.05).

5. Discussion

Conventional treatments, such as surgery, chemotherapy, and radiotherapy for BC have several side effects and considerably decrease the patient's quality of life [22]. In recent years, using natural Compounds is more common compared to chemical agents. In this study, Piperine was used as an anticancer drug. [23,24]. Research has shown that piperine has been used to treat many cancers [25]. In addition, this substance affects signaling pathways and is involved in apoptosis induction [26,27]. In this study, piperine as inducing apoptosis was used in Mcf-7 cancer cells.

As a brief review, In Mcf-7 cells, piperine strongly suppressed cell proliferation and caused apoptosis through caspase-3 activation, and also, inhibited *HER2* gene expression at the transcriptional level [28]. Park et al showed that piperine reduces adipocyte differentiation by suppressing *PPARγ* expression, thus, this action of piperine leads to potential treatment of obesity-related diseases [29]. In another research, PTX combined with PIP showed synergistic effects in SKOV-3 cells modulated by pro and anti-apoptotic genes, which can compensate for the side effects and toxicity of PTX [30]. Also, Qi and colleagues showed that piperine increased the chemosensitivity of 143B and U2OS cells against DOX. Also, in vivo and in vitro findings indicated the dramatic suppression of tumor growth and cell proliferation in the combined treatment group than in the monotherapy group. Apoptosis assessment showed that PIP augmented DOX-related cell apoptosis by up-regulating the *P53* and *bax* expression, and a decrease in *bcl-2* expression. Also, PIP could attenuate the start of the *PI3K/AKT/GSK-3β* signaling pathway in osteosarcoma cells through changes in the P-PI3K, P-AKT, and *P-GSK3β* expression levels [31]. But in the research done in Iran, Zare et al, investigated the effect of piperine on MCF-7 cells, and the *MMP-9, VEGF*, and *E*-cadherin expression. According to the MTT assay, treatment with piperine (5, 10, 25, 50, 75, and 100 μ M) for 24 hours could inhibit cell viability of MCF-7 cells than the control group. Also, *VEGF*, *MMP-9*, and *E*-cadherin expression levels were dose-dependently inhibited by piperine (5, 10, and 25 μ M) (P<0.05; P<0.01). Piperine at 5, 10, and 25 μM could significantly inhibit *MMP-9* protein expression following 24 hours of treatment (P<0.01) [32]. Also, Fattah and colleagues investigated the effect of piperine and cisplatin on the apoptosis pathway and results showed cisplatin (5 μM) and Piperine (20 μ M) for 24 h induced apoptosis strongly by a reduction in *bcl-2* expression and an elevation in *caspase 9*, *caspase 3*, *p53*, and *bax* expression [33].

Compared to the research, in this study, we investigated the effect of piperine on Mcf-7 cells and the expression of bax gene. According to the MTT assay, treatment with piperine (5, 10, 15, 20, and 25 μ M) for 24, 48, and 72 hours could inhibit cell viability of MCF-7 cells than the control group. The Flow cytometry results indicated that 58.3% of the cells were apoptotic at IC50 concentration and 48-hour treatment. Also, Real-time PCR results showed an elevation in *bax* expression than the control group in MCF-7 cells at IC50 concentration and 48 hours of treatment.

5-1. Conclusion

In this study, piperine showed an excellent inhibitory effect on the MCF-7 cell line. Also, piperine increased the expression of the *bax* (as a pro-apoptotic) gene and induced apoptosis. Therefore, piperine could be a potential carrier for treating breast cancer as the next generation of drug delivery systems.

Acknowledgements

The authors at thankful to the Active Pharmaceutical Ingredients Research Center (APIRC), Tehran Medical Sciences, and Islamic Azad University for providing them with the necessary equipment and laboratory services.

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Conflicts of interest

One of the authors of this article is a member of the committee board of the Journal of Human Genetics and Genomics.

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