

Original Research Article

Effect of capecitabine and melatonin on HER2+ (SK-BR-3) and HER2- (MCF7) human breast cancer cell lines

Sepideh Shayan¹, Laya Takbiri Osgoei^{2*}, Fatemeh Javani Jouni³

¹Department of Biochemistry, Faculty of Biological Sciences, ²Department of Microbiology, Faculty of Biological Sciences, North Tehran Branch, ³Department of Biomedical Engineering, Faculty of Health, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

*For correspondence: **Email:** L_takbiri@iau-tnb.ac.ir; **Tel:** +98-905-7241495

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Abstract

Purpose: To determine the effect of melatonin in combination with capecitabine on the proliferation and induction of apoptosis in MCF-7 and SK-BR-3 breast cancer cell lines.

Methods: The MTT assay (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) was performed to investigate the effect of capecitabine and in combination with melatonin on cell lines and to determine the half-maximal concentration (IC₅₀) and combination index. Expression of apoptotic markers, Bax, Bcl-2, and caspase-3 were measured by polymerase chain reaction (PCR) after treatment.

Results: The IC₅₀ of melatonin and capecitabine in MCF-7 and SK-BR-3 cell lines were 4.52 mM and 619.36 µg/mL, and 5.1 mM and 679.51 µg/mL, respectively. The combined use of melatonin and capecitabine significantly reduced IC₅₀. Also, combination index (CI) values were < 1, indicating that the combination of capecitabine and melatonin has a synergistic effect. The results of gene expression also showed enhancement in the Bax/Bcl-2 ratio in melatonin-capecitabine combination compared with capecitabine alone in both cell lines.

Conclusion: Melatonin-capecitabine-induced cell death is controlled by caspase-3 and Bax/Bcl-2-dependent apoptosis. The combination of melatonin and capecitabine has a synergistic effect on both HER2+ and HER2- cells.

Keywords: Melatonin, Capecitabine, Breast cancer, MCF7, SK-BR-3, Apoptosis

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INTRODUCTION

In recent years, cancer has become the leading cause of death in many countries, including developed countries such as the United States. There were 18.1 million new cancer diagnoses and 9.6 million cancer-related deaths recorded globally in 2018 [1]. High mortality rates had been reported for breast cancer (BC), acute

lymphocytic leukemia, gastric cancer, lung cancer, prostate cancer, colon cancer, and brain cancer [2,3]. Breast cancer is caused by many mutations in epithelial cells and ultimately creates cancers with complex genomic and biological heterogeneity. Breast cancer is classified into five main categories based on innate gene expression profile: luminal A, luminal B, basal-like, HER2 (overexpression of human

epidermal growth factor 2), and normal-like [4,5]. Many factors have a direct effect on the prognosis and response to breast cancer, including the estrogen receptor (ER), progesterone receptor (PR), HER2 level, lymph node metastasis, histological grade, size, and type of tumor [6-10].

Capecitabine is an oral fluorouracil drug used for adjunctive chemotherapy in gastric cancer. It has also been approved for adjuvant therapy of metastatic breast cancer after the use of anthracyclines and taxanes. Capecitabine has been used in combination with other drugs such as pyrotinib, neratinib, and lapatinib to treat HER2⁺ metastatic breast cancer. Also, a combination of capecitabine with bevacizumab has been used to treat HER2⁻ cancer with reduced mortality in patients [6-7].

Melatonin or N-acetyl-5-methoxytryptamine is a physiological hormone that is secreted in mammals by the intestine, pineal gland, immune system, and brain. The pineal gland secretes melatonin into the bloodstream, then melatonin interacts with various cellular kinases through two membrane melatonin receptors, creating some of its biological effects. This hormone has a wide range of physiological actions, including sleep regulation and circadian rhythm, as well as affecting bones, reproductive system, immune system, and aging [8].

Melatonin's capacity to control apoptosis-related processes is thought to be the cause of its reported anti-tumor effects. Some of the anti-cancer effects of melatonin are mediated by binding to two membrane-bound G-binding protein receptors (GPCRs), namely melatonin receptor MT1 (encoded by MTNR1A gene) and melatonin receptor MT2 (encoded by MTNR1B) gene [9]. This study was aimed at investigating the combined effect of capecitabine and melatonin on HER2⁻ and HER2⁺ in breast cancer cells in humans.

EXPERIMENTAL

Cell culture and mycoplasma contamination test

The cells (SK-BR-3 and MCF-7) were prepared by Pasteur Institute (Tehran, Iran) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal bovine serum (FBS), 1 % L-glutamine, and 100IU/mL penicillin-streptomycin in a humidified air incubator at 37 °C, 95 % humidity, and 5 % CO₂.

Mycoplasma presence was determined using an EZdetect™ PCR kit (HiMedia Laboratories, Mumbai, India). DMEM media supplemented with 10 % FBS was used to cultivate cells without antibiotic solutions. Thereafter, a microtube was filled with 2 mL of the supernatant from the cultivated cells, and centrifuged for 30 min at 13000 rpm.

The pellet was dissolved in 50 µL of 1 x TE buffer, vortexed, and heated at 95 °C for 10 min. Supernatant from second centrifugation of the microtube was employed as a PCR template. Polymerase Chain reactions (PCR) were conducted with reagents under cycling conditions and visualized using 2 % agarose gel and a UV gel documentation system. The reaction included a positive and negative control.

Cytotoxic evaluation

A rapid approach called MTT, (3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) test, relies on the breakdown of yellow tetrazolium salt into purple formazan crystals in metabolically active cells by mitochondrial enzymes. The cells (MCF-7 and SK-BR-3) were grown logarithmically in 96-well plates at a rate of 10,000 cells per well. The cells were treated separately after 24 h with various capecitabine doses (75, 150, 300, and 600 µg/mL) (Cas No. 154361-50-9) and melatonin (0.5, 1, 2, and 4 mM) (CAS no. 73-31-4) as well as in combination. After treatment for 24 h, 20 µL of MTT (5 g/L) was added to each well and incubated for 4 h. Culture media was removed and thereafter 150 µL of dimethyl sulfoxide (DMSO) was added. At 545 nm, the absorbance was measured with an ELISA reader (Awareness, USA). Finally, IC₅₀ value was calculated and the percentage of viable cells (C) in different treatments was determined using Eq 1.

$$C (\%) = (At/Ac)100 \dots\dots\dots (1)$$

where At is the mean absorbance of treated cells and Ac is mean absorbance of control cells.

Evaluation of apoptosis

Plasma membrane changes in apoptosis were examined by double staining with Annexin V-FLUOS and Propidium Iodide (PI). The Annexin V-FLUOS kit was used to measure the apoptosis of treated and control cells. The cells (10⁶) were added to 100 µL of a solution composed of 2 µL of annexin-V-FLUOS, 2 µL of propidium iodide, and 1 ml of incubation buffer. Each tube was diluted with buffer and the cells were analyzed

using flow cytometry (AQUIOS CL, USA). The cells were then divided into four sections: necrosis cells, late apoptosis, primary apoptosis, and living cells. This experiment was performed in three replications.

Determination of expression of caspase-3, Bax and Bcl-2 genes

Cellular RNA was extracted using GeneAll kit (South Korea) and treated with DNase enzyme to ensure that no DNA was present in the product. The extracted RNA (10 μ L) with 1 μ L of enzyme buffer and 2 μ L of Dnase enzyme was kept at 37 °C and 80 °C for 5 min each. The RNA sample (0.2 μ g) was converted to cDNA using the Yekta Tajhiz Azma kit (Tehran, Iran), which utilizes Oligo primer (dT) 18 reverse cDNA transcript. Synthesized cDNA was heated at 85 °C for 5 secs incubated at 42 °C for 60 min, and inactivated at 85 °C for 5 min.

Reverse transcriptase-polymerase chain reaction (RT-PCR) was used on the cDNA sample to assess the expression of the caspase-3, *Bax*, and *Bcl-2* genes using specific primers. Finally, the data were analyzed by $2^{-\Delta\Delta Ct}$ relative expression assay. This experiment was performed in triplicates. The sequence of all primers used in this study was designed by Primer3 software (Table 1).

Table 1: Primers used to investigate *Bax*, *Bcl-2*, and *Caspase3* gene expression

Gene	Primer sequence
Bax-F	5'- TGCTTCAGGGTTTCATCCA -3'
Bax -R	5'- GACTCTCGCTCAGCTTCTTG -3'
Bcl-2-F	5'-GATAACGGAGGCTGGGTAGG-3'
Bcl -2-R	5'-CCAGAGGAGGAGGTAGGGAC-3'
Casp3-F	5'-GTTTGAGCCTGAGCAGAGACAT-3'
Casp3-R	5'-CCAGAGGAGGAGGTAGGGAC-3'
GAPDH-F	5'- AGGGCTGCTTTAACTCTGG -3'
GAPDH-R	5'- CCCCACTTGATTTGGAGGG -3'

Evaluation of lipid peroxidation index (MDA) and cell necrosis

Cell MDA levels were measured by Nalondi TM lipid peroxidation assay kit (NID) (Navand

Salamat, Iran). The study is based on the interaction of MDA with TBARS (reactants of thiobarbituric acid) and absorbance at 532 nm was measured using a spectrophotometer. Cell LDH levels were measured by the colorimetric method with lactate dehydrogenase (LDH) assay kit (ab102526, Abcam, Cambridge, MA, USA). Assays were carried out following the manufacturer's instructions. Assay was performed in triplicates.

Statistical analysis

For statistical analysis of quantitative variables, one-way ANOVA test and Tukey tests were used. The IC₅₀ value was calculated by GraphPad Prism V8 software for each combination. Combination index (CI), computed by CompuSyn software, was also utilized to assess the synergistic effects of drug compounds. Combination index (CI) quantitatively shows synergism (CI < 1), additive effect (CI = 1), and antagonism (CI > 1).

RESULTS

Cytotoxic effects of melatonin-capecitabine on MCF-7 and SK-BR-3 cells

The cytotoxic effects of melatonin, capecitabine alone, and their combination on MCF-7 and SK-BR-3 were determined by MTT assay. The results showed that IC₅₀ of melatonin for MCF-7 and SK-BR-3 cells was 4.52 mM and 5.1 mM, respectively. Also, the IC₅₀ of capecitabine for MCF-7 and SK-BR-3 cells was 619.36 μ g/mL and 679.51 μ g/mL, respectively.

The results of the combined use of melatonin and capecitabine demonstrated that the percent of MCF-7 cells that survived in the presence of melatonin (concentrations of 0.5, 1, 2, and 4 mM) and capecitabine (concentrations of 75, 150, 300, and 600 μ g/mL) were 71.68 \pm 1.1, 51.9 \pm 0.49, 42.84 \pm 0.4 and 32.45 \pm 0.56 %, respectively, and IC₅₀ of melatonin (1.59 μ g/mL) and capecitabine (239.4 μ g/mL) was lower than each of them.

Table 2: Calculated CI for the melatonin-capecitabine compound at different doses on MCF-7 and SK-BR-3 cells

Melatonin concentration (mM)	Capecitabine concentration (μ g/mL)	CI values for MCF-7 cell lines	CI values for SK-BR-3 cell lines
0.5	75	0.384	0.553
1	150	0.133	0.151
2	300	0.129	0.141
4	600	0.11	0.166

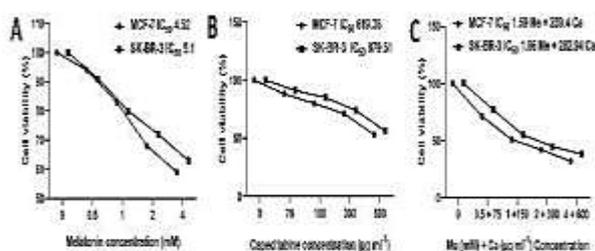


Figure 1: Cytotoxic effects of melatonin (A) and capecitabine (B) on SK-BR-3 and MCF-7 cells in combination and individually (C), Me: melatonin, Ca: capecitabine

On the other hand, the results of the combination of melatonin and capecitabine on SK-BR-3 cells showed that cell viability percentage in the presence of melatonin and capecitabine concentrations was 77.12 ± 0.8 , 55.53 ± 1.38 , 45.52 ± 0.68 , 38.55 ± 0.32 %, respectively. Also, the IC₅₀ of melatonin (1.86 μg/mL) and capecitabine (282.94 μg/mL) was lower than IC₅₀ of each of them. Combination of melatonin and capecitabine had a synergistic effect (CI < 1) on both cell lines (Table 2).

Effects of melatonin and capecitabine on SK-BR-3 and MCF-7 cell apoptosis

Melatonin at IC₅₀ concentration alone led to a significant increase in apoptosis up to 35.37 % and 37.19 % in MCF-7 and SK-BR-3 cell lines, respectively. Capecitabine at IC₅₀ concentration also increased apoptosis to 37.03 % and 36.3 % in MCF-7 and SK-BR-3 cell lines, respectively. The combined effect of these two compounds synergistically led to an increase in apoptosis so that at lower concentrations, apoptosis increased to 48.54 and 53.43 % in MCF-7 and SK-BR-3 cell lines, respectively.

Caspase-3, Bax, and Bcl-2 gene expressions

Cell treatment with melatonin and capecitabine increased apoptosis by altering apoptosis-related gene expression. Expression of genes implicated in cellular apoptosis is shown in Figures 3 A and B.

The results of MCF-7 cell line showed a non-significant increase in Bax gene expression following treatment with melatonin and capecitabine alone ($p > 0.05$). In this cell line, melatonin and capecitabine alone led to a significant decrease in Bcl-2 gene expression, capecitabine significantly increased Caspase-3 expression, while melatonin did not significantly increase Caspase-3 expression. The effect of the melatonin-capecitabine combination significantly increased Bax and Caspase-3 in MCF-7 cells,

but a decrease in Bcl-2 expression compared to capecitabine was not significant. Melatonin or capecitabine, on the other hand, caused an increase in Caspase-3 and Bax genes and a decrease in Bcl-2 in SK-BR-3 cell line.

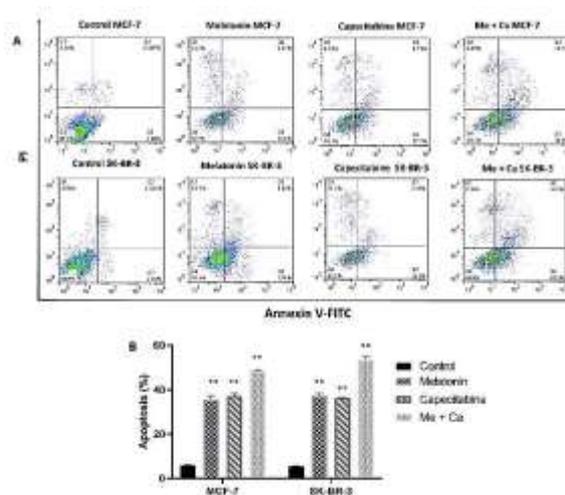


Figure 2: Effects of melatonin and capecitabine alone and in combination on MCF-7 and SK-BR-3 cell apoptosis by flow cytometry (A), cell apoptosis percentage (B), Annexin V-PI analysis in cells during the 24 h incubation period for the compounds at IC₅₀ concentration, four zones are identified on the graph, and they are as follows: percentages of necrotic cells (Q1) late apoptotic cells (Q2); primary apoptotic cells (Q3) and viable cells (Q4). **Significant at $p < 0.01$. Me: melatonin, Ca: capecitabine

Furthermore, capecitabine plus melatonin therapy resulted in a considerable rise in the expression of the Caspase-3 and Bax genes and a significant reduction in Bcl-2 SK-BR-3 ($p < 0.01$).

Figure 3 C shows the Bax/Bcl-2 ratio. This ratio increased significantly in the case of combination drug treatments compared to their use alone in SK-BR-3 cells ($p < 0.01$), but the increase in MCF-7 cells was not statistically significant ($p > 0.05$).

Determination of lipid peroxidation index (MDA)

Lipid peroxidation refers to the oxidation of lipids by free radicals [10]. The results revealed that MDA as an indicator of lipid peroxidation in cells treated with capecitabine, melatonin, and melatonin + capecitabine significantly increased compared to the control group ($p < 0.01$). In both cell lines, the level of MDA in capecitabine + melatonin treatment was significantly greater than when used alone ($p < 0.01$).

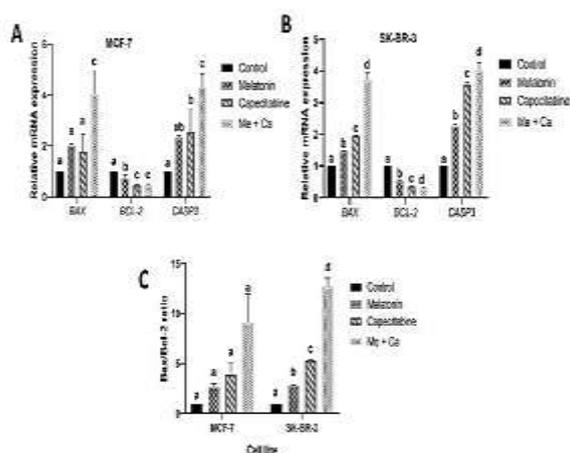


Figure 3: Expression of *Bax*, *Bcl-2*, and *Caspase-3* genes in MCF-7 (A) and SK-BR-3 (B), *Bax/Bcl-2* ratio (C) cell lines, different letters show a significant relationship at $p < 0.05$, Me: melatonin, Ca: capecitabine

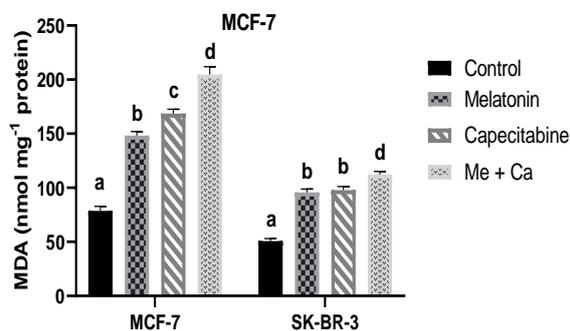


Figure 4: Effect of capecitabine, melatonin, and their combination on lipid peroxidation in MCF-7 and SK-BR-3 cells. Cells were exposed to IC₅₀ concentrations of compounds. Different letters indicate a significant relationship at $p < 0.05$, Me: melatonin, Ca: capecitabine

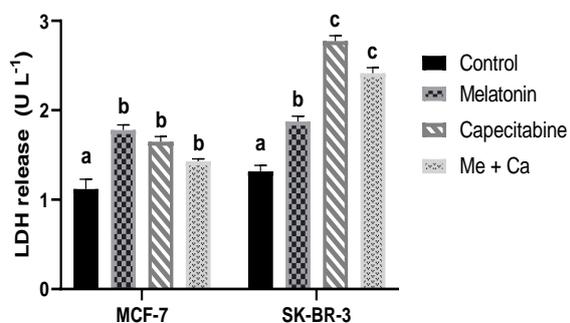


Figure 5: Released lactate dehydrogenase following treatment of SK-BR-3 and MCF-7 cells with capecitabine, melatonin, and their combination. Cells were exposed to IC₅₀ concentrations of compounds. Different letters show a significant relationship at $p < 0.05$, Me: melatonin, Ca: capecitabine

Investigation of cell necrosis released by LDH

Lactate dehydrogenase (LDH) levels in cellular supernatants were measured after treatment with the compounds. The results showed that melatonin and capecitabine significantly increase LDH. The combination of melatonin and capecitabine also increased LDH compared with the control group, but melatonin and capecitabine alone significantly decreased LDH.

DISCUSSION

A prevalent malignant disease in women across the world is breast cancer. Women's cancer rates are higher in less developed countries. The first line of therapy against breast cancer is surgery. In addition, people with breast cancer are advised to have chemotherapy, radiation treatment, and estrogen-based therapy as well. Combination therapy is also recommended because it reduces resistance to chemotherapy as well as the risk of relapse [11].

Numerous cell, animal, and human culture investigations have shown that melatonin has growth suppressant and pre-apoptotic characteristics. The safety of melatonin has also been shown. Melatonin has also been reported to be useful as an adjunct drug and has improved survival rates. According to Kosar et al. [11], melatonin (0.3 mM) in the MCF-7 cell line was found to enhance doxorubicin-induced apoptosis. The effects of induction of arsenic trioxide apoptosis in SK-BR-3 and MDA-MB-231 cell lines were also increased by melatonin (1 mM) [12]. On the other hand, capecitabine is a drug used to treat various cancers, including breast cancer [13]. There have been no reports of the anti-cancer effect of the combination of capecitabine and melatonin on breast cancer. This effect was investigated on SK-BR-3 and MCF-7 cancer cells. This research has provided the first evidence of the synergistic cytotoxic effects of melatonin and capecitabine on SK-BR-3 and MCF-7 cells.

The concentration of IC₅₀ inhibiting the proliferation and viability of each compound alone and in combination was investigated and the results showed a significant decrease in IC₅₀ of capecitabine when used concomitantly with melatonin, which can help reduce its side effects. Apoptosis is programmed cell death that maintains cell homeostasis between cell division and cell death [14]. The external receptor pathway or death and internal or mitochondrial pathway are the two main mechanisms for triggering apoptosis, and both appear to be engaged alone or in combination in melatonin-

induced cell death. The mitochondrial pathway is activated by proteins in the B cell leukemia/lymphoma 2 family (Bcl-2). However, because Bax is a pro-apoptotic protein, the release of cytochrome C from mitochondria to the cellular cytoplasm is regulated by the ratio of Bax/Bcl-2, which serves as a marker of the mitochondrial apoptotic pathway [15].

The present study showed that melatonin decreased the expression of Bcl-2 anti-apoptotic gene and increased the expression of Bax and Caspase-3 genes in the MCF-7 and SK-BR-3 cells. Although these changes were not significant in MCF-7 cells ($p > 0.05$) but were significant in SK-BR-3 cells ($p < 0.05$). It therefore means that expression of apoptotic genes was changed by melatonin, which thus has anti-cancer effects. Similarly, studies by Yun *et al* [12] stated that melatonin causes enhanced cellular death in MDA-MB-231 and SK-BR-3 breast cancer cells by modifying the expression of the Bcl-2 and Bax genes.

The results also showed that melatonin-capecitabine led to a significant increase in Bax / Bcl-2 ratio compared to capecitabine treatment alone, which was consistent with findings from other studies in various cancer cells, including MCF-7 [16].

Caspase-3 protein is required for DNA fragmentation and morphological changes associated with apoptosis [14]. Melatonin stimulates the activation of caspase-3 to induce cell death in malignant cells of the pancreas [17]. There was an increase in caspase-3 gene expression, which is a mechanism for the innate pathway of apoptosis. Increased expression of caspase-3 is associated with the mechanism responsible for the intrinsic pathway of melatonin apoptosis. Furthermore, melatonin-capecitabine combination resulted in a further increase in caspase-3 gene expression than capecitabine or melatonin alone. Therefore, melatonin in combination with capecitabine synergistically increases apoptosis through caspase. Flow cytometry was used to investigate cell apoptosis and the results revealed a significant rise in apoptosis in groups when compared to one another, which confirms results of gene expression. In comparison with MCF-7 cells, SK-BR-3 cells' Bax/Bcl-2 ratio, and caspase-3 gene expression were not significantly different.

Melatonin-capecitabine combination increased apoptosis to the same extent in HER2⁺ and HER2⁻ cells. The release of lactate dehydrogenase (LDH) from cellular cytoplasm is a good indicator of cell necrosis. Lactate

dehydrogenase was significantly increased in treated groups compared to the control group. Level of lactate dehydrogenase in cells treated with melatonin-capecitabine combination was not significantly different from those treated with capecitabine alone. Lipid peroxidation is also involved in regulated cell death. Lipid breakdown products have been shown to induce apoptosis. Lipid peroxidation has also been reported to be the main stimulus for ferroptosis, a regulated necrotic cell death [18]. In addition, melatonin-capecitabine increased MDA in breast cancer cells (MCF-7 and SK-BR-3). This suggests that melatonin-capecitabine may be a good combination for the treatment of breast cancer because due to the oxidative nature of cancer cells.

CONCLUSION

The significant difference between cell inhibitory capacity of capecitabine and melatonin-capecitabine combination will ultimately to the reduction of the dose of capecitabine, thus lowering its adverse effects. Melatonin-capecitabine combination has a synergistic apoptotic effect. Also, the combination of melatonin-capecitabine has similar antiproliferative properties on both HER2⁺ and HER2⁻ cell types. Although further study of the cytotoxic effect *in vivo* is necessary, these results indicate the potential of melatonin-capecitabine combination as a therapeutic agent for the treatment of breast cancer.

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Contribution to the paper is as follows. Study conception and design: Sepideh Shayan, Laya Takbiri Osgoei; data collection: Sepideh Shayan, Laya Takbiri Osgoei; analysis and interpretation of results: Laya Takbiri Osgoei, Fatemeh Javani Jouni; draft manuscript preparation: Y Sepideh Shayan, Laya Takbiri Osgoei, Fatemeh Javani Jouni. All authors reviewed the results and approved the final version of the manuscript for publication.

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