RESEARCH ARTICLE

Editorial Process: Submission:02/19/2023 Acceptance:06/23/2023

Induction of Unfolded Protein Response by Tannic Acid Triggers Apoptosis in MDA-MB-231 Breast Cancer Cells

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Abstract

Introduction: Endoplasmic reticulum (ER) stress can reduce cell survival and enhances the apoptosis of cancer cells. Plant polyphenols like tannic acid trigger ER stress and apoptosis and therefore can be a novel agent for the treatment of cancer. In this study, we investigated the effect of tannic acid on survival, migration, colony formation, ER stress pathway, and apoptosis of the MDA-MB-231 breast cancer cells. **Methods:** The MTT assay was performed to investigate the effect of tannic acid on the cell survival of breast cancer cells. We used the qPCR method to reveal the effect of tannic acid on the Bak, CHOP, ATF4, P21, MMP-2, and Bcl-2 expression. Also, colony formation, cell migration, and Hoechst staining assays were employed. **Results:** The results of the MTT test showed that tannic acid reduced the cell survival rate. In the qPCR assay, we found that tannic acid decreased the expression levels of MMP-2, Bcl-2, ATF4, and CHOP genes, paradoxically, enhanced the expression of Bak and P21 genes. The colony formation and cell migration assays indicated that tannic acid significantly diminished breast cancer cell proliferation and migration, respectively. In the apoptosis assay, tannic acid increased the number of apoptotic cells. **Conclusion:** Tannic acid increases the rate of cell death but decreases viability and cell migration. Moreover, tannic acid induces apoptosis in breast cancer cells. Overall, our study demonstrates that tannic acid induces ER stress by increasing the genes which are playing role in ER stress pathway. These results show that tannic acid can be used as an effective agent for breast cancer treatment.

Keywords: ATF4- Breast cancer- CHOP- Endoplasmic reticulum- Tannic acid

Asian Pac J Cancer Prev, **24 (6)**, 2029- 2035

Introduction

Breast cancer is the most common cancer among women, which causes death to approximately 500,000 people every year worldwideBarzaman et al., (2020); (Wang et al., 2021). Although chemotherapy and radiotherapy are potential treatments for breast cancer, the problems of resistance to treatment and recurrence of the disease are still significant (Kim et al., 2019; Kim and Ko, 2021). Drugs obtained from plants are considered valuable resources in the treatment of diseases, especially cancers, because of their less toxic effect, bioactive substances, and availability (Nie et al., 2016; Dalil et al., 2022).

Tannic acid is a polyphenolic tannin existing in plants like green tea, coffee, and fresh fruits such as pomegranate and grapes (Jing et al., 2022). Tannic acid has anti-oxidant and antitumor properties (Sp et al., 2020). Numerous studies have shown that tannic acid inhibits EGFR, JAK/STAT, TGF-β, and NF-Kβ signaling pathways, arresting the cell cycle, and metastasis, and inducing apoptosis in all molecular subtypes of breast cancer (Ngobili et al., 2015; Darvin et al., 2017; Kim et al., 2019; R et al., 2021). Also, tannic acid increases the anticancer effects of several chemotherapy agents such as doxorubicin, paclitaxel, and cisplatin in different categories such as breast, ovarian, and pancreatic cancer without affecting normal human epithelial cells. These results suggest tannic acid is a strong anti-cancer agent (Tikoo et al., 2011; Chowdhury et al., 2019).

The endoplasmic reticulum (ER) is a cell organelle that has several functions such as synthesis, folding, maturation, and transfer of proteins (Storm et al., 2016). ER stress causes the accumulation of unfolded proteins that can be destroyed by proteolytic mechanisms such as ubiquinone/proteasome and autophagy (Díaz-Villanueva et al., 2015). Disturbances in ER homeostasis cause the accumulation of unfolded protein, which is known as the UPR phenomenon, involved in the metastasis of several cancers (Corazzari et al., 2017). UPR activation is beneficial for cell survival, but long-term ER stress leads to cell death (Yadav et al., 2014).

The three stress sensors located on the membrane of

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the endoplasmic reticulum include activating transcription factor 6 (ATF6), inositol-requiring protein-1α (IRE1), and protein kinase RNA- like endoplasmic reticulum kinase (PERK). The main chaperone of the endoplasmic reticulum (Bip) is attached to the luminal domains of these stress sensors. Tannic acid causes apoptosis and stops the cell cycle by inducing ER stress (Yadav et al., 2014; Corazzari et al., 2017; Nagesh et al., 2018). During stress, Bip is disassociated from these three endoplasmic reticulum sensors and causes oligomerization, trans-phosphorylation, and activation of these sensors. ATF6 enters the Golgi apparatus and secretes specificity protein 1 (SP1) and specificity protein 2 (SP2) into the cytosol and activates the transcription factors. Activated IRE1 degrades several genes encoding pro-apoptotic factors (Bak, Bim) and activates jun amino-terminal kinase (JNK) protein through TNF receptor-associated factor 2 (TRAF2)and apoptosis signal-regulating kinase 1 (ASK1)(Corazzari et al., 2017). Finally, the activated PERK inhibits eIF2-α, which is a transcription factor, which leads to the entry of ATF4 into the nucleus, which affects the downstream target genes, such as enhancement of CHOP expression. CHOP overexpression causes cell cycle arrest in the G1/S phase or induces apoptosis (Rozpedek et al., 2016; Tang et al., 2020; Shi et al., 2021). CHOP has this effect by increasing pro-apoptotic proteins and decreasing anti-apoptotic proteins (Lei et al., 2017). Inhibiting eIF2-α and reducing its expression level causes suppression of cyclinD1 synthesis, which leads to cell cycle arrest. Previous studies proved that treatment with tannic acid arrests the cell cycle in G1/S by reducing the expression of cyclinD1 and enhancing the expression of P18 and P21 in cancer cells (Nagesh et al., 2018). Therefore overexpression of CHOP causes a halt in growth and survival, induces apoptosis, and causes damage to DNA.

Investigations demonstrated that tannic acid increased the expression of caspase-3, the cleavage of PARP, and the expression of pro-apoptosis proteins like Bim and Bak. Also, these compounds decreased the expression of anti-apoptosis proteins like Bcl-2 and Bcl-xL (McCullough et al., 2001; Nagesh et al., 2018). Moreover, tannic acid inhibits the epithelial-to-mesenchymal transition (EMT) process, by reducing mesenchymal markers such as MMP-2 and MMP-9 and increasing epithelial markers such as E-cadherin (Du and Shim, 2016; Nagesh et al., 2018).

Although tannic acid inhibits miscellaneous oncogene signaling cascades (Darvin et al., 2017; Avila-Carrasco et al., 2019), the impact of this compound on the response of unfolded proteins (UPR) that occur in the endoplasmic reticulum (ER), has rarely been investigated. Moreover, the exact molecular mechanisms of the effect of tannic acid on breast cancer cells are still unknown. This study aims to investigate the effect of tannic acid on the ER stress pathway, survival, migration, colony formation, and the apoptosis of the MDA-MB-231 breast cancer cells.

Materials and Methods

Cell culture

The human MDA-MB-231 breast cancer cell line was obtained from the Iranian Pasteur Institute (Tehran, Iran). Cells were cultured in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 1% antibiotics (100 µg/ml streptomycin, 100 IU/mL penicillin,) (Sigma-Aldrich), 1% Glutamine, 20% heat-inactivated fetal bovine serum (FBS) (Sigma Aldrich). The cell line was incubated at 37°C under a humidified atmosphere with 5% CO_2 (Figure 1).

MTT assay for cytotoxicity

The methyl thiazolyl tetrazolium (MTT) assay was used to measure the cytotoxicity of tannic acid on MDA-MB-231 cells. The assay was divided into 3 groups: blank control, solvent control, and tannic acid. Briefly, all cells were seeded at a density of 1×10^4 cells in 96well plates. After 24 h, the cells were treated with different concentrations of tannic acid (Sigma-Alderich, Figure 2) and incubated for 24 h. Then, 10 µL MTT solution was added to the wells and incubated for 4 h at 37 ºC. Subsequently, the supernatants were carefully discarded and 150 µL of DMSO was added to the cells. The absorbance for each well was read at 540 nm. The survival rate (SR) was determined according to the equation as follows: SR (%) = (A Experiment /A Control) × 100%. The concentrations that reduced the 50% and 30%of the survival rate (IC_{50} and IC_{30}, respectively) were measured by GraphPad software (GraphPad Software Inc, San Diego, CA, USA). In the next experiments, the treatment was done with the IC_{30} and IC_{50} doses.

QRT-PCR assay

Total RNA was isolated by the total RNA extraction kit (Parstous, Tehran, Iran) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 µg of purified total RNA by use of a cDNA synthesis kit following the manufacturer's instruction (Parstous). QRT-PCR was performed on a Light Cycler 96 System (Roche Diagnostics GmbH, Mannheim, Germany) by use of SYBR premix Ex Taq (Parstous). RT-PCR reactions were performed in a total volume of 20 µL containing 1 µL cDNA, 10 µL of SYBR green reagent, 0.2 µM of each of the oligonucleotides primers, and 8µL of distilled water. The primer sequences were listed in Table 1. The qRT-PCR parameters were as follows: initial denaturation at 95ºC for 5 min, then 40 cycles of denaturation at 95ºC for 10 seconds annealing at 55ºC for 30 sec. Relative quantification of gene expression was calculated with the $2^{-(\Delta\Delta Ct)}$ method (Alamdari-Palangi et al., 2020; Shahverdi et al., 2020; Shahverdi et al., 2021), using β-actin as a reference gene.

Wound healing assay

The MDA-MB-231 cells (1×10^5 cells/ml) were seeded in a 6-well plate and incubated for 48 h. The cells were scratched with a 200 ml pipet when the cells had confluency of 90-100%. The plate was washed with PBS and replaced with 1 ml fresh RPMI-1640. The cells were

treated with the IC_{30} and IC_{50} doses of tannic acid for 24 h. Cell migration into the wound area was photographed at consecutive 24h time points over 3 days, using an inverted microscope (Nikon, Japan).

Colony formation assay

The MDA-MB-231 cells were seeded in 12 well plates (1×10^5 per well) and incubated overnight at 37°C for 24 h. Then, the cells were exposed to the IC_{30} and IC_{50} doses of tannic acid for 24 h. The medium was replenished with a fresh culture medium. After 5 days, colonies were fixed for 20 min with acetic acid/methanol and stained with 2% crystal violet. Finally, the MDA-MB-231 was photographed.

Hoechst33342 staining assay

MDA-MB-231 cells were seeded at a density of 1×10^5 cells in a 6well plate for 24 h and then treated with IC_{30} and IC_{50} concentrations of tannic acid. After 24 h, the cells were washed twice with PBS, fixed in 3.7 % formaldehyde for 30 minutes, and then stained with Hoechst 33342 (Sigma-Alderich) for 30 min. The cells were observed under a fluorescence microscope (Nikon, Japan). Apoptotic morphological features and fragmented nuclei were determined.

Statistical analysis

Quantitative data are presented as mean ± standard deviation (SD). All data were analyzed by the ANOVA and t-test using GraphPad Prism software. A p-value less than or equal to 0.05 was accepted as statistically significant.

Results

Tannic acid suppressed the survival of the MDA-MB-231 cells

To explore the toxic effect of tannic acid on breast cancer cells, the MTT assay was performed. The results of the MTT assay showed that treatment with tannic acid significantly reduced the survival rate of MDA-MB-231 cells in a dose-dependent manner ($p< 0.05$, relative to the blank control). The IC_{30} and IC_{50} doses of tannic acid for 24 h treatment were 44.8 and 59.2 µM, respectively (Figure 1). There was no difference in cell survival rate among solvent control and blank control groups ($p> 0.05$).

Tannic acid altered the expression of metastatic, apoptotic, and ER stress-related genes

To investigate the effect of tannic acid on the expression of apoptotic, metastatic, cell cycle regulators and ER stress-related genes, qPCR was performed. The results demonstrated that the IC_{30} dose of tannic acid enhanced the expression of Bak, CHOP, and ATF4, compared to the control group ($p< 0.05$, Figure 2). Moreover, tannic acid reduced the expression of Bcl-2, P21and MMP-2 ($p< 0.05$, Figure 2). Moreover, the IC_{50} dose of tannic acid had a bigger effect on gene expression related to the

Table 1. The Primer Sequences Used in qPCR

Genes name	Sequences(5′ →3′)
β-actin FW	GACATCCGCAAAGACCTGTA
β-actin RV	GGAGCAATGATCTTGATCTTCA
Bak FW	GTTTTCCGCAGCTACGTTTTT
Bak RV	GCAGAGGTAAGGTGACCATCTC
MMP-2 FW	ATACCATCGAGACCATGCG
MMP-2 RV	CCAATGATCCTGTATGTGATCTG
Bcl-2 FW	GGATGCCTTTGTGGAACTG
Bcl-2 RV	CAGCCAGGAGAAATCAAACAG
P21 FW	TGGAGACTCTCAGGGTCGAAA
P21 RV	CGGCGTTTGGAGTGGTAGAA
CHOP FW	GCCTTTCTCCTTTGGGACACTGTCCAGC
CHOP RV	CTCGGCGAGTCGCCTCTACTTCCC
ATF4 FW	ATGACCGAAATGAGCTTCCTG
ATF4 RV	GCTGGAGAACCCATGAGGT

FW, Forward; RV, Reverse

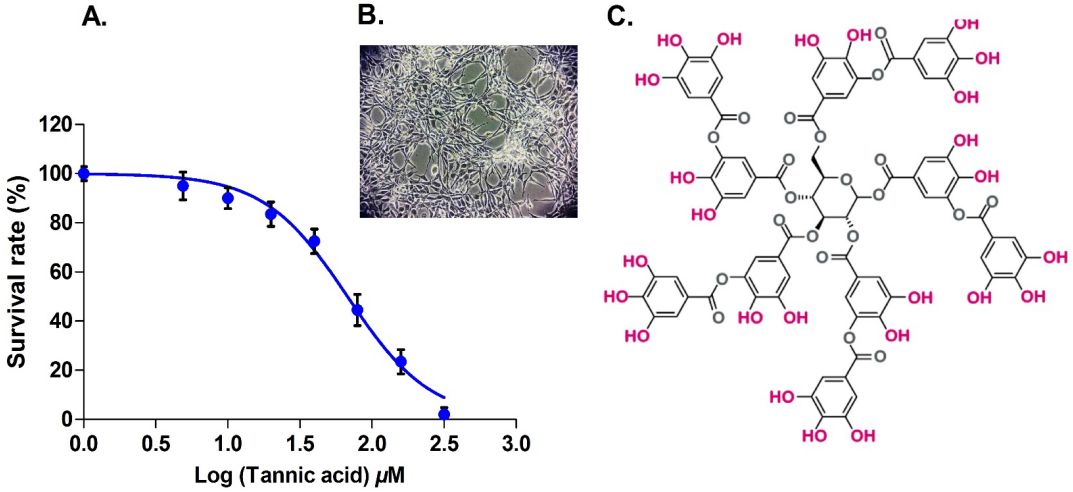

Figure 1. Effect of Tannic Acid on Cell Survival. The MDA-MB-231 cells were treated with tannic acid at indicated concentrations. Twenty-four hours after treatment, the cell survival was measured by the MTT assay. The cell survival curves were plotted using Graph Pad 6.1 software. The data are expressed as mean ± SD (n=3). (A) MTT graph, (B) cell morphology, (C) tannic acid structure.

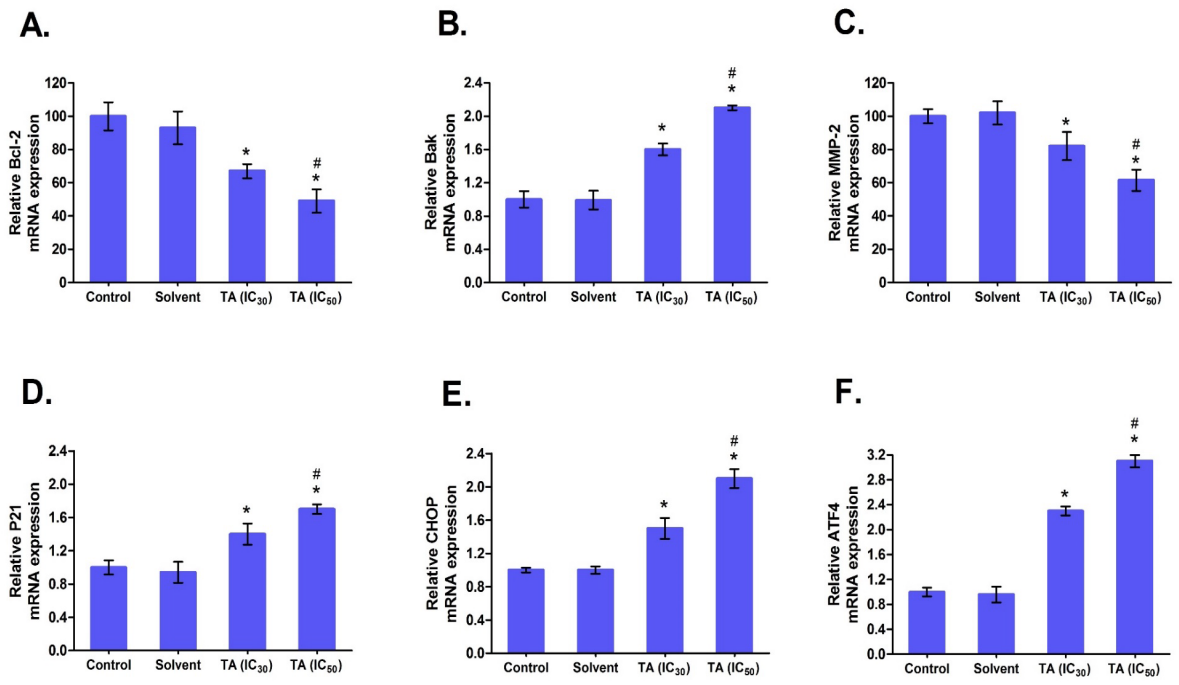

Figure 2. Gene Expression Analysis of MDA-MB-231 Cells Treated with Tannic Acid. The cells were treated with tannic acid for 24 h. Relative Bcl-2 (A), Bak (B), MMP-2 (C), P21 (D), ATF4 (E), and CHOP (F) mRNA expression was measured by RT-qPCR using the $2^{-(\Delta\Delta Ct)}$ method, and β-actin as an endogenous control. Data are presented as mean ± SD (n=3). *p< 0.05 relative to blank control; #p< 0.05 relative to IC30. TA, tannic acid.

IC_{30}. As we expected, there were no significant changes in gene expression, among the solvent control and the blank control groups (p> 0.05, Figure 2).

Tannic acid suppressed the migration of the MDA-MB-231 breast cancer cells

The wound healing assay was employed to evaluate the anti-metastatic effect of tannic acid on MDA-MB-231 cells. The results indicated that tannic acid (IC_{30}, IC_{50}) reduced the breast cancer cell migration dose-dependently. After 24h of treatment, the number of migration cells in treated groups decreased significantly compared to the blank control group (Figure 3). These results suggest that tannic acid can effectively suppress the migration of breast cancer cells.

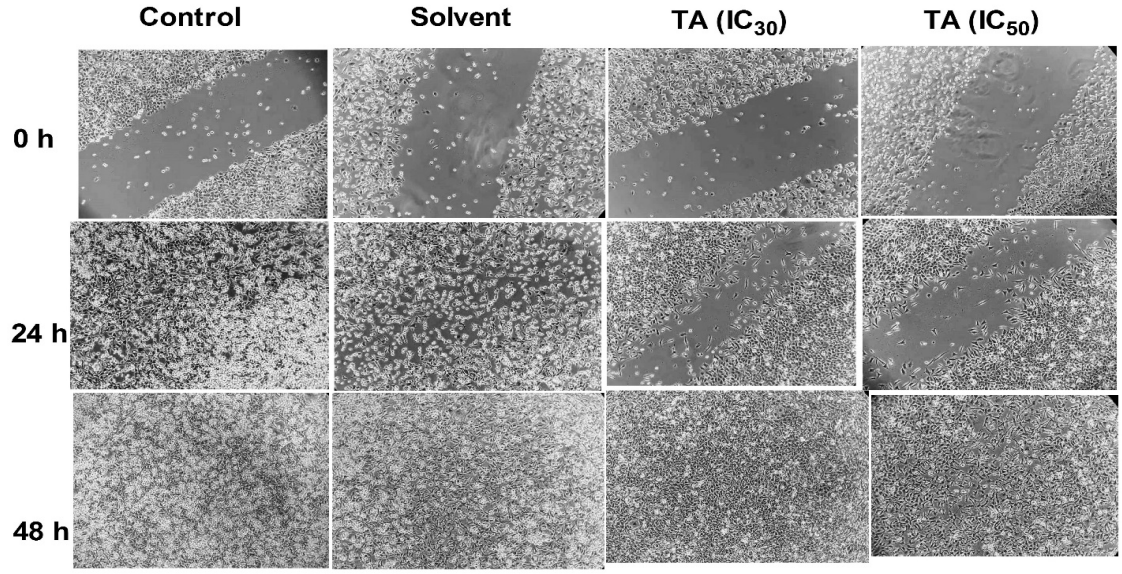

Figure 3. The Effect of Tannic Acid on Breast Cancer Cell Migration. MDA-MB-231 cell cultures were scratched by a sterile micropipette tip and the cells were treated with various IC_{30} and IC_{50} doses of tannic acid for 24 h. Cells migrated into the wounded region were photographed after 0, 24, and 48 hours of treatment. TA, tannic acid.

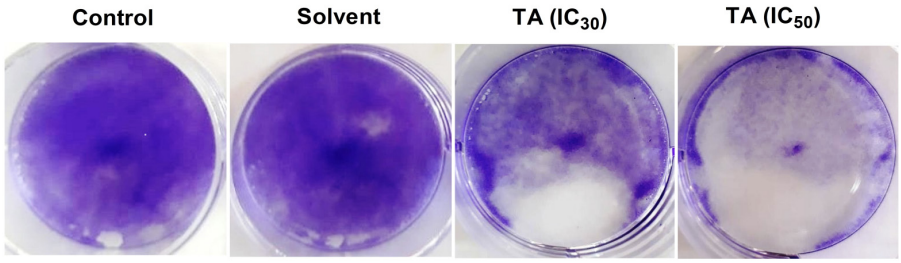

Figure 4. Tannic Acid Inhibited the Colony Formation Ability of Breast Cancer Cells. MDA-MB-231 cells were treated with tannic acid (IC30 and IC50) for 24 h. The cells were fixed and then stained with crystal violet and the cell colonies were photographed. TA, tannic acid.

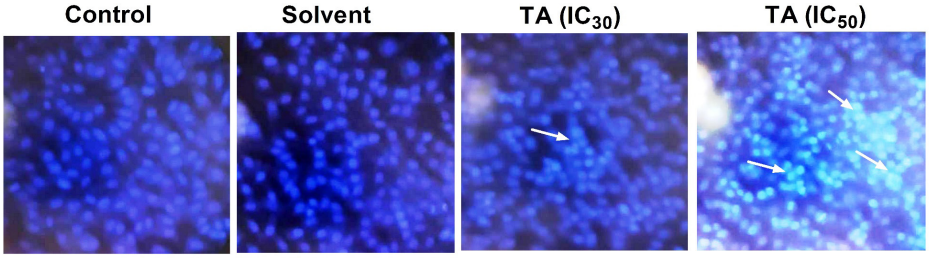

Figure 5. Induction of Apoptosis by Tannic Acid in MDA-MB-231 Cells. Cells were treated with IC_{30} and IC_{50} doses of tannic acid for 24 h. Nuclear morphological changes were observed using Hoechst33342 staining and a fluorescence microscope. Arrows indicate condensed and fragmented nuclei. TA, tannic acid.

Inhibition of the colony formation in breast cancer cells by tannic acid

The capability to form colonies in cancer cells is a prominent feature. The colony formation assay was performed to determine the effect of tannic acid on the growth and proliferation of MDA-MB-231 cells. Results showed that tannic acid (IC_{30}, IC_{50}) dose-dependently reduced the number of colonies compared to the blank control group (Figure 4). No marked difference between the solvent and blank control groups was observed.

Induction of apoptosis in breast cancer cells by tannic acid

Hochest33342 staining was used to investigate the induction of apoptosis based on the morphologic change of the nucleus on the MDA-MB-231 breast cancer cells. As shown in Figure 5, after 24 h of treatment, the tannic acid treated cells had more nuclear fragmentation and chromatin condensation in contrast with the blank control group. The IC_{50} dose of tannic acid had a stronger effect on apoptosis relative to the IC_{30}. Moreover, no difference was observed between the apoptotic features of the blank control and solvent control groups.

Discussion

Although potential treatments for breast cancer are chemotherapy, radiotherapy, recurrence, and metastasis are the main problems. Studies showed that activation of ER stress inhibits proliferation and metastasis and induces apoptosis in tumor cells. So far, the effect of tannic acid on ER stress in breast cancer cells has not been determined. In this study, the effect of tannic acid on ER stress and UPR the signaling pathway was investigated. Moreover, colony formation, cell migration, and, apoptosis of breast cancer cells were assessed.

Our results demonstrated that tannic acid significantly suppressed the ny formation and cell migration and triggered apoptosis in MDA-MB-231 breast cancer cells. Our study also showed that the gene expression levels of Bak and P21 were enhanced and the expression of Bcl-2 was decreased during tannic acid treatment. In addition, tannic acid reduced the expression of mesenchymal marker (MMP-2), which inhibited the EMT process. Moreover, the expression levels of ER stress-related genes ATF4 and CHOP were increased. So far, several studies have investigated the effect of tannic acid on cancer cells. For example, Nie et al., (2016) in a study investigated the effect of tannic acid on breast cancer cells. Their results showed that tannic acid reduced the expression of fatty acid synthetase and induced cell apoptosis. In another study conducted by Sun et al., (2012), they showed that treatment with tannic acid induced cell apoptosis by activating caspase-3, and increased the sensitivity of human ovarian cancer cells to cisplatin. The results of another study showed that tannic acid decreased survival and increased apoptosis in liver cancer cells. An increase in the production of reactive oxygen species and DNA fragmentation was also observed after treatment with tannic acid Mhlanga et al., (2019). A study by Nagesh et al., (2018) showed that tannic acid activated the UPR pathway in prostate cancer cells and increased the expression of ER stress proteins such as ATF4 and Bip. Activation of the UPR pathway was associated with a decrease in anti-apoptotic proteins (Bcl-xL and Bcl-2), cell cycle proteins (cyclin D1), and metastatic proteins (MMP-2 and MMP-9). Also, the expression level of pro-apoptotic proteins Bim and Bak increased. The results of the study by Darvin et al., (2015) determined that tannic

acid inhibited cellular cyclins as well as proteins Bcl-2 and Bcl-xL by inhibiting the transcription factor STAT3, and caused the cell cycle arrest and induced apoptosis in YD-38 gingival cancer cells. The study of Sp et al., (2020) showed that the growth and invasion of A549 lung cancer cells were reduced by treatment with tannic acid and the mitochondrial apoptosis pathway induced. In another study conducted by Pyrko et al., (2007), they showed that inhibition of Bip expression by siRNA is associated with increased expression of CHOP, activation of caspase-7, and decreased resistance of glioblastoma cells to temozolomide. The above data are consistent with the results of our study and confirm that the anti-growth and apoptosis inducing effect of tannic acid is mediated through the ER and UPR stress pathway.

Mechanistically activation of the ER stress leads to the accumulation of unfolded proteins in ER and the activation of the UPR pathway (Chowdhury et al., 2019; Baldwin and Booth, 2022). When the cell stress occurs, the Bip chaperon is disassociated from the sensor proteins such as PERK and activates it (Corazzari et al., 2017). Activated PERK then phosphorylates eIF-2α, and inhibits protein translation, leading to the entrance of ATF4 to the nucleus and enhancement of CHOP expression. CHOP protein then stimulates apoptosis by altering the expression of pro-apoptotic and anti-apoptotic proteins such as Bak and Bcl-2 (Lei et al., 2017; Nagesh et al., 2018). Our results are consistence with these data and show that tannic acid can induce apoptosis by up-regulation of the CHOP expression through UPR signaling pathway.

In conclusion, overall, our study suggests that tannic acid is a compound with anticancer properties, which prevents the proliferation, and metastasis of breast cancer cells. Additionally, tannic acid is correlated with ER stress and increases the expression of stress regulatory proteins such as CHOP and ATF4 which lead to the activation of the intrinsic pathway of apoptosis. These data demonstrate that tannic acid can be as a beneficial candidate for the prevention and treatment of breast cancer via inducing ER stress and UPR pathway.

Author Contribution Statement

Study concept and design: HK; Acquisition of data: VDC; Analysis and interpretation of data: HK, VDC and YA; Drafting of the manuscript: HK, VDC and YA; Critical revision of the manuscript for important intellectual content: HK and YA; Funding recipients: HK..

Acknowledgements

This work is part of M.Sc. thesis of Vida Dehghani Champiri that was supported by a grant from the Molecular and Medicine Research Center, Arak University of Medical Sciences (AUMS), Arak, Iran. The authors thank the Molecular and Medicine Research Center for providing the necessary equipment for this work.

Funding Statement

This work was supported by a grant from the Molecular and Medicine Research Center from AUMS of Iran [Grant number 6671].

Ethical approval

This research was ethically wise approved from Deputy of research and technology, Arak University of Medical Sciences, Arak, Iran [Number 6671].

Conflict of interest

The authors have no conflict of interest to declare.

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