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*Original Article*

# Combination of metformin and gallic acid induces autophagy and apoptosis in human breast cancer cells

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## *Abstract*

**Background and purpose:** Breast cancer is the most common type of cancer and one of the major causes of death among women. Many reports propose gallic acid as a candidate for cancer treatment due to its biological and medicinal effects as well as its antioxidant properties. This study aimed to assess the effects of metformin and gallic acid on human breast cancer (MCF-7) and normal (MCF-10) cell lines.
**Experimental approach:** MCF7 and MCF-10 cells were treated with various concentrations of metformin, gallic acid, and their combination. Cell proliferation, reactive oxygen species (ROS), as well as cell cycle arrest were measured. Autophagy induction was assessed using western blot analysis.
**Findings/Results:** Metformin and gallic acid did not cause toxicity in normal cells. They had a stronger combined impact on ROS induction. Metformin and Gallic acid resulted in cell cycle arrest in the sub-G1 phase with G1 and S phase arrest, respectively. Increased levels of LC3 and Beclin-1 markers along with decreased P62 markers were observed in cancerous cells, which is consistent with the anticancer properties of metformin and gallic acid.
**Conclusion and implications:** The effects of metformin and gallic acid on cancerous cells indicate the positive impact of their combination in treating human breast cancer.

***Keywords:*** Apoptosis; Autophagy; Breast cancer; Gallic acid; Metformin; ROS.

## INTRODUCTION

Cancer is a major global cause of death. The census shows that 8 million people died from cancer in 2008, and the death toll is expected to rise to 11 million by 2030. Breast cancer is the most common type of cancer and one of the major causes of death among women (1). Two molecular targets are significant in breast cancer's pathogenesis. The first one is the estrogen receptor alpha (ERα) which is a transcription factor activated by estrogen and is expressed in 70% of invasive cancers. Expressed progesterone receptor (PR) is also a sign of ERα signaling. The second molecular target is the epidermal growth factor 2 (ERBB2 or HER2) which is a transmembrane receptor tyrosine kinase overexpressed in about 20% of breast cancer cases. Triple-negative breast cancer constituting about 15% of breast tumors is diagnosed with the absence of ER, PR, and HER2 molecular targets (2). Breast cancer types are divided into invasive and non-invasive categories based on the location of the tumor. The size, type, and spread of cancer cells in other tissues determine the stages of breast cancer on a scale of zero to four. Understanding these stages is important in the treatment of breast cancer (3).

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The treatment methods are selected based on the type and stage of cancer and the patient's preference. Using herbal treatments is a natural alternative, given the cancer-treating properties of several plant species (4,5). Furthermore, the resistance to chemotherapy is another reason to look for additional treatment options (6).

Gallic acid (3,4,5-trihydroxybenzoic acid) is a common phenolic acid in plants observed as white or light-yellow crystals and found in grapes, wine, mangoes, green and black tea, as well as edible mushrooms (7). Gallic acid and its derivatives (such as lauryl gallate, propyl gallate, octyl gallate, tetradecyl gallate, and hexadecyl gallate) are natural antioxidants that are used in the food industry to prevent the oxidation of oils and fats by inhibiting the free radicals (8). Numerous studies have reported the biological and medicinal application of these phytochemicals with a focus on their antioxidant, antimicrobial, anti-inflammatory, anticancer, cardioprotective, and neuro-protective properties (9,10).

Metformin is the first-line treatment for type 2 diabetes. Owing to its broad mechanisms of action, it is proposed to be used in various illnesses. The safety, cost, and tolerance of metformin make it a subject of study for other treatment targets. Several pre-clinical and clinical studies reported its effect in treating various types of cancer (11). The anticancer mechanism of metformin might be through the autophagy-lysosome pathway. Metformin targets mitochondria, directly reducing ATP synthesis. The reduced energy pressure provides access to downstream AMP-activated protein kinase (AMPK). In addition, AMPK signaling pathway activation inhibits the mammalian target of the rapamycin pathway resulting in autophagy (12).

Autophagy is a complex process in which a cell destroys its faulty and old parts and recycles them for metabolism (13). Autophagy-related genes (ATG) code the required proteins. Autophagy is completed in three phases. First, nucleation and induction of autophagy-isolation membrane occur followed by isolation membrane elongation and completion of autophagosome. Finally, lysosomal fusion and cargo degradation complete the process (14).

Measuring microtubule-associated light chain 3 (LC3), P62, and Beclin-1 is the main marker for autophagy as they are the major proteins involved in the process. P62 protein is an adaptor with multiple binding motifs with a role in the formation of autophagy protein complexes (15). Beclin-1 protein forms a part of the class 3 phosphatidylinositol 3-phosphate kinase complex which participates in autophagy activation (16). Recent studies have shown that autophagy is significant in the pathophysiology of many conditions such as cancer, neurodegenerative diseases, autoimmune diseases, aging, cellular death, cardiovascular diseases, and infections. Autophagy's potential in regulating cellular death makes it the target of cancer treatment (12). Autophagy is believed to prevent cancer. However, once cancer develops, the autophagic flux stimulates the survival and the promotion of tumor cells (17). Combination therapy is a new and effective strategy in cancer treatment. Several studies show that using metformin in combination with other chemotherapy or non-chemotherapy medications can positively affect the prevention and treatment of breast cancer (18-20).

Considering the consequences of breast cancer and the side effects of current treatment options, we investigated the anticancer effects of metformin and gallic acid on normal (MCF-10) and cancer (MCF-7) human breast cell lines. Additionally, cell cycle arrest, apoptosis, and autophagy induction were evaluated as possible mechanisms of action.

## MATERIALS AND METHODS

### *Material*

Gallic acid, 98% purity, was purchased from Sigma-Aldrich (St. Louis, Missouri, United States) and dissolved in phosphate-buffered saline (PBS) at a concentration of 3 mg/mL at 40 °C. Metformin (1,1-Dimethylbiguanide hydrochloride; 98% purity) was purchased from Exir Pharmaceutical Company (Iran) and dissolved in PBS 1 M. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) powder, 2-7 dichlorofluorescein diacetate (DCFH-DA), and propidium iodide (PI) were procured from Sigma-Aldrich (St. Louis,

Missouri, United States). RNase A was purchased from Yekta Tajhiz Azma, Iran. Antibodies against LC3-B (3868S), P62 (88588S), and Beclin-1 (3495S) were obtained from Cell Signaling Technology (CST; Danvers, Massachusetts, United States), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; sc-47724) were obtained from Santa Cruz Biotechnology (California, USA). BMG Spectro Nano Elisa Reader (Germany) and BD FACSCalibur (USA) flow cytometers were also used.

***Cell culture and treatment***

MCF-7 and MCF-10 cell lines were obtained from the Cancer Research Center affiliated with Shiraz University of Medical Sciences. MCF-7 and MCF-10 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and DMEM/HamsF12 (Asagene, Iran), respectively, with high glucose and supplemented with 10% fetal bovine serum (FBS; Gibco, USA) and 1% penicillin-streptomycin antibiotic (Gibco, USA) in a humid incubator at 37 °C and 5% $CO_2$. Cells were treated with metformin (6.25, 12.5, 25, 50, and 100 mM), gallic acid (15, 30, 60, 90, and 120 µg/mL) and their combinations for 48 h.

***Cytotoxicity assay***

The MTT method was developed based on the cleavage of a yellow tetrazolium salt to purple formazan crystals by mitochondrial enzymes in metabolically active cells as previously described (21,22). MTT assay was used to study the level of cytotoxicity of metformin, gallic acid, and their combination on MCF-7 and MCF-10 cells. For this purpose, $1.5 \times 10^4$ MCF-7 and MCF-10 cells were seeded separately in 96-well plates. After 16 h, treatment with different concentrations of metformin (6.25, 12.5, 25, 50, and 100 mM) and gallic acid (15, 30, 60, 90, and 120 µg/mL) were selected based on previous studies (18,23) and their combination were applied on both cell lines for 48 h. Also, untreated cells were considered a negative control group. Then, 20 µL of MTT stock solution with a final concentration of 0.5 mg/mL was added to each well and incubated for additional 4 h. The culture medium was removed and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance of the samples was read with the ELISA Reader (BMG Spectro Nano, Germany) at 570 and 630 nm wavelengths, and the percentage of alive cells was calculated. In the same way, three independent repetitions were also done for each group. In the pilot study, the treatment of cancer cells was studied for 24 h. Due to the toxicity of the effective concentration of the compound to normal cells, the treatment duration was increased to 48 h by decreasing the concentration. Half maximal inhibitory concentration ($IC_{50}$) was afterward calculated.

***Detection of intracellular reactive oxygen species production***

DCFH-DA staining was used to detect reactive oxygen species (ROS) production in MCF-7 cells (24). The total populations of $5 \times 10^4$ cells were pre-cultured in a 6-well culture plate following incubation with metformin, gallic acid, and their combination. After incubation for 48 h, the culture medium was removed, and cells were trypsinized. The medium containing cells was centrifuged at 1200 rpm for 3 min at 4 °C. Then the cells were stained with PBS containing DCFH-DA (10 µM, 500 µL) at 37 °C for 60 min. Finally, at an excitation wavelength of 488 nm, the fluorescence intensity of differently treated cells was determined using a flow cytometer (BD FACSCalibur, USA)

***Cell cycle analysis by flowcytometry***

The cell cycle test was performed on the MCF-7 cell line. The cell cycle was investigated by pre-culturing $5 \times 10^4$ cells in each well in a 24-well plate. After 16 h incubation, different concentrations of gallic acid, metformin, and their combination were introduced to the cells in a fresh media. After 48 h, cells were harvested with 0.25% trypsin, followed by fixation in 4 mL of ice-cold ethanol 70% for 1 h. After centrifugation at 4000 rpm for 5 min, the cells were re-suspended in 500 µL PBS containing 0.25% Triton® X100 and incubated for an additional 15 min on ice cold water. Then, cells were spun down for 2 min at 4000 rpm. The cells were finally re-suspended in 500 µL PBS containing

10 µg/mL of RNase A and 20 µg/mL of PI for 30 min. The cell cycle analysis was studied using a BD FACSCalibur flow cytometer (24).

***Western blotting analysis***

MCF-7 cells were seeded at a density of $10^6$ in 25-mL flasks. After incubation, cells were treated with metformin and gallic acid at selected and combined concentrations for 48 h. Cells were then lysed in NP-40 lysis buffer and protein concentrations were determined using a bicinchoninic acid assay kit for normalization. Then, the proteins were electrophoresed on a sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel. Membranes were blocked with 5% nonfat milk and coated with primary antibodies to P62, LC3, Beclin-1, and GAPDH (as an internal control) (1:1000 dilution) in a cold room overnight. Then the membranes were washed with 1X TBST buffer three times for 20 minutes and covered with horseradish peroxidase-conjugated secondary antibodies (1:2000 dilution) for 2 hours at room temperature and then using luminescence reagents to enhance the spots for 2 min. Quantitative spots were photographed using the ChemiDoc MP imaging system (Bio-Rad, USA). To quantify the band's intensity, a densitometer was used, and the images were analyzed with Image Lab version 5.2.1 software (Bio-Rad, USA) (25).

***Statistical analysis***

All experiments were repeated three times, and the results were presented as mean ± SEM of triplicate experiments. One-way and two-way ANOVA tests determined variations between independent groups followed by Tukey's multiple comparison test. *P*-values less than 0.05 were accepted as statistically significant. Biostatistical analyses were performed using GraphPad Prism 9 software.

## RESULTS

***The effects of metformin and gallic acid on the proliferation of breast cancer cells***

Inhibition of cell proliferation was investigated by the MTT test and as shown in Fig. 1, the viability of cancerous cells was inhibited in a concentration-dependent manner. Based on MTT tests over a 48-h treatment, all concentrations of metformin (Fig. 1A) inhibited the MCF-7 cells proliferation significantly. The value of $IC_{50}$ for 48 h metformin treatments was 34.46 mM. All concentrations of gallic acid except the 15 µg/mL (Fig. 1B) inhibited the proliferation of MCF-7 cells significantly. The value of $IC_{50}$ of gallic acid treatments after 48 h was 47.71 µg/mL. According to MTT results, the $IC_{50}$ concentration of metformin (35 mM) and the non-toxic concentration of gallic acid were considered for co-treatment. As shown in Fig. 1C, the viability of MCF-7 cells co-treated with metformin and gallic acid was reduced significantly compared to the single treatment. All the concentrations of gallic acid (15, 30, and 60 µg/mL) + metformin 35 mM led to reduced MCF-7 cell viability by 27.4%, 48.5%, and 50.7% in comparison with metformin 35 mM, respectively. This indicated that metformin increased the effect of gallic acid on reducing the viability of cancerous cells. Therefore, it can be concluded that metformin and gallic acid cause toxicity in MCF-7 cells and amplify each other's effect on inhibiting the proliferation of cancerous cells.

As shown in Fig. 1D metformin in low concentrations has no significant toxicity on MCF-10 cells. In the metformin treatment on MCF-10 cells, the $IC_{50}$ value was calculated at 75.28 mM which is more than the $IC_{50}$ value in MCF-7 cells. As a result, it can be considered that the toxicity of metformin for the normal cell line is much lower than that of the cancerous cell line.

Figure 1E exhibited that gallic acid does not have toxicity on MCF-10 cells at 15 and 30 µg/mL. However, at the concentrations of 60, 90, and 120 µg/mL cell viability decreased significantly. The $IC_{50}$ value for gallic acid treatments was > 120 µg/mL.

The co-treatment of gallic acid (15, 30, and 60 µg/mL) + metformin (35 mM) considerably resulted in decreased normal cells viability by 18%, 27%, and 42% compared to the metformin-treated group as shown in Fig. 1F. Therefore, due to gallic acid 60 µg/mL + metformin 35 mM considerable toxicity in normal cells, this combination was excluded from further assays of this research.

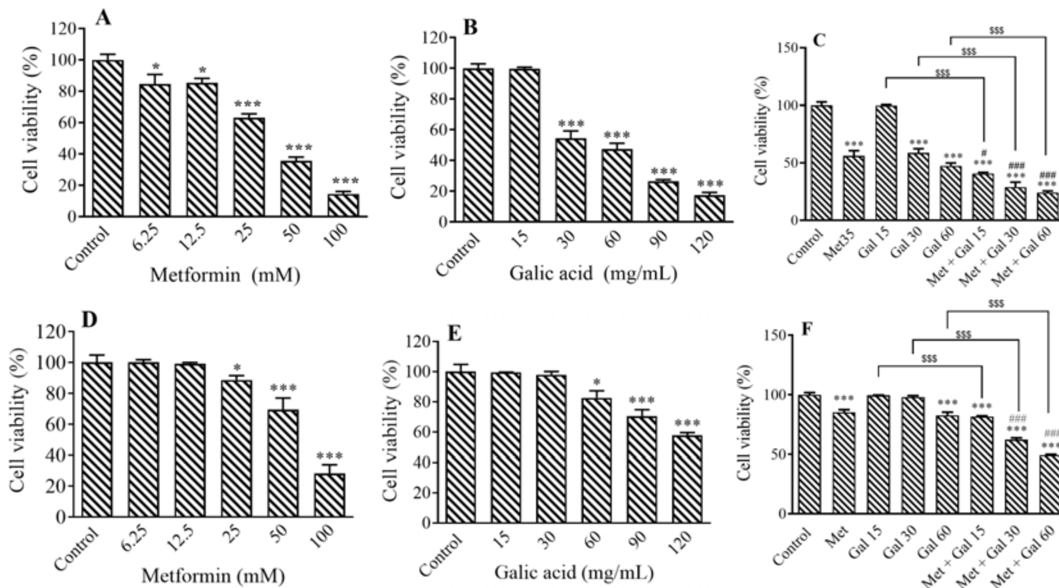


**Fig. 1.** Effect of metformin, gallic acid, and their combinations on the viability of MCF-7 and MCF-10 cell lines. Cell viability was measured using an MTT assay. MCF-7 cells were treated for 48 h with (A) metformin (6.25, 12.5, 25, 50, and 100 mM); (B) gallic acid (15, 30, 60, 90, and 120 µg/mL); or (C) metformin (35 mM) and gallic acid, alone, or their combination. (D) MCF-10 cells were treated for 48 h with (D) metformin (6.25, 12.5, 25, 50, and 100 mM); (E) gallic acid (15, 30, 60, 90, and 120 µg/mL); or (F) metformin (35 mM) and gallic acid, alone, or their combination. Data are shown as a percentage of three independent tests (mean ± SEM). Met, Metformin; Gal, gallic acid. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate significant differences compared with the control group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus the group treated with metformin at 35 mM (Met); and $^{\$\$\$}P < 0.001$ between the defined groups.

***The impact of metformin and gallic acid on the production of ROS***

The ROS production in cancerous cells treated with metformin and gallic acid was investigated. Metformin enhanced the ROS level in MCF-7 cells compared to the control group as shown in Fig. 2.

According to the results, ROS production was not observed in the cells treated with gallic acid at 15 µg/mL. Also, the results of the MTT test showed that gallic acid at 15 µg/mL is not able to inhibit the proliferation of cancer cells, so the antioxidant activity of gallic acid was concluded. However, gallic acid at 30 µg/mL significantly elevated the ROS production in cancerous cells compared to the control group.

It was demonstrated that metformin and gallic acid co-treatment leads to a noticeable overproduction of intracellular ROS when compared to gallic acid at 15 and 30 µg/mL or metformin at 35 mM. Specifically, the production of ROS in the combination of metformin and gallic acid 30 µg/mL was more than each compound used alone, therefore, metformin and gallic acid reinforce each other's effect in inducing ROS production.

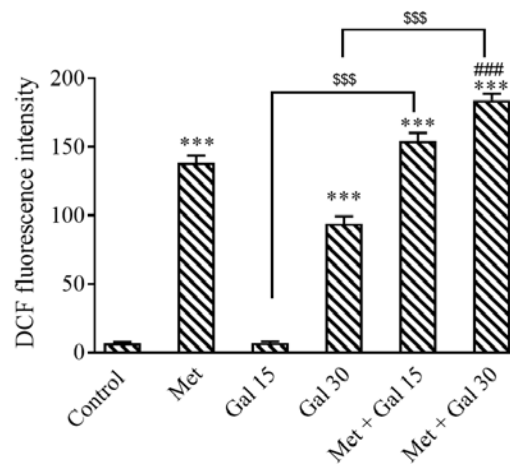


**Fig. 2.** The effect of different concentrations of metformin, gallic acid, and their combinations on the production of reactive oxygen species. MCF-7 cells were treated with metformin (35 mM) and gallic acid (15 and 30 µg/mL), alone or in combination, for 48 h. Data are shown as a percentage of three independent tests (mean ± SEM). Met, Metformin; Gal, gallic acid. $^{***}P < 0.001$ indicates significant differences compared with the control group; $^{\#\#\#}P < 0.001$ versus the group treated with metformin at 35 mM (Met); and $^{\$\$\$}P < 0.001$ between the defined groups.

***The effect of metformin and gallic acid on inducing cell cycle arrest***

The results of the cell cycle test using PI staining revealed that metformin, gallic acid, and their combination considerably increase apoptosis in MCF-7 cells (Fig. 3A and B).

Flow cytometry test revealed that metformin increases the number of cancer cells in the sub-G1 phase, which means that apoptosis occurs. Also, metformin treatment elevated the population of cells in the G1 phase resulting in cell cycle arrest in this phase. In the same way, the number of cells in the S and G2/M phases decreased in metformin treatment, and the cells could not pass through the G1 phase.

Besides, treatment of MCF-7 cells with gallic acid at 15 and 30 μg/mL increased the cell population in the sub-G1 phase. Thus, gallic acid induced apoptosis in this cell line at these concentrations. In addition, the population of cells in the G1 phase significantly reduced in the group treated with gallic acid at 30 μg/mL. In contrast, the accumulation of MCF-7 cells in the S phase indicates cell cycle arrest in this phase. Moreover, gallic acid at 15 and 30 μg/mL did not induce any remarkable change in G2/M phases.

In co-treatment groups, the population of cells in the sub-G1 phase was noticeably increased compared to each of the compounds alone. While a significant decrease was observed in the G1 and S phases compared to the metformin, an increasing proportion of cells in the G2/M phase was seen. Based on these results, it can be asserted that metformin and gallic acid cause cell cycle arrest and apoptosis induction in MCF-7 cells with different mechanisms.

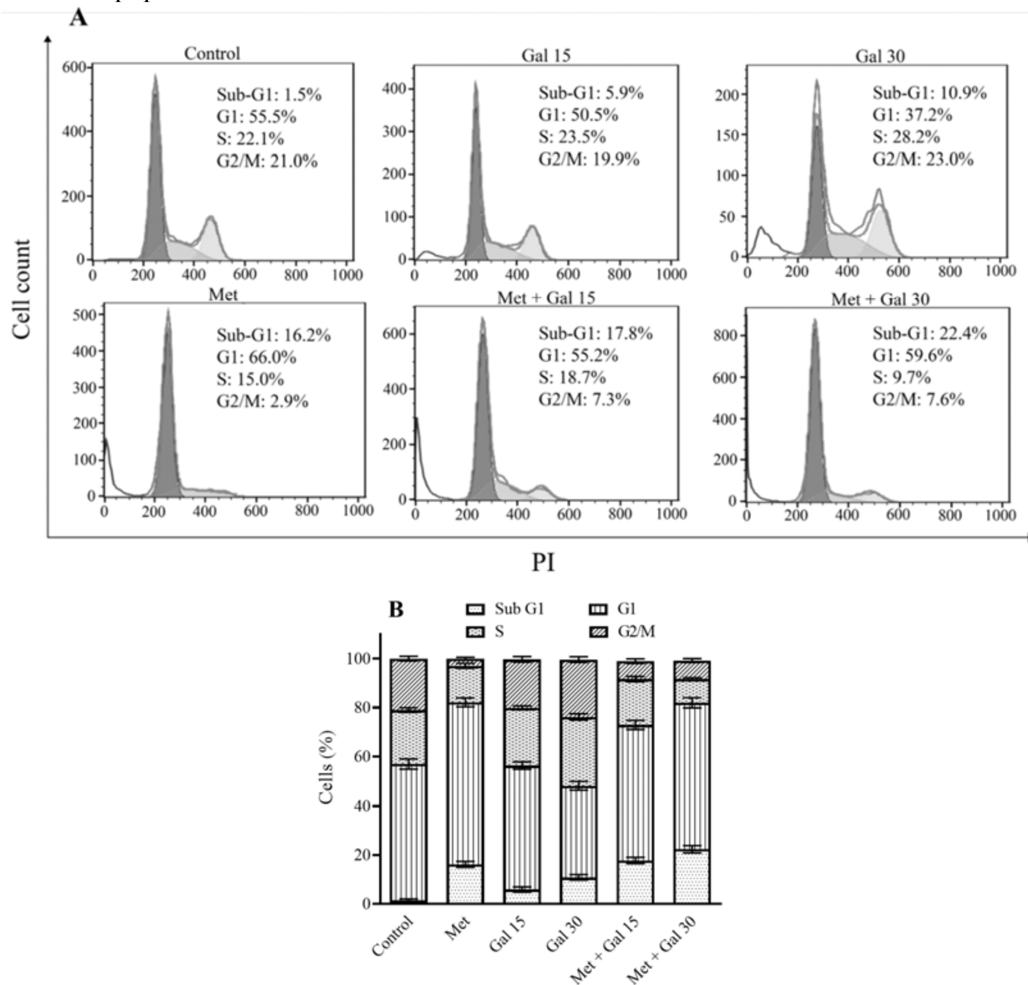


**Fig. 3.** The effect of metformin (35 mM) and gallic acid (15 and 30 μg/mL), alone or in combination on the cell cycle of MCF-7 cells. (A) Cell cycle analysis histograms and (B) the percentage of different cell-cycle phases. Data are shown as three independent tests (mean ± SEM). Met, Metformin; Gal, gallic acid; PI, propidium iodide.

***The effect of metformin and gallic acid on the induction of autophagy in cancer cells***

Western blot analysis was conducted to investigate autophagy induction. As shown in Fig. 4A-D, MCF-7 cells treatment with metformin at 35 mM considerably elevated the expression of Beclin-1, reduced P62, and did not change LC3-II protein expression which indicated autophagy induction. In gallic acid treatments, the changes in measured autophagy markers showed that autophagy is inhibited or failed. The LC3-II and Beclin-1 protein levels did not change in the treatment with 15 µg/mL of gallic acid, while P62 increased significantly indicating failure in the autophagy process. While, gallic acid at 30 µg/mL only reduced the level of LC3-II significantly, which demonstrates autophagy inhibition.

Furthermore, it was found that the co-treatment of metformin and gallic acid changed autophagy markers. Increasing Beclin-1 and decreasing P62 levels in metformin 35 mM + gallic acid 15 µg/mL were observed. In addition, metformin 35 mM + gallic acid 30 µg/mL increased LC3-II protein expression, along with a decrease in the level of P62 considerably.

In the co-treatment of metformin + gallic acid 15 µg/mL, a reduction in Beclin-1 protein level compared to metformin treatment alone was observed. Also, P62 protein was decreased significantly compared to metformin and gallic acid treatments. In the combination of metformin + gallic acid 30 µg/mL, LC3-II protein level increased compared to metformin and gallic acid treatments. While the P62 protein level was diminished compared to the gallic acid treatment alone.

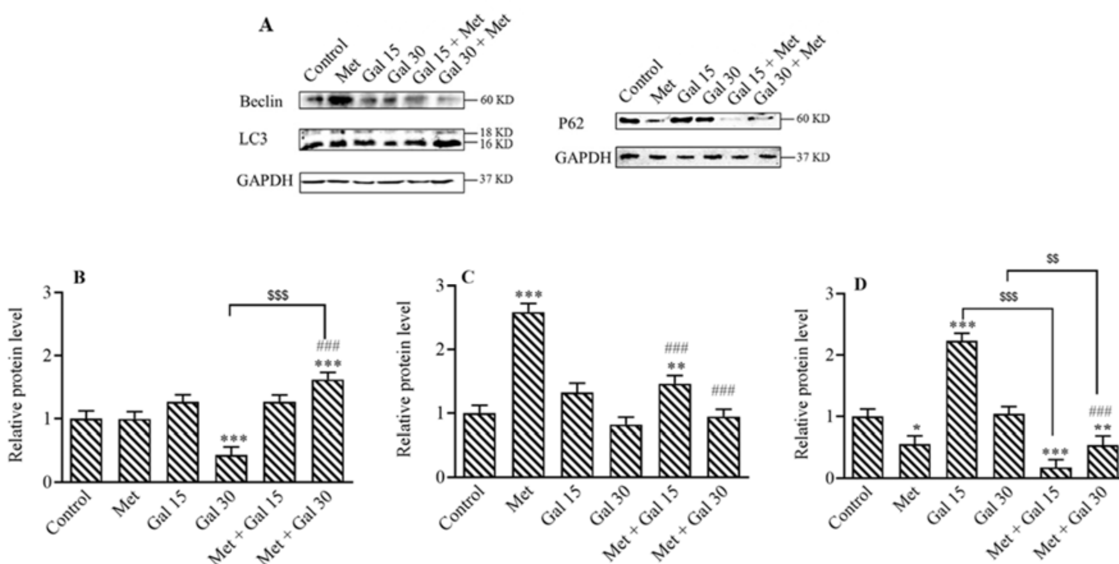


**Fig. 4.** The effect of metformin (35 mM) and gallic acid (15 and 30 µg/mL), alone or in combination, on autophagy. (A) Representative images from the western blot analysis of LC3-II, Beclin-1, and P62 proteins in MCF-7 cells. GAPDH was used as a loading control. Western blotting analysis of the expression of (B) LC3-II, (C) Beclin-1, and (D) P62 proteins in treated MCF-7 cells. Protein expression has been calculated in proportion to the control group. Data are shown as a percentage of three independent tests (mean ± SEM). Met, Metformin; Gal, gallic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. $^{*}P < 0.05$ and $^{***}P < 0.001$ indicate significant differences compared with the control group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ versus the group treated with metformin at 35 mM (Met); and $^{\$\$\$}P < 0.001$ between the defined groups.

## DISCUSSION

Breast cancer is the most common type of cancer and the leading cause of death in women. The undesirable side effects of current cancer treatments require the search for new treatments. Metformin is commonly used to treat type 2 diabetes. However, its unique characteristics make it a candidate for other treatments. Many preclinical and clinical studies have investigated the effectiveness of metformin in the treatment of cancer. Also, gallic acid is a known polyphenol that is commonly available in food sources. Many reports have studied the biological and medicinal effects of gallic acid with attention to its antioxidant, pro-oxidant, and anticancer therapeutic properties. This research investigated the combined effects of metformin and gallic acid on normal (MCF-10) and cancerous (MCF-7) human breast cell lines.

A previous study has reported that breast cancer cells treated with metformin exhibited decreased mitochondrial respiration and proliferation, and increased glycolysis (26). The MTT assay results in this study showed that treating breast cancer cells with metformin reduced cell proliferation in a concentration-dependent manner. Meanwhile, metformin treatment was not cytotoxic on normal MCF-10 cells at low concentrations. Additionally, metformin caused less toxicity at higher concentrations in normal cells than in cancer cells. As confirmed by other studies, the lack of toxicity of metformin in normal breast cells, suggests that Metformin is an option in cancer treatment (27-29).

Furthermore, treatment of normal and cancerous human breast cell lines with gallic acid revealed its selective cytotoxic effect on the cancer cell line. The calculated $IC_{50}$ for the normal human cell line (> 120 µg/mL) was significantly higher than the $IC_{50}$ for the cancer cell line (47.71 µg/mL) indicating the low toxicity of gallic acid to the normal breast cell line. These results indicated that gallic acid selectively inhibits cancer cell proliferation while remaining safe for normal human breast cells. However clinical studies are needed to confirm this claim. Various studies have shown the selective effect of gallic acid on liver cancer cells (30), and another study indicated that gallic acid is not toxic to normal human breast cells (31).

In the co-treatment of metformin and gallic acid, the concentrations were optimized to produce minimal toxicity on normal cell line while reducing cancer cell survival. We observed that the combination of gallic acid and metformin significantly reduced the MCF-7 cell viability compared to the treatment with each compound alone. This was especially evident when the addition of metformin to 15 µg/mL gallic acid reduced cancer cell viability, whereas gallic acid at 15 µg/mL did not affect cancer cells. In a study by Fatehi *et al.,* metformin enhanced the anticancer effects of resveratrol on breast cancer cells (18). Rezaei-Seresht et al. studied the cytotoxicity of gallic acid and caffeic acid on breast cancer cells and showed that each polyphenol causes cytotoxicity by a different mechanism (32).

ROS plays a pivotal role in regulating several cell survival and death pathways as well as in the mechanisms of action of many anticancer compounds, because cancer cells have a weaker antioxidant capacity, and some chemotherapeutic drugs induce apoptosis of cancer cells by generating ROS (33). Our observations of ROS levels on MCF-7 cells indicated that metformin markedly induced ROS in these cells. A previous study conducted by Queiroz *et al.* stated that metformin increased ROS production in MCF-7 cells which was associated with an increased anti-proliferative effect (34).

According to our results, gallic acid at 30 µg/mL significantly induced ROS production and reduced breast cancer cell viability. However, the concentration of 15 µg/mL had no pro-oxidant effect on MCF-7 cells. Gallic acid plays cytotoxic and antitumor roles by establishing an antioxidant/pro-oxidant balance. In some cases, gallic acid may control the carcinogenic effects of ROS by activating superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase, or by reducing lipid peroxidation and ROS generation. In other cases, gallic acid can induce cell cycle arrest, autophagy, and apoptosis by activating the caspase pathway and generating ROS (9).

Also, the ability of gallic acid to protect cells against oxidative stress or pro-oxidant activity depends on the type of cell line, concentration, or exposure time (35). Furthermore, our results showed that the ROS generation in the MCF-7 cells co-treated with 30 µg/mL gallic acid and metformin was higher than that of treated with metformin or gallic acid alone.

The cell cycle represents a series of tightly integrated events that allow cells to grow and proliferate. Agents abrogate cell cycle checkpoints at critical times, making the tumor cell vulnerable to apoptosis. Understanding the cell cycle is necessary to understand how best to develop these agents clinically, both as single agents and in combination with chemotherapy (36).

Cell cycle distribution was analyzed to investigate how the combination of metformin and gallic acid affects the growth of MCF-7 cells. We found that metformin treatment increased the number of cells in the sub-G1 phase, indicating apoptosis, as well as the number of cells in the G1 phase causing cell cycle arrest. Previous research showed that metformin promotes G1 cell cycle arrest in a cyclin G2-dependent manner in the estrogen-sensitive breast cancer cell line MCF-7. Furthermore, cyclin G2 is negatively regulated by estrogen-bound ER (E2), and overexpression of cyclin G2 induces p53-dependent cell cycle arrest in G1-phase (37). Additionally, it is widely accepted that the inhibitory effects of metformin on growth are related to the stimulation of AMPK which detects energy levels in various eukaryotic cell types. Activation of AMPK leads to the arrest of the G1 phase of the cell cycle through the upregulation of the p53-p21waf1 axis (38). As mentioned previously, the anti-proliferative effects of metformin are associated with elevated oxidative stress and the stimulation of AMPK and FOXO3a, ultimately leading to cell cycle arrest or apoptosis (34). Our findings are consistent with previous studies indicating that metformin can induce apoptosis by arresting the cell cycle at the sub-G1 phase in various cancer cell types, such as lung cancer (39), nasopharyngeal carcinoma (40), and myeloma (41).

Gallic acid increased cell population in the sub-G1 phase and cell cycle arrest in the S phase, suggesting induction of apoptosis in MCF-7 cells. Consistent with our research, previous studies have shown that gallic acid induces mitochondrial dysfunction *via* a decrease in the mitochondrial membrane potential, an increase in the Bax/Bcl-2 ratio, and cytochrome C release into the cytosol, as well as activation of caspase-9 in MCF-7 cells (42). Also, gallic acid induces the accumulation of the cells in the sub-G1 phase in MDA-MB-231 breast cancer (43) and Caco-2 colon cancer (44) cell lines. In addition, it has been found that oxidative stress can trigger cell cycle arrest in the S phase (45). Therefore, we hypothesized that ROS generation promotes apoptosis induction in the MCF-7 cancer cell line.

Furthermore, simultaneous metformin and gallic acid treatment increased the number of cells in the sub-G1 phase more than any individual treatment while decreasing cell numbers in the S and G2/M phases. This indicates that they have enhanced cell cycle arrest and apoptosis through different mechanisms. Therefore, identifying any chemical compound that causes cell cycle arrest is highly valued in cancer treatment research (46).

Autophagy can be triggered to maintain cellular hemostasis by various internal and external stimuli (such as chemotherapy, oxidative stress, starvation, and so on) (47). Autophagy normally helps cancer cells cope with nutrient deprivation and hypoxia. Autophagy modulation can have opposing roles in tumor suppression and promotion. Although autophagy modulators have been used as a novel anticancer strategy, how to manipulate autophagy to improve the treatment of established cancers remains unclear (48).

Based on western blot results, the changes in autophagy markers, an increased Beclin-1 protein level, and a decreased P62 confirmed that metformin can induce autophagy in MCF-7 cells. This result, besides generating ROS and inhibiting the proliferation of cancerous cells, may also support the occurrence of autophagic cell death through metformin treatment. Other studies support that metformin by inhibiting complex 1 of the mitochondrial electron

transport chain, reduces ATP production, increases the AMP/ATP ratio, triggers apoptosis *via* accumulation of unfolded or misfolded proteins, and also, activates unfolded protein response-mediated apoptotic death signaling and autophagic cell death (49).

However, treatment of the same cells with 15 µg/mL of gallic acid significantly increased the P62 levels, without notable changes in LC3-II and Beclin-1 levels, as mentioned previously, which may be due to disturbances in the autophagy process (50). Moreover, treatment with gallic acid 15 µg/mL increased autophagy but accumulated dysfunctional autophagic vesicles. It appears that the disruption of autophagy may be due to the antioxidant effects of gallic acid, which prevents cell death by activating other autophagic pathways. Also, in 30 µg/mL gallic acid treatment, changes in autophagy markers and autophagy inhibition were consistent with previous studies showing that autophagy inhibition enhances the anticancer effects of chemotherapy drugs or induction of cell death by apoptosis (51-53).

Cell viability and ROS assay, as well as cell cycle analysis, showed the effectiveness of the co-treatment of metformin and gallic acid in reducing cancer cells' survival and inhibiting their proliferation. ROS are capable of causing severe damage to cellular molecules, including DNA, RNA, and proteins. A growing body of evidence suggests that several important factors, such as p53, PARP-1, and FoxO3A, involved in DNA repair mechanisms, may also influence the regulation of autophagy. After DNA damage, the transcriptional program of FoxO3A was activated, leading to the upregulation of various ATGs, such as LC3, GABARAPL1, ATG12, BNIP3, and BNIP3L (54).

The results of this study showed changes in autophagy markers indicating the occurrence of autophagy in cancer cells. Initiation of autophagy was observed by an increase in LC3-II protein levels in the co-treatments. Increased Beclin-1 protein levels are another indication of the progression of autophagy and autophagosome formation that we observed in combination treatment. Finally, by reducing the levels of P62 protein, the autophagy process is completed and P62 is hydrolyzed. Similar findings regarding changes in autophagy markers have been confirmed in previous studies of breast cancer cells (51,55,56). Therefore, in the co-treatment of metformin and gallic acid, the autophagy was completed, and according to the rate of cell death in this treatment, it can be said that the induction of autophagy was in line with the anticancer effects of metformin and gallic acid. Therefore, the autophagy induction mechanism is proposed as a cancer treatment method (57). Recently, Kumar *et al.* showed a synergistic chemoprotective effect of metformin and gallic acid against breast cancer in a rat model with possible interference of inflammatory mediators (58).

The effectiveness of chemotherapeutic medication combinations in successfully treating various tumors is currently being studied in clinical trials. The use of natural substances/phytochemicals in combination with metformin as a chemotherapeutic drug has attracted increasing attention because it targets cancer cell metabolism and induces energy stress with minimal side effects, both as a neoadjuvant and adjuvant treatment modalities (49).

Our limited understanding of changes in apoptotic factors and autophagy signaling regulation pathways limits our ability to characterize cell death events. The variations in dosage and exposure duration having been reported in the studies may be considered additional difficulties associated with polyphenol therapy.

## CONCLUSION

We have demonstrated that co-treatment of the effective and safe drug metformin with the natural polyphenol gallic acid, found in many food sources, can inhibit the proliferation and progression of breast cancer cells. Furthermore, our study showed that the applied concentrations did not cause toxicity in the normal breast cell line (MCF-10). The combination of metformin and gallic acid can induce apoptosis of cancer cells. We suggest that this combination may be an effective option for treating breast cancer with fewer side

effects. Finally, induction of autophagy-mediated cell death by a co-treatment of metformin and gallic acid may be identified as a new target in cancer therapy, requiring further pre-clinical and clinical studies.

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***Conflict of interest statement***

The authors declared no conflicts of interest in this study.

***Authors' contributions***

M. Haghshenas and M. Rashedinia, and N. Firouzabadi conceived and designed the work, analyzed the data, wrote and revised the article. M. Haghshenas and A.R. Akbarizadeh collected and analyzed the data. The finalized article was approved by all authors.

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