

Effect of Ellagic Acid and Cryptotanshinone on Cell Viability/Cytotoxicity, Metastasis, and Oxidative Stress in Triple-Negative Breast Cancer Cells

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Cite this article as: Yilmaz U, Seyhan MF. Effect of ellagic acid and cryptotanshinone on cell viability/cytotoxicity, metastasis, and oxidative stress in triple-negative breast cancer cells? *Experimed* 2024; 14(1): 46-53.

ABSTRACT

Objective: Triple-negative breast cancer (TNBC) has the highest rate of metastases and relapses as well as the worst overall survival of all breast cancers. Here, we aimed to investigate the effects of ellagic acid and cryptotanshinone, which are known to have antioxidant, antimutagenic, anticancer, and apoptotic effects, on cell viability/cytotoxicity, metastasis, and oxidative stress in MDA-MB-231 cells.

Materials and Methods: The effects of various concentrations of ellagic acid and cryptotanshinone on cell viability or cytotoxicity in TNBC cells were determined by WST-1. A scratch assay was performed to determine the effects of ellagic acid and cryptotanshinone on cell migration and metastasis, and a DCF-DA test was performed to determine the reactive oxygen species (ROS) levels.

Results: MDA-MB-231 cells exposed to cryptotanshinone exhibited reduced cell proliferation by approximately 50%, particularly at 20 µg/mL after 48 h. The cell viability decreased by 75% at 20 µg/mL after 72 h of cryptotanshinone exposure. After 48 h of exposure to ellagic acid at 40 µg/mL, the scratch in the MDA-MB-231 cells closed. In addition, treatment with cryptotanshinone at 25 µg/mL covered the scratch after 72 h. Ellagic acid (40 µg/mL) induced oxidative stress at 24 h, and cryptotanshinone (25 µg/mL) at 48 and 72 h. Furthermore, the fluorescence intensity of MDA-MB-231 cells was increased by exposure to ellagic acid (40 µg/mL) and cryptotanshinone (25 µg/mL) after 24 h compared to the negative control.

Conclusion: Ellagic acid and cryptotanshinone may inhibit cell proliferation, suppress cell migration, and induce the accumulation of intracellular ROS in MDA-MB-231 cells.

Keywords: Triple-negative breast cancer, MDA-MB-231, cell culture, ellagic acid, cryptotanshinone

INTRODUCTION

Breast cancer (BC) tumors can become resistant to treatment, and with the formation of resistance, the BC tumors are not limited to the primary area and can metastasize to other regions (1). Triple-negative breast cancer (TNBC) is a type of BC with high rates of metastasis and recurrence and has the worst overall survival rates of all BC subtypes. Estrogen, progesterone, and human epidermal growth factor receptor 2 (HER-2) receptors are absent in TNBC (2). Considering that TNBC does not respond to chemotherapy and radiotherapy, its high

incidence and mortality rates, and the limitations in treatment effectiveness, have resulted in the need to find alternative treatments for TNBC (3).

In recent years, natural plant phenolic and flavonoid compounds have attracted attention because of their anticarcinogenic, antioxidant, and antiinflammatory activities and their potential to be used as an adjunct to cancer treatment (3). Furthermore, phytochemicals have been among the alternative cancer treatments in recent years because of their anticarcinogenic, antiinflammatory, and antitumor effects. Phytochemicals play an active

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Submitted: 02.02.2024 **Revision Requested:** 04.03.2024 **Last Revision Received:** 28.03.2024 **Accepted:** 01.04.2024



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role in many steps of cancer suppression. They protect DNA from oxidative damage, activate carcinogen metabolism and detoxification, prevent cell proliferation, and induce cellular cytotoxicity (4).

Ellagic acid is a phytochemical polyphenol compound with antioxidant properties that is found in fresh berries, such as raspberries, strawberries, and pomegranates, and shelled fruits, such as walnuts, chestnuts, and hazelnuts (5). Ellagic acid has antioxidant, antimutagenic, anticancer, and apoptotic effects. Ellagic acid can clear cancer-causing chemicals from the serum; thereby, it prevents carcinogens from binding to DNA, acts as an antioxidant, limits carbon tetrachloride toxicity and lung fibrosis, stimulates the immune system, and induces the death of cancer cells (6). It has previously been shown to suppress the development of some tumors caused by carcinogens in cell culture and animal experiments (7).

In addition, ellagic acid plays a protective role against cancer because of its antiproliferative, antiangiogenic, and antimetastatic effects and by inhibiting the cell cycle, regulating several intracellular signaling pathways, and inducing apoptosis (8, 9). Ellagic acid has exhibited antitumor activity in BC cell lines by affecting signaling pathways such as PI3K/Akt, NFκ-B, CDK6, TGF-β/Smad3, and Akt/mTOR. Moreover, ellagic acid blocks vascular endothelial growth factor (VEGF), a protein involved in angiogenesis (10).

Cryptotanshinone is a lipophilic compound that is extracted from the roots of the plant species *Salvia miltiorrhiza Bunge* (Danshen), which is used in China for the treatment of cardiovascular diseases, hepatitis, diabetes, and chronic liver failure (11). This compound has the potential to prevent ischemia and atherosclerosis and also has antiinflammatory, anticancer, antioxidative, and antiaggregant effects (12). Cryptotanshinone plays a role in the treatment of angiogenesis-related diseases by inhibiting the proliferation of endothelial cells (13). In addition to its antipermeability and antiangiogenic effects, cryptotanshinone has antiinflammatory properties, as demonstrated by its interaction with other cytokines and chemokines (14). Cryptotanshinone has been shown to induce apoptosis (15) and function as an antimetastatic agent (16) in some cancer cell culture studies. Cryptotanshinone suppresses estrogen receptor (ER)-α-mediated transcriptional activity, glycolysis, cell proliferation, migration, and invasion in BC cell lines. In addition, cryptotanshinone treatment causes cell cycle arrest, induces apoptosis, and increases sensitivity to chemotherapeutic drugs in BC cell lines (17). Cryptotanshinone exerts these effects by downregulating the PKM2/β-catenin and ERα-dependent IGF-1/Akt/mTOR signaling pathways, reducing CCNA2 and CDK1 expression, downregulating the GPER-mediated PI3K/Akt signaling pathway, or inducing the mitochondria-derived ROS/FOXO1 pathway.

The efficacy of ellagic acid is demonstrated by arresting the cell cycle of BC cells in the G0/G1 phase, stimulating apoptosis through TGF-β/Smad3 signaling, inhibiting the CDK6 or PI3K/

Akt pathway, and suppressing angiogenesis-related activities, including proliferation (10). Similarly, cryptotanshinone arrests the cell cycle of BC cells in the G2/M phase, reduces the expression of cyclin D, or suppresses the PI3K/Akt signaling pathway (18). Ellagic acid and cryptotanshinone have been reported to induce apoptosis via similar pathways in BC cells. However, the effects of these two antioxidants on oxidative stress-mediated cell death in TNBC have not been comprehensively examined. Here, the effects of ellagic acid and cryptotanshinone were investigated on cell viability/cytotoxicity, metastasis, and oxidative stress levels in TNBC (MDA-MB-231) cells, which are BC cells that are resistant to chemotherapy and radiotherapy.

MATERIALS AND METHODS

Cell Culture

In this study, the MDA-MB-231 (ER/PR-Her2/neu-) BC cell line (ATCC, Rockville, MD, USA) was used to determine the effectiveness of ellagic acid and cryptotanshinone. MDA-MB-231 cells were grown in a medium containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM-F12), 10% fetal bovine serum (FBS) (v/v), and 1% penicillin/streptomycin (v/v) at 37°C under 5% CO₂ conditions. The experiments began when MDA-MB-231 cells reached confluency.

Determination of the Effectiveness of Ellagic Acid and Cryptotanshinone on Cell Viability

A WST-1 assay was used to determine the effect of ellagic acid and cryptotanshinone on cell proliferation in MDA-MB-231 cell lines. For the WST-1 assay, MDA-MB-231 cells were seeded into 96-well plates at a density of 1×10^4 cells/well in 100 μL. The MDA-MB-231 cells were then incubated for 24 h to observe cell proliferation and differentiation. The ellagic acid and cryptotanshinone compounds were dissolved in dimethyl sulfoxide (DMSO) to prepare intermediate dilutions of 0.1 mg ellagic acid and 0.05 mg cryptotanshinone. The cytotoxic effect of the agents on the cells was determined by applying them to the cells over a range of doses. The most effective doses were determined, and the cells were exposed to these final concentrations. Following the concentration that induced the appropriate conditions in the cells were observed under the microscope and were 5, 10, 15, 20, 25, 40, 50, 75, 90, and 100 μg/mL of ellagic acid, and 2.5, 5, 7.5, 10, 20, 25, 37.5, 45, and 50 μg/mL of cryptotanshinone, which were then applied to MDA-MB-231 cells at the various doses. The proliferation of MDA-MB-231 cells was analyzed using a Cell Proliferation Reagent WST-1 kit (Roche, USA). The WST-1 compound (10 μL) was added to each well, and after 3 h of incubation at 37°C, measurements at a wavelength of 450 nm (reference wavelength 620 nm) were taken at 24, 48, and 72 h using a multiscan spectrophotometer device (MultiScan Go, Thermo Scientific, USA). The absorbance of control cells that were not exposed to ellagic acid or cryptotanshinone was determined to be 100%, and the percentages of the applied doses were

calculated by comparing them with the control. The experiment was performed in four replicates.

Determination of Cell Migration Rates Using the *In Vitro* Scratch Test Assay

To measure the migration speed, the medium was adjusted to contain 3% FBS (v/v). MDA-MB-231 cells were seeded into 6-well plates at a density of 5×10^5 cells/well and incubated for 24 h. After the cells covered the plate in a single layer, a straight line was drawn in the middle of the plate using a 10 μ L pipette tip. Thereafter, the medium was removed, and the plate was rinsed with 1–2 mL of fresh medium to remove any residue. The selected doses of ellagic acid and cryptotanshinone were applied before the medium was added to the cells, and then 5 mL of the scratch assay medium (containing 3% FBS) was added to the cells. Images were obtained by scanning under a fluorescence microscope with a 10 \times magnification objective at 0, 24, and 48 h, and the rate of scratch closure was calculated using Image J software. The scratch closure rate was compared quantitatively to the control and expressed as a percentage (19).

Detection of the ROS Levels in Cells

2',7'-Dichlorofluorescein diacetate (DCF-DA) (Merck, Germany) is a widely used ROS indicator that is used to detect intracellular peroxides. DCF-DA enters the cell via passive diffusion and undergoes oxidation in the presence of intracellular ROS to form DCF, which is a strong fluorescent substance and can be easily detected under a fluorescence microscope. To determine the intracellular ROS levels, the ellagic acid and cryptotanshinone doses were applied to the MDA-MB-231 and control cells in 12-well plates containing round coverslips and washed once with phosphate-buffered saline (PBS). Then 0.5 mL of DCF-DA solution was prepared in serum-free medium and added to the cells, with a final concentration of 10 μ M. This was kept in the dark at 37°C for 30 min. After quickly and gently washing three times with PBS, it was fixed with paraformaldehyde and viewed under a fluorescent microscope (Carl-Zeiss, Axio Observer, Germany). For the positive control, cells were exposed to the same conditions as the tested cells but were incubated with 250 μ M of H₂O₂ (20).

Statistical Analyses

A two-way analysis of variance test with the Tukey post-hoc test was used for the comparison of cell viability in the MDA-MB-231 cell lines with the control. The statistical analysis of all experiments was performed using the GraphPad Prism 6 program. At least three independent replicates were performed for all experiments. A value of $p < 0.05$ was considered statistically significant.

RESULT

Ellagic Acid and Cryptotanshinone may Inhibit Cell Proliferation in MDA-MB-231 Cells

The cytotoxic effect of ellagic acid at doses of 5, 10, 15, 20, 25, 40, 50, 75, 90, and 100 μ g/mL for 24, 48, and 72 h was investigated in the MDA-MB-231 cell line (Figure 1a). After the MDA-MB-231 cells were exposed for 24 h to ellagic acid, cell proliferation was observed to increase compared to the control at concentrations of 5, 10, 15, 20, and 25 μ g/mL ($p < 0.05$). However, under the same conditions, a decrease in cell proliferation was observed at the doses ≥ 40 μ g/mL, but with no statistical significance ($p > 0.05$). There was no observed effect on cell viability or cytotoxicity in the MDA-MB-231 cell line after 48 and 72 h of exposure to ellagic acid ($p > 0.05$). This may be because ellagic acid is sensitive to light and has a short half-life.

The cytotoxic effect of cryptotanshinone at concentrations of 2.5, 5, 7.5, 10, 20, 25, 37.5, 45, and 50 μ g/mL for 24, 48, and 72 h was investigated on the MDA-MB-231 cell line (Figure 1b). It was revealed that exposure of MDA-MB-231 cells to cryptotanshinone for 24 h did not affect cell viability at all doses. However, exposure of MDA-MB-231 cells to cryptotanshinone at doses of 20, 25, 37.5, and 45 μ g/mL for 48 h reduced cell viability by approximately 50% ($p < 0.05$).

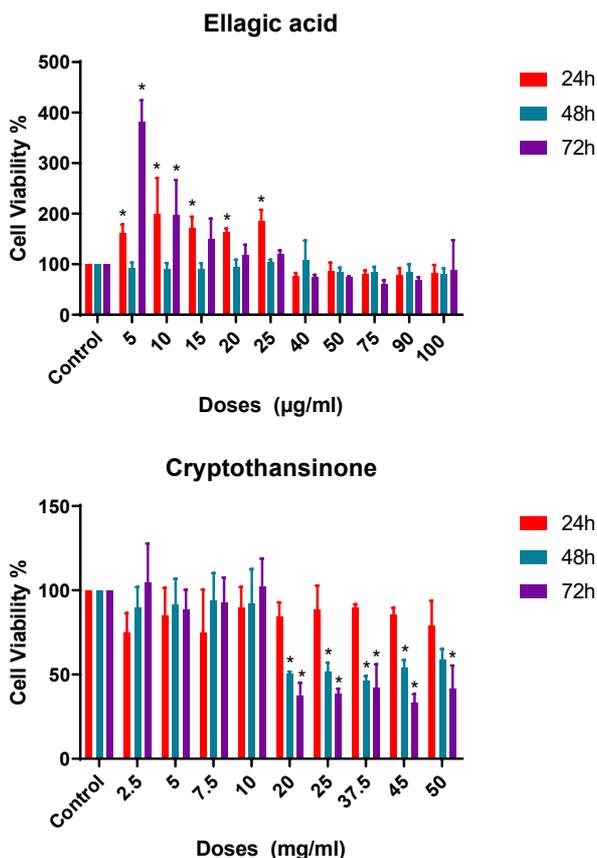


Figure 1a. Effects of ellagic acid and b. Cryptotanshinone on cell proliferation in MDA-MB-231 cells.

Moreover, cell viability was decreased by 75% after 72 h of exposure to cryptotanshinone at doses of 20, 25, 37.5, 45, and 50 $\mu\text{g/mL}$ ($p < 0.05$). Low concentrations of cryptotanshinone had no cytotoxic effect on the MDA-MB-231 cells.

The half-maximal inhibitory concentration (IC_{50}) values of ellagic acid and cryptotanshinone are shown in Table 1. According to the results of the WST-1 cell proliferation assay, ellagic acid at 40 $\mu\text{g/mL}$ and cryptotanshinone at 25 $\mu\text{g/mL}$ were selected as the most effective concentrations, and these concentrations were used to determine cell migration and detect ROS levels.

Ellagic Acid and Cryptotanshinone may Suppress the Migration of MDA-MB-231 Cells

In the scratch test assay, MDA-MB-231 cells were examined at 0, 24, and 48 h after being treated with ellagic acid and cryptotanshinone, and their images were recorded. The *in vitro* scratch test assay was performed on MDA-MB-231 cells, and it was observed that the scratch healed within 24 h. Exposure of the MDA-MB-231 cell line to ellagic acid at a concentration of 40 $\mu\text{g/mL}$, resulted in the scratch being covered after 48 h. Interestingly, after exposure to 25 $\mu\text{g/mL}$ of cryptotanshinone, it was found that scratch was covered after

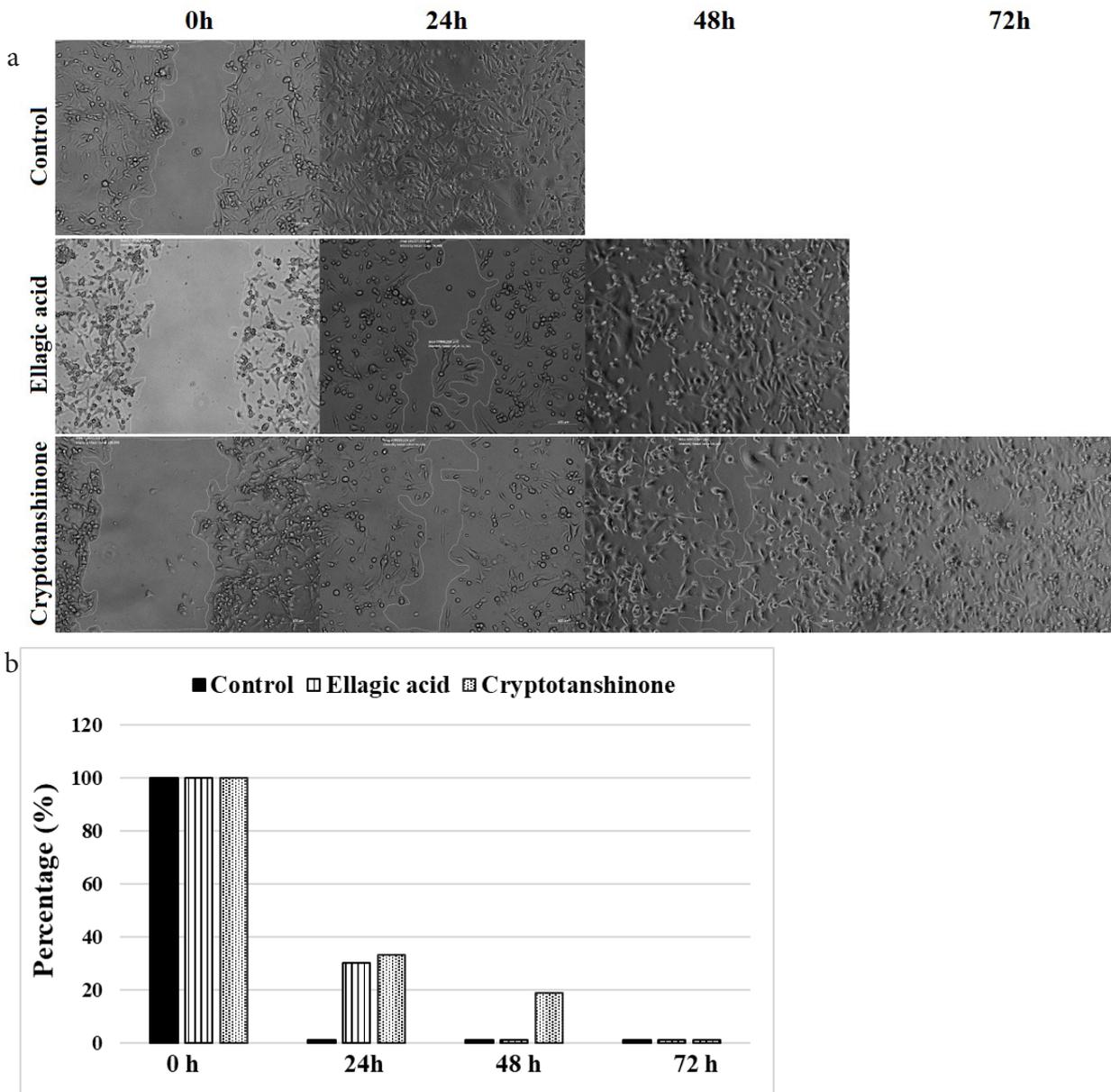


Figure 2. Ellagic acid and cryptotanshinone may suppress the migration of MDA-MB-231 cells.

a. Images were obtained by scanning with a fluorescence microscope at a 10 \times magnification objective. b. The closure areas of the cells in the scratch test assay are given as a percentage compared to the control.

Table 1. Half-maximal inhibitory concentration (IC₅₀) values of ellagic acid and cryptotanshinone

	24 h	48 h	72 h
Cryptotanshinone (µg/mL)	Unstable	41.98	25.90
Ellagic acid (µg/mL)	141.2	372.4	103.8

72 h; therefore, according to these results, it can be inferred that cryptotanshinone is the most effective material in terms of inhibiting cell migration. The closure areas of the cells in the scratch test assay are given as percentages compared to the control (Figure 2).

Ellagic Acid and Cryptotanshinone may Induce the Generation of ROS in MDA-MB-231 Cells

ROS is a key regulator of many cellular activities, including cellular metabolism, the cell cycle, and programmed cell death. Fluorescent DCF-DA was used to determine the effect of ellagic acid and cryptotanshinone on the accumulation of intracellular ROS in the MDA-MB-231 cell line. After treatment with ellagic acid and cryptotanshinone for 24, 48, and 72 h, the cells were imaged using an inverted microscope with a fluorescent attachment. According to the DCF-DA cellular ROS assay experiment, minimum fluorescence was observed in the control group at 24 and 48 h. However, there was a statistically significant increase in fluorescence intensity in MDA-MB-231 cells after 24 h of treatment with ellagic acid (40 µg/mL) compared to the negative control ($p < 0.0001$), but no statistically significant difference in fluorescence intensity was observed after 48 and 72 h of exposure to ellagic acid. After exposure to cryptotanshinone at a concentration of 25 µg/mL, a higher fluorescence intensity was measured in the MDA-MB-231 cells compared to the control at 24 h, and this difference is statistically significant ($p < 0.0001$). However, cryptotanshinone exposure for 48 and 72 h revealed no statistically significant difference. As a result, it was determined that ellagic acid and cryptotanshinone induced oxidative stress, and the 24 h fluorescence intensity was higher than that of the 48 and 72 h of exposure (Figure 3a, b).

DISCUSSION

TNBC is a subtype of BC in which the estrogen, progesterone, and HER-2 receptors are not involved. TNBC is associated with a higher grade and more aggressive biological characteristics, and it accounts for approximately 20% of all BC cases. These features make TNBC one of the most challenging diseases to treat in clinical practice (2, 3).

Ellagic acid, a small molecular polyphenol, is a powerful antioxidant that acts either directly as an antioxidant or by inducing antioxidant cellular enzyme systems (21). In addition, ellagic acid has anticarcinogenic properties, as it inhibits tumor cell proliferation, induces apoptosis, and disrupts angiogenesis (22). Ellagic acid suppressed BC cell growth,

migration, and invasion and blocked tumor initiation and metastasis by increasing the activity of the PI3K signaling pathway inhibitor (23). In addition, ellagic acid inhibited the proliferation and migration of BC cells *in vivo* and *in vitro* (MDA-MB-231) and exhibited antiangiogenesis effects through the VEGFR-2 signaling pathway by inhibiting the expression of P-VEGFR2 (24). In MCF-7 cells, ellagic acid significantly enhanced radiation-induced cytotoxicity, inhibited cell growth by blocking the cell cycle, and enhanced apoptosis (25). Ellagic acid inhibited cell proliferation by inducing the TGF-β/Smad3 pathway in MCF-7 cells (26), arrested the cell cycle in the G0/G1 phase, inhibited proliferation, and induced apoptosis (27). Yousuf et al. reported that ellagic acid reduced the proliferation and colonization of MCF-7 and MDA-MB-231 cells and induced apoptosis through the inhibition of CDK6 (28). The results of this study are consistent with previous studies, and our results showed that ellagic acid inhibited cell proliferation, suppressed cell migration, and induced the accumulation of intracellular ROS in MDA-MB-231 cells. Ellagic acid may reduce cell proliferation by increasing the accumulation of intracellular ROS and inducing apoptosis.

Cryptotanshinone is a lipid-soluble diterpenoid derivative found in plants of the genus *Salvia*, of which *S. miltiorrhiza* Bunge, known as Danshen, is rich in diterpenes. Cryptotanshinone may inhibit the growth of tumor cells in various cancer types through its antitumor activity and enhanced antitumor immunity (29). Cryptotanshinone exhibits anticancer activity in different cancer cells because of its strong STAT3 inhibitor attribute. Cryptotanshinone showed immunomodulatory and antitumor effects by inhibiting the JAK2/STAT3 pathway and regulating the secretion of related cytokines in an experimental BC model (30). In addition, cryptotanshinone inhibited tumor growth in both the animal model of BC and the MCF-7 cell line (31). Cryptotanshinone has been shown to be chemically homogeneous with estrogen, and the combination of arsenic and cryptotanshinone induced endoplasmic reticulum stress and apoptosis in MCF-7 cells (32). In addition, ROS generated by cryptotanshinone induced apoptosis in the MCF-7 cell line by causing endoplasmic reticulum stress (33). The ability of cryptotanshinone to inhibit BC cells is dependent on ER-α (34). Cryptotanshinone effectively inhibited the gene expression of ER-α as well as transactivation and suppressed the growth of ZR-75-1 and MCF-7 cells (35). MCF-7 cells are more sensitive to cryptotanshinone than MDA-MB-231 cells, and cryptotanshinone inhibits the proliferation and metastasis of ER-positive cancer cells (36). There are a few studies that have investigated the effects of cryptotanshinone on MDA-MB-231 cells. Cryptotanshinone reduced glycolysis-related proteins and PKM2/β-catenin signaling in MCF-7 and MDA-MB-231 cells. In addition, cryptotanshinone demonstrated anticancer activity in MCF-7 and MDA-MB-231 cells by inhibiting cell proliferation, migration, and invasion (37). Cryptotanshinone inhibits the proliferation of the SKBR-3 BC cells through G-protein-coupled ER-mediated PI3K/Akt pathway inhibition (38). In addition, Shi et al. showed that cryptotanshinone exerts its antiproliferative

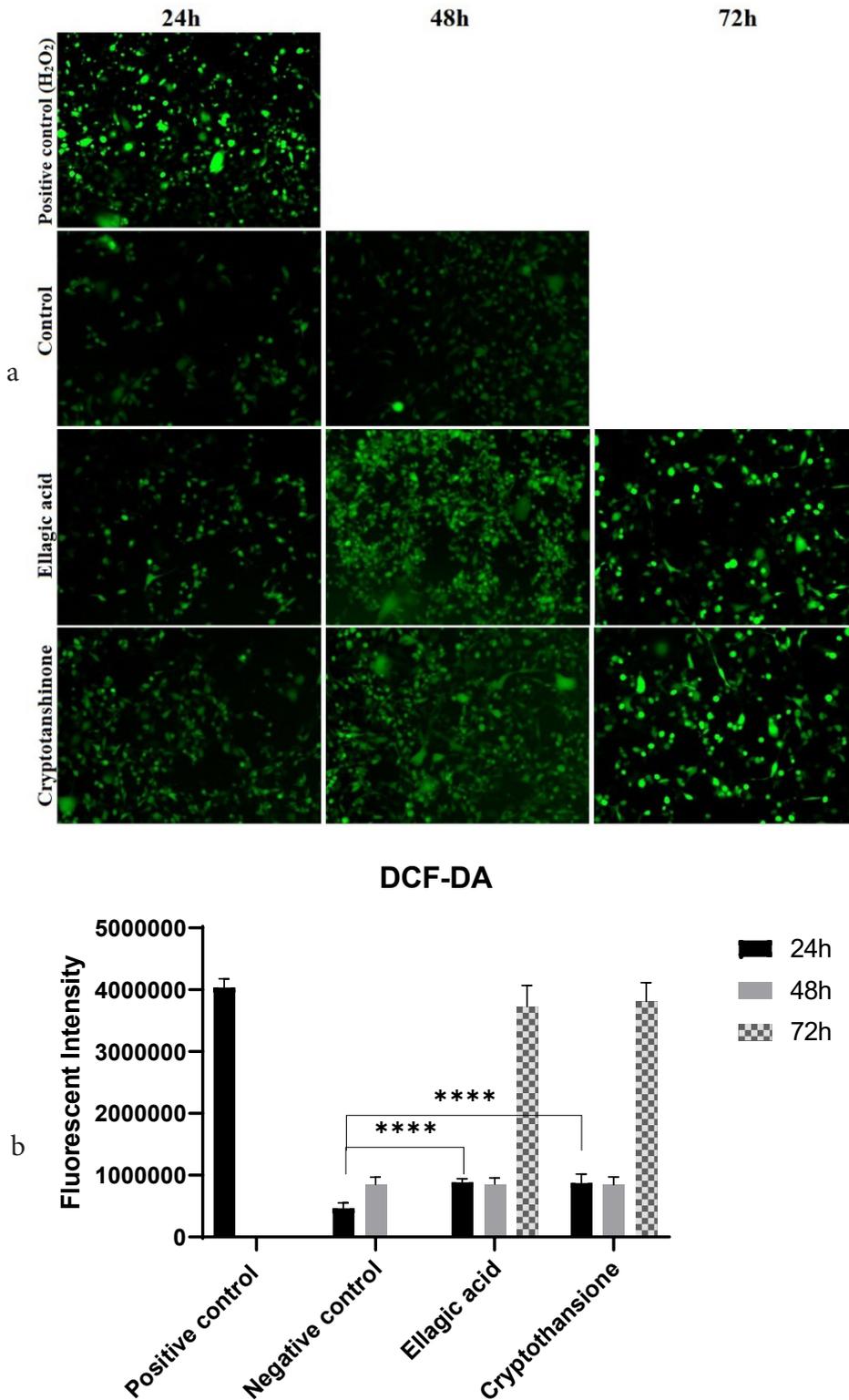


Figure 3. Ellagic acid and cryptotanshinone may induce the generation of reactive oxygen species in MDA-MB-231 cells. a. Fluorescence microscopic image of cells. b. Fluorescence intensity measurement which indicates the enhancement of ROS in cells treated ellagic acid and cryptotanshinone. Groups with the asterisk symbol (****) on the graph indicate that they are statistically different from the control group ($p < 0.0001$).

effect on MCF-7 cells by inhibiting the G-protein-coupled ER-mediated PI3K/Akt pathway (18). In addition, tanshinone-I suppressed the proliferation and angiogenesis of MCF-7 and MDA-MB231 cells (39). In the results of this study, we found that cryptotanshinone inhibited cell proliferation and migration and increased the accumulation of intracellular ROS in MDA-MB-231 cells. Cryptotanshinone may reduce cell proliferation through an increase in the accumulation of intracellular ROS and the induction of apoptosis. There are a few studies that have investigated the effects of cryptotanshinone on TNBC cells. In this regard, our study provides preliminary data for future studies.

Phytochemicals are potential candidates because of their diverse pharmacological activities. Mitochondria are the main source of intracellular ROS generation, and intracellular ROS levels play an important role in cancer processes. It has been reported that high levels of intracellular ROS activate oxidative stress and apoptosis, which leads to cell death, and low levels of intracellular ROS activate angiogenesis, which initiates the spread and metastasis of tumor cells (40). In the results of this study, we found that ellagic acid and cryptotanshinone inhibited cell proliferation, suppressed cell migration, and induced the accumulation of intracellular ROS in MDA-MB-231 cells. These results demonstrate that ellagic acid and cryptotanshinone may be effective for the treatment of TNBC, and suggest that further *in vitro* studies of ellagic acid and cryptotanshinone should target molecular pathways to develop treatment options for TNBC.

Ethics Committee Approval: Since a commercial cell line was used in the study, there is no need for an ethics committee.

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- U.Y., M.F.S.; Data Acquisition- U.Y., M.F.S.; Data Analysis/ Interpretation- U.Y., M.F.S.; Drafting Manuscript- U.Y.; Critical Revision of Manuscript- U.Y.; Final Approval and Accountability- U.Y., M.F.S.

Conflict of interest: The authors declare that they have no conflict of interest.

Financial Disclosure: This work was supported by the Research Fund of Karabuk University [Project No. 22-DS-118].

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