

Evaluation of kinetic effects of Gliotoxin in different breast cancer cell lines

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ABSTRACT

In the present research, the antiproliferative properties of Gliotoxin, which is obtained from marine fungus and thought to be a promising metabolite, on MCF-7 and MDA-MB-231 breast cancer cells, which have different molecular subtypes, were evaluated. Different cell kinetic parameters were employed for this aim. In experiments, cell viability, cell index, mitotic index, BrdU labeling index, and apoptotic index were assessed. Gliotoxin concentrations of 1.5625 μ M, 3.125 μ M, and 6.25 μ M were used in studies for both cell lines. As a result of the values obtained from cell viability and xCELLigence Real-Time Cell Analysis (RTCA) System, 1.5625 μ M concentration was determined as IC₅₀ dose. This concentration was applied to all other parameters and anticancer activities were observed.

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Introduction

Cancer, which is formed by the combination of mutations, affects events such as the life cycle of the cell and differentiation. Depending on the phenotype of the cell from which they originate, they uncontrollably proliferate and tend to spread to the surrounding tissues (1).

Breast cancer accounts for 30% of all cancer types in women and is the most prevalent malignancy among them worldwide (2, 3). It is one of the heterogeneous cancer types with a high level of complexity regarding cellular origin, histology, mutations, metastasis, clinical history, and family history (4, 5). Since the course of the disease varies in patients diagnosed with breast cancer, prognostic factors are used to make a definitive diagnosis. Currently, axillary lymph node metastases, tumor diameter, tumor proliferation rate, hormone receptors, tumor type and histological evaluation, and molecular prognostic factors are used to determine the biological and clinical differences between patients and those at high risk for breast cancer to develop rapidly (6).

In addition to the cancer drugs currently used in the clinic, the search for new and effective active substances continues increasingly. Developing anticancer drugs from natural sources is one of the most important strategies in the production of novel cancer therapies. Due to their extensive biodiversity, marine organisms represent a tremendous library of bioactive compounds. Marine-originated compounds have gained great interest as anticancer agents (7). In this context, a secondary metabolite called Gliotoxin (GTX), which is thought to be a promising anticancer agent, is derived from *Aspergillus fumigatus*, a marine fungus. It is an epipolyiodioxopiperazine characterized by a disulfide bridge across a piperazine ring (8). Marine-

derived fungi has been proven to be a prolific source of secondary metabolites with interesting structural properties and biological activities (9-11). GTX has antifungal, antiviral, anti-inflammatory and antitumor effects (12-16). GTX and some of its analogs have previously been shown to exhibit potential cytotoxic effects in different cell lines (17).

In this experimental study, it was aimed to evaluate the effects of Gliotoxin, on estrogen receptor-positive breast cancer cells MCF-7 and triple-negative breast cancer cells MDA-MB-231 at the cellular level.

Materials and Methods

Cell culture and Reagents

MCF-7 and MDA-MB-231 cell lines were supplemented in Dulbecco's Modified Eagle's Medium (DMEM) (high blood glucose) (Gibco Co) with 10% (v/v) fetal bovine serum (FBS, Gibco Co, Grand Island, NY, USA) at 37 °C in a humid atmosphere with 5 % CO₂. GTX (Cat. No. 2637) was purchased from Tocris Bioscience.

Cell viability analysis

To determine the effect of GTX (1.5625 μ M, 3.125 μ M, and 6.25 μ M) on proliferation cell lines, 10⁴ cells were seeded into 96-well plates. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide) assay was performed at 24 h. The absorbance values of all experimental groups were measured with a spectrophotometer at 490 nm.

Cell Index (CI)

100 μ l of DMEM was added to each well of 16-well e-plates containing microelectrodes and background read-

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ing was performed. Then, cell count was performed and 1000 cells for MCF-7 and 5000 cells for MDA-MB-231 were seeded in each well. Cells were treated with different concentrations of GTX (1.5625 μ M, 3.125 μ M, and 6.25 μ M) after 24 hours. Changes in cell proliferation xCELLigence in E-Plates incubator (Roche Diagnostics GmbH, Penzberg, Germany) have been observed.

Mitotic index (MI)

The Feulgen method was used to determine the MI. In order to determine the MI, 3000 cells were counted as dividing and non-dividing cells at the determined concentration and the number of dividing cells was used for the MI.

Mitotic index (%) = (Number of dividing cells / Total cell number) x 100 formula was used.

BrdU (5-bromo-2'-deoxyuridine) Labelling Index

BrdU assay was performed in accordance with the manufacturer's protocol to determine the proportion of cells in the synthesis phase in cells treated with GTX at IC₅₀ concentration.

Apoptotic index (AI)

After the GTX application, the DAPI staining method was applied to determine the presence of apoptotic cells. At the end of the experimental period, the cells were fixed and then incubated in DAPI, a fluorescent dye, for 20 min. After staining, apoptotic cells were identified by counting under a fluorescent microscope.

Statistical Evaluation

In the statistical analysis of the experimental parameters, the one-way Analysis of Variance (ANOVA) test and DUNNETT's test were applied, respectively. Statistical significance was determined at a $p < 0.05$ level.

Results

Cell viability

The absorbance values of the MCF-7 cell line for GTX were $410,5 \times 10^{-3}$ for control, $210,8 \times 10^{-3}$ for 1,5625 μ M, $198,12 \times 10^{-3}$ for 3,125 μ M and $89,286 \times 10^{-3}$ for 6,25 μ M for 24 h (Figure 1). These values were $441,25 \times 10^{-3}$ for control, $230,96 \times 10^{-3}$ for 1,5625 μ M, $215,8 \times 10^{-3}$ for 3,125 μ M and $71,5 \times 10^{-3}$ for 6,25 μ M for MDA-MB-231 cells for 24 h (Figure 2).

When these absorbance values were examined, com-

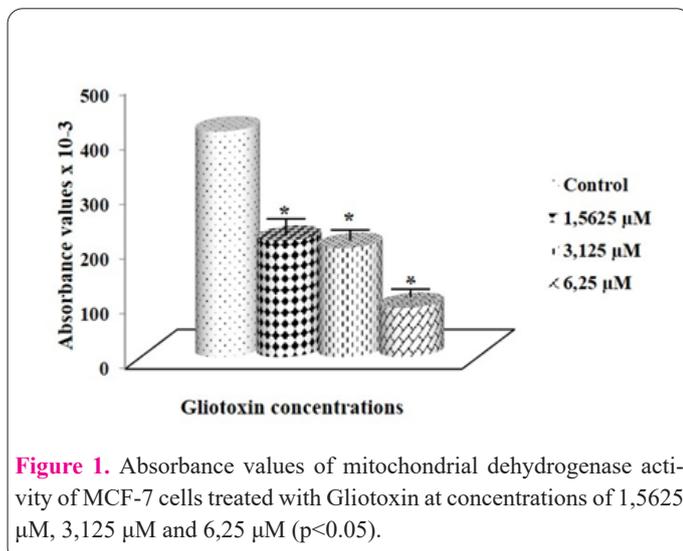


Figure 1. Absorbance values of mitochondrial dehydrogenase activity of MCF-7 cells treated with Gliotoxin at concentrations of 1,5625 μ M, 3,125 μ M and 6,25 μ M ($p < 0.05$).

pared to the 100% accepted control group for MCF-7 cells, it was observed that 1,5625 μ g/ml GTX concentration decreased cell viability to 51,35%, 3,125 μ g/ml GTX concentration decreased cell viability to 48,26% and 6,25 μ g/ml GTX concentration decreased cell viability to 21,75% (Figure 3). For MDA-MB-231 cells, it was observed that 1,5625 μ g/ml GTX concentration decreased cell viability to 52,34%, 3,125 μ g/ml Gliotoxin concentration decreased cell viability to 48,9% and 6,25 μ g/ml GTX concentration decreased cell viability to 16,2% (Figure 4). According to the data obtained, it was seen that 1,5625 μ g/ml GTX concentration for both cell lines was the IC₅₀ concentration.

Cell Index (CI)

The data obtained from the RTCA system in cells treated with GTX at different concentrations showed that GTX application caused a decrease in cell proliferation in both hormone-sensitive and insensitive cell lines. The curves of the graph suggest that all applied GTX concentrations cause DNA-damaging effects in both cell lines (Figure 5 and 6).

Mitotic index (MI)

To determine the changes in the percentage of cells in the mitotic phase, 1,5625 μ M GTX concentration was applied for both cell lines for 0-72 h. MI values decreased from 6,27 % to 3,33 % at 24 h; from 6,96 % to 2,21 % at 48 h and from 7,11 % to 1,16 % at 72 h for the MCF-7

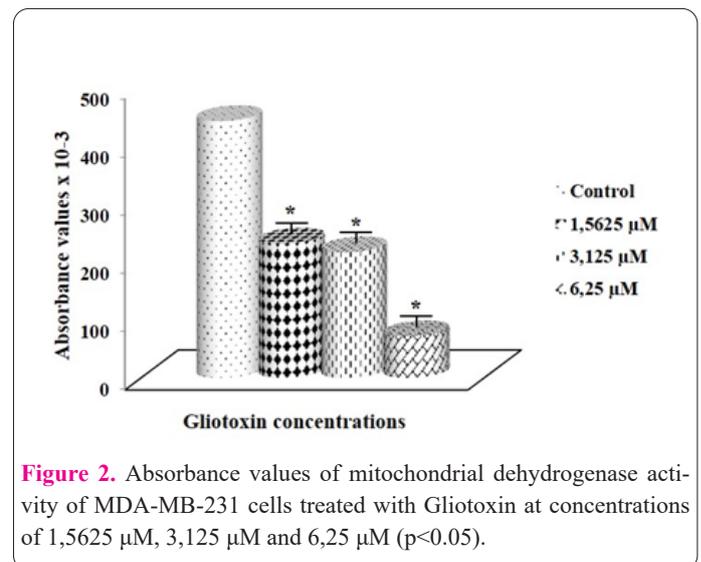


Figure 2. Absorbance values of mitochondrial dehydrogenase activity of MDA-MB-231 cells treated with Gliotoxin at concentrations of 1,5625 μ M, 3,125 μ M and 6,25 μ M ($p < 0.05$).

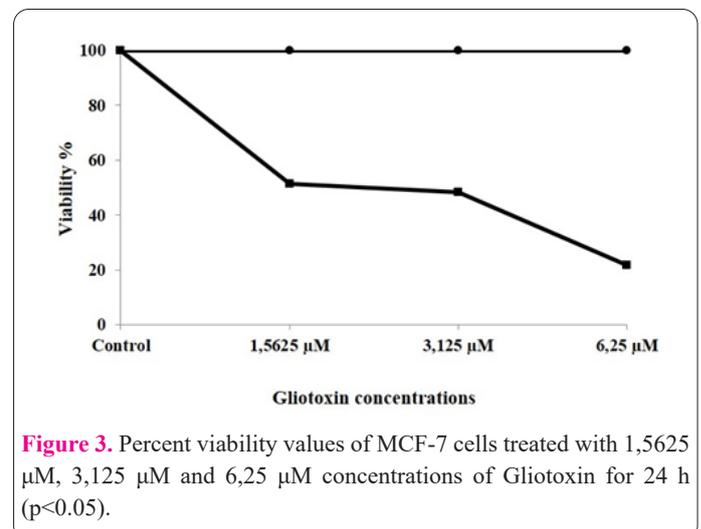
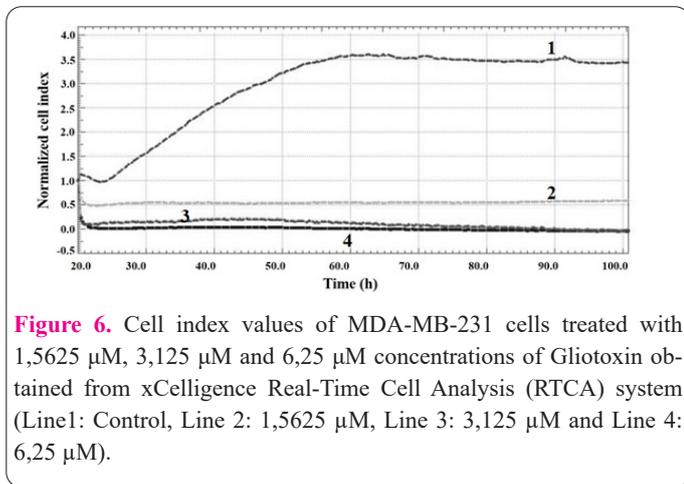
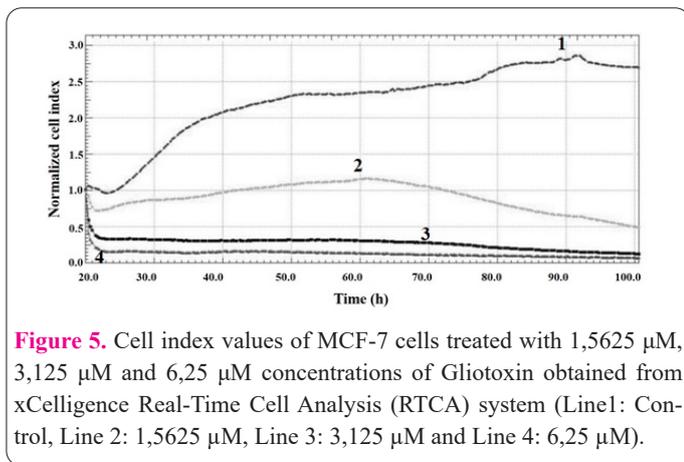
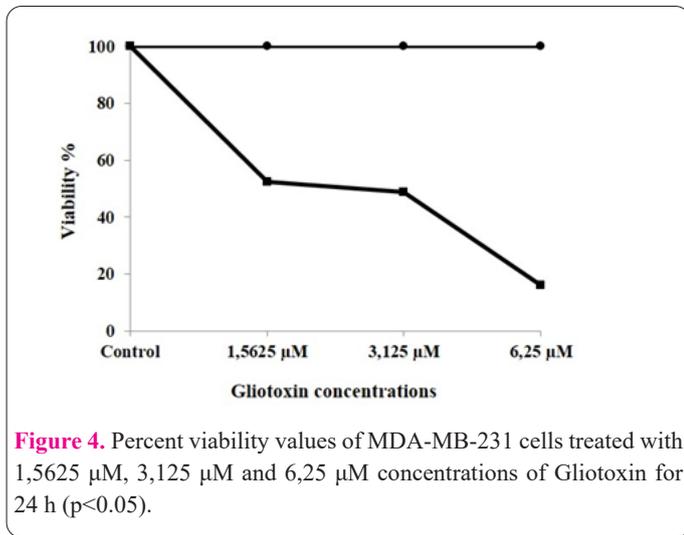


Figure 3. Percent viability values of MCF-7 cells treated with 1,5625 μ M, 3,125 μ M and 6,25 μ M concentrations of Gliotoxin for 24 h ($p < 0.05$).



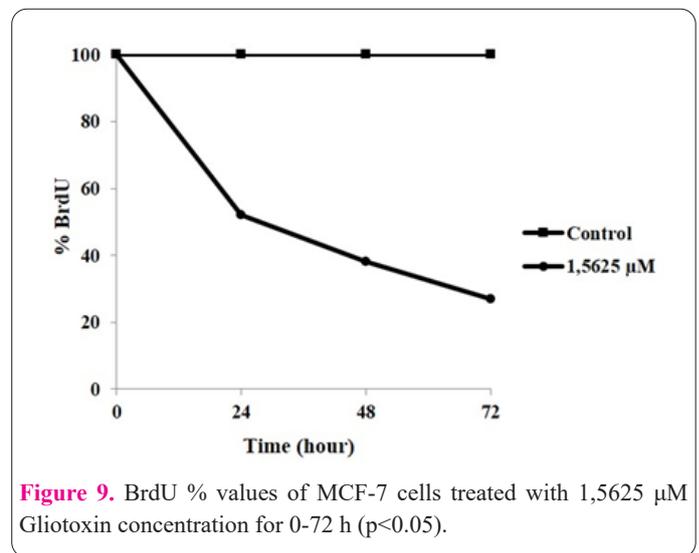
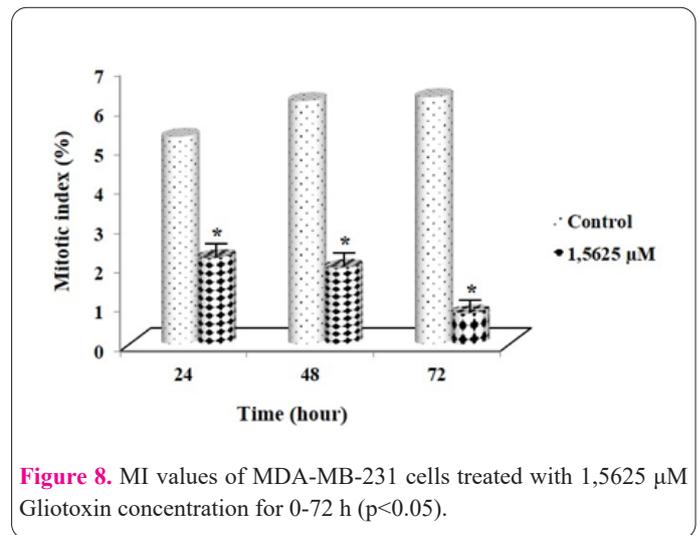
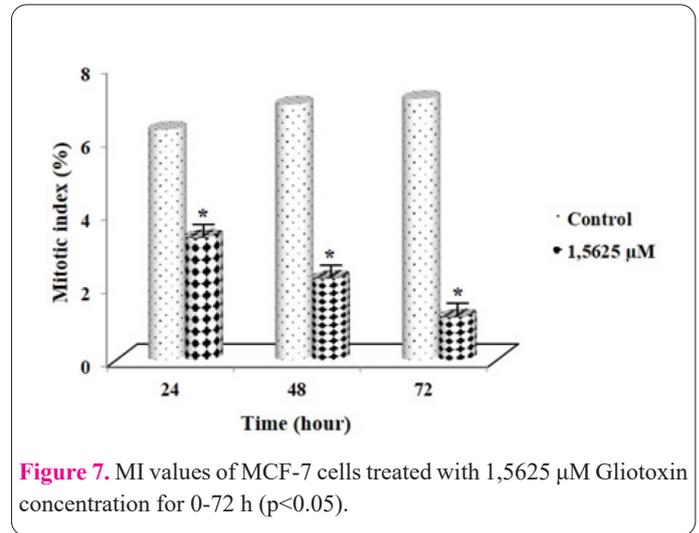
cell line (Figure 7). For MDA-MB-231 cells MI values decreased from 5,27 % to 2,19 % at 24 h; from 6,18 % to 1,94 % at 48 h and from 6,28 % to 0,78 % at 72 h (Figure 8).

BrdU Labelling Index

GTX concentration of 1,5625 µg/ml determined for both cell lines was applied for 0-72 h. For the MCF-7 cell line, BrdU% readings were 52%, 38%, and 27% compared to the control groups, which were taken to be 100%. (Figure 9). In comparison to the control groups, which were deemed 100%, these values for the MDA-MB-231 cell line were found to be 48%, 36%, and 23% (Figure 10).

Apoptotic index (AI)

In order to determine the apoptotic effect of GTX on MCF-7 and MDA-MB-231 cells, 1,5625 µM concentration of GTX was applied for 0-72 h. AI values increased from 2,14 % to 7,69 % at 24 h; from 2,67% to 15,96 % at 48 h and from 3,13% to 21,34 % at 72 h for the MCF-7 cell line (Figure 11). For MDA-MB-231 cells AI values increased from 2,15 % to 8,96 % at 24 h; from 2,37% to 12,94 % at 48 h and from 3,16 % to 19,43 % at 72 h (Figure 12).



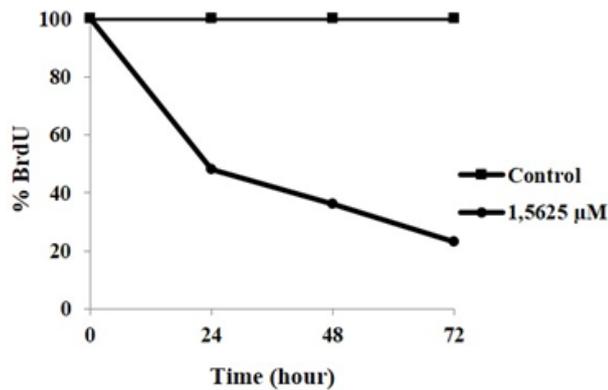


Figure 10. BrdU % values of MDA-MB-231 cells treated with 1,5625 µM Gliotoxin concentration for 0-72 h ($p < 0.05$).

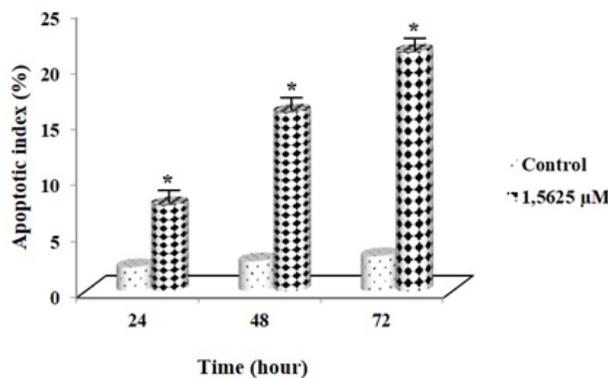


Figure 11. AI values of MCF-7 cells treated with 1,5625 µM Gliotoxin concentration for 0-72 h ($p < 0.05$).

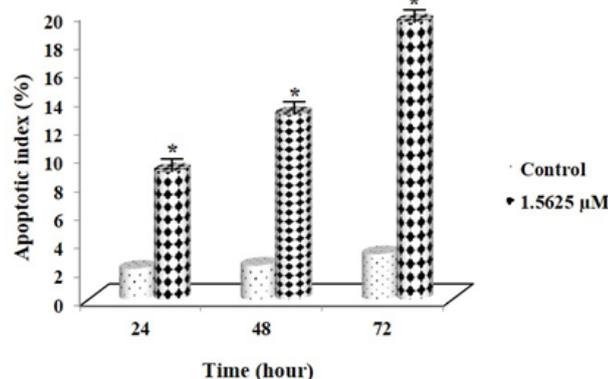


Figure 12. AI values of MDA-MB-231 cells treated with 1,5625 µM Gliotoxin concentration for 0-72 h ($p < 0.05$).

Discussion

Breast cancer poses a great threat to women (15). The incidence of breast cancer is increasing worldwide, and considering the historical incidence of developed countries, it is due to reasons such as delayed reproductive age, low breastfeeding duration, obesity developing with malnutrition, and low physical activity (16). Approximately 20% of these cases constitute the 'triple negative' subtype. This subtype is negative for human epidermal growth factor, progesterone receptor and estrogen receptor and is

more aggressive than other subtypes (18). In contrast, the luminal A breast cancer subtype, which has very high hormone sensitivity due to its high ER expression, is the most common breast cancer molecular subtype (19).

The increase in the frequency of cancer cases in the community necessitates the discovery of alternative active substances to treat the disease (20). One of the most crucial methods used in the creation of novel cancer therapies in recent years is the production of anticancer medications from natural sources. Sea creatures with a wide biodiversity represent an enormous source of active substances biologically. Marine-derived compounds are of great interest as anticancer agents (21). One of these agents is Gliotoxin.

In addition to the immunosuppressive, antibacterial and antiviral effects of Gliotoxin, its anticancer effects have also been demonstrated in recent years (14, 21-26). In various studies it was shown that Gliotoxin promotes apoptosis suggesting that it can be used as an anticancer agent. The cytotoxic effects of Gliotoxin in SH-SY5Y, rat Kupffer cells and human hepatic stellate have also been demonstrated (27-29). Gliotoxin was shown to suppress cell growth and promote apoptosis in HeLa and SW1353 cell lines (14). In a study examining changes in MCF-7 cells when treated with gliotoxin, typical apoptotic changes were observed in MCF-7 cells after administration (26). Gliotoxin has been shown to inhibit cell growth of the adriamycin-resistant non-small cell lung cancer cell line A549/ADR while promoting apoptosis (30). Gliotoxin activates the Bak gene, a member of the Bcl-2 family. Activation of the Bak gene initiates processes that ultimately cause the cell to undergo apoptosis (31).

Gliotoxin has also been shown to function as an inhibitor of several signaling pathways that contribute to cancer formation and progression. It has been shown that gliotoxin promotes β -catenin degradation and, as a result, suppresses the Wnt signaling pathway. It also promotes apoptosis while inhibiting proliferation in colorectal cancer cells where the Wnt signaling pathway is over-activated (15). Gliotoxin has also been shown to inhibit the NOTCH2 signaling pathway involved in the formation of various types of cancer (32).

In this current study, the values obtained from the cell viability and cell index parameters in this study showed that 1.5625 µM Gliotoxin was the IC_{50} concentration for both cell lines. The IC_{50} concentration of Gliotoxin showed a significant decrease, especially in the mitotic index values for both cell lines, at the 24th hour, while it approached zero at the 48th h and 72nd h. These data support the idea of mitotic catastrophe and mitotic arrest. In addition, the increase in the apoptotic index parameter in both cell lines supports the studies mentioned above.

All data obtained from this experimental study showed that Gliotoxin is effective in breast cancers with different molecular subtypes, regardless of hormone dependence. This result suggests that Gliotoxin may have positive contributions to the treatment of various cancer types as well as breast cancer. In addition, more detailed studies are needed.

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Conflicts of interest

The author certifies that there is no conflict of interest.

Author's contribution

All authors are responsible for the manuscript equally.

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