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Running Title: Effect of Flaxseed on Breast Cancer Cells
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Flaxseed Extract Can Modulates Apoptotic Genes to Promote Cell Death in Breast Cancer Cells

Ehsan Zare Mehrjardi[*], MSc, Shadi Montazerolhojeh[**], MSc, Masoud Dehghan Tezerjani[***], MSc, Safieh Dehshiri[**], MSc, Mahmood Dehghani Ashkezari[**], PhD, Seyed Morteza Seifati[**♦], PhD

[*]*Department of Industrial Environmental and Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran*
[**]*Medical Biotechnology Research Center, Ashkezar Branch, Islamic Azad University, Ashkezar, Yazd, Iran*
[***]*Abortion Research Centre, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran*

♦Corresponding Author
Seyed Morteza Seifati, PhD
Medical Biotechnology Research Center,
Ashkezar Branch, Islamic Azad University,
Ashkezar, Yazd, Iran
Email: seifati@gmail.com

Abstract
Background: Breast cancer is a multifactorial malignancy with uncertain treatment outcomes that make its challenging treatment. Balanced level of genes expression, including *BAX*, *BCL2L1 (bcl-xl) and BCL-2* are needed to regulate apoptosis pathway in cancers. The aim of this study was to examine the impact of flaxseed extract on the expression of *BAX*, *BCL-2*, and *BCL2L1* (*bcl-xl*) gens in BT-474 and MCF-7 breast cancer cells.
Method: This in vitro experimental study investigated the impact of flaxseed extract on apoptosis and the proliferation of BT-474 and MCF-7 breast cancer cells. MTT assay was conducted on flaxseed extract treated BT-474 and MCF-7 cells at concentrations 3.90628, 7.8125, 156.125, 312.5, 125, 2, 1000, and 2000 µg/ml for 24, 48 and 72h. IC50 (µg/ml) was calculated for flaxseed extract in both cells. The expression of *BCL2L1* (*bcl-xl*), *bcl-2* and *Bax* genes were evaluated at these concentrations using real time Polymerase Chain Reaction (PCR). The data from different groups were analyzed using the Student's t-test.
Results: Flaxseed extract reduced the multiplication and growth of BT-474 and MCF-7 cells in a concentration-dependent manner over 24, 48, and 72 hours of incubation. In both flaxseed-extract exposed BT-474 and MCF-7 cell lines, the *BAX* gene expression increased ($P < 0.05$); however, the gene expression of *BCL2L1 (bcl-xl) and BCL-2* genes decreased ($P < 0.05$).
Conclusion: Incubating BT-474 and MCF-7 with flaxseed extract repressed their growth and induced apoptosis. These results may provide precious information for developing a plant-based agent to prevent or treat breast cancer.
Keywords: Breast Neoplasms, Apoptosis, Plant Extracts, IC50, Cell Line

Introduction

GLOBOCAN 2020, an international organization that investigates cancer occurrence and mortality, reported that 6.9% of cancer-related fatalities in women are attributed to breast cancer. Breast cancer, which accounts for 11.7% of all cancer cases, has surpassed lung cancer as the most prevalent neoplasm.[1] It is a complex malignancy with almost five molecular subtypes. In addition to genetic risk factors, ethnicity, environment, and lifestyle also contribute to the development of breast cancer.[2, 3] Various therapeutic approaches have been developed for breast cancer treatment.[4, 5] However, no single therapeutic strategy can be considered the most effective. To avoid the side effects and drug resistance associated with current therapeutic strategies, exploring new antitumor agents is vital.

Cancers have some basic characteristics known as "hallmarks of cancer", one of which is evading apoptosis.[6] To preserve appropriate cell densities in tissues, damaged or undesired cells are naturally eliminated through the process of apoptosis.[7] There are two distinct routes that cause apoptosis: the extrinsic death receptor pathway and the internal mitochondrial pathway. Members of BCL-2 family (*BAX*, *BCL*-2, *bcl-xl*) can alter the mitochondrial route.[8]

Tumor cells use various molecular mechanisms to repress apoptosis. For example, they acquire resistance to apoptosis through mutations or reduced production of pro-apoptotic proteins like BAX, and by increasing the expression of anti-apoptotic protein like bcl-xl and BCL-2 (9). *BCL-2* is up-regulated in almost 70% of ER^+ breast tumors.[10] In the ductal carcinoma in situ breast cancers, *bcl-xl* overexpression has also been reported.[11] Besides, increased levels of *bcl-xl* and *BCL-2* have contributed to drug resistance in breast cancer.[12] These anti-apoptotic proteins are believed to collaborate with pro-proliferative signals to facilitate the establishment and development of breast cancer.[13] Thus, the proteins of BCL-2 family can be alluring targets for therapeutic intervention in breast cancer treatment.

Significant evidence has demonstrated that a traditional herbal drug, flaxseed (*Linum usitatissimum*), possesses anti-obesity, anti-diabetic and anti-neoplastic properties.[14] Flaxseed has a suppressive effect on the growth of human breast xenografts in athymic mice. Furthermore, it was discovered to reduce the development of tumors in women with postmenopausal breast cancer.[15,16] Flaxseeds are rich in α-linolenic acid oil and a significant source of phytoestrogens lignan, secoisolariciresinol diglycoside (SDG). In the human or animal gut, SDG is metabolized into enterolactone (ENL) and enterodiol (END), which are mammalian lignans.[17] SDG can initiate apoptosis in various human cancerous cells by inducing of an intrinsic apoptotic mechanism. The apoptotic effects of SDG apoptotic effect were assessed in the cells of SW480 colon cancer, where treatment with lignan significantly increased the levels and gene expression of AIF and caspase-3.[18] Studies have demonstrated that both flaxseed and its ingredients have the ability to inhibit cell growth, prevent the formation of new blood vessels, and promote programmed cell death in living organisms. As a result, these substances can decrease in tumor size, number, and the spread of cancer to other parts of the body.[19, 20] Furthermore, numerous researches have indicated the anti-cancer features of flaxseed and its components on breast cancer BT-474 cells.[21, 22] Therefore, flaxseed can be an effective drug or supplement to prevent and treat breast cancer. However, the exact effects of flaxseed on breast cancer cells are still not fully understood. In this study, we assessed the influence of flaxseed extract on the apoptosis and survival of BT-474 and MCF-7 breast cancer cells.

Materials and Methods

Ethical consideration

This in vitro experimental study received ethical approval from the Ethics Committee of Ashkezar Branch, Islamic Azad University with the number IAU.REC1401.27.

Flaxseed extract production

Flaxseed extraction was carried out using the soxhlet method. The flaxseeds were finely ground, and 25 gr of flaxseed powder were placed into a filtration thimble and inserted the main chamber of the soxhlet extractor. A distillation flask containing 250 cc (10 times the weight of the dry flaxseed powder) of 70% ethanol (solvent) was placed under the Soxhlet extractor. A reflux condenser was placed atop the extractor. The flask was heated to 50-60^0c to initiate reflux. The solvent vapor reached the main chamber, and the condenser cooled the vapors. The liquid solvent then penetrated the flaxseed powder, extracting the compounds.

Cell culture

Human breast cancer cell lines BT-474 (human epidermal growth factor receptor 2 (HER2+) and estrogen receptor (ER) & progesterone receptor (PR+) and MCF-7 (ER&PR+, HER2-) were recruited from Pasteur Institute (Tehran, Iran). They were cultured in a high glucose medium (DMEM, thermofisher – GIBCO, USA) supplemented with fetal bovine serum, 10% (v/v) (Thermofisher – GIBCO, USA), 1% penicillin (Thermofisher – GIBCO, USA), 1% streptomycin (Thermofisher – GIBCO, USA). The cells were incubated at 37°C with 5% CO2 and 95% humidity. To achieve 80–90% confluence, 0.25% trypsin-EDTA (1X) (Thermofisher – GIBCO, USA) was used to pass cells. Flaxseed extract was made in concentrations 3.90628, 7.8125, 156.125, 312.5, 125, 2, 1000, and 2000 µg/ml. Flaxseed extract was dissolved in Dimethyl sulfoxide (DMSO) (Pars tous - Iran) and then diluted with DMEM medium.

MTT assay

The vitality of the cells was investigated using the MTT test (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). Cells were cultured on a 96-well plate at 1×10^4 cell/ well concentration, with six repeats for each extract concentration. Cancer cells with flaxseed extract at concentrations 3.90628, 7.8125, 156.125, 312.5, 125, 2, 1000, and 2000 µg/ml were incubated for 24, 48 and 72h. Subsequently, 20 µl MTT (Thermofisher – GIBCO, USA) and Phosphate-buffered saline (PBS) (0.5 mg/ml) was added to each well and subjected to a four-hour incubation period. After the incubation period, the MTT solution was removed, and 100 µl of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using an ELISA reader (Biotek ELx800).

Assessment of genes expressions by (Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR))

The RNeasy Plus Mini kit, Qiagen, was used to extract total RNA from flaxseed extract-treated BT-474 and MCF-7, as well as from untreated cells, following the guidelines provided by the manufacturer. The RNA content was assessed employing a Nanodrop (Thermo Scientific, USA) at 260/280 nm and 260/230 nm wavelength. Additionally, the integrity of the RNA was checked using gel electrophoresis. The synthesis of cDNA was carried out using 1 µg of RNA, random hexamers primers, and the RevertAidTM First strand cDNA synthesis Kit (Fermentas, USA) based to the manufacturer's guidelines. mRNA levels were quantified Using RealQ Plus 2x Master Mix Green /High Rox (amplicon, Denmark) and the Applied Biosystems® StepOnePlus™ system, *BAX*, *BCL-2*, *bcl-xl* and *GADPH* primers were selected from the primer bank database. The primer sequences are listed in table 1. A total reaction volume of 20 µl was prepared by combining 1 10 µl of Master Mix Green, 0.5

µl of each primer, 1 µl of cDNA, and 8 µl of ddH2O. The thermal cycling conditions were set as follows: an initial holding stage at 95 °C, 15 minutes, 35 cycles at 95 °C for 15 seconds and 58 °C for 1 min. The melt curve stage included 95 °C for 15 seconds, 58 °C for 1minute and 95 °C for 15 seconds. The qRT-PCR experiment was duplicated for each sample, including a non-template control. After amplification, melting curve analysis was conducted, showing one distinct peak for each primer set. The expression of genes in each sample was normalized using the $2^{-\Delta\Delta Ct}$ method, with *GADPH* gene expression serving as an internal standard.

Statistical analysis

The Student's t-test was used to analyze the data from different experimental groups using IBM SPSS Statistics, version 20 (SPSS Inc., Chicago, IL, USA). Statistical significance was determined with p-values reported as follows: $P < 0.05$ was considered statistically significant. Data from all experimental groups were expressed as mean ± standard deviation (SD).

Results

Flaxseed extract diminished BT-474 and MCF-7 viability

The cytotoxicity impact of flaxseed extract (FE) was assessed on BT-474 and MCF-7 cells in different concentrations, ranging 3.90628, 7.8125, 156.125, 312.5, 125, 2, 1000, and 2000 µg/ml in 24, 48 and 72 hours. As shown in figure 1, the expansion of BT-474 and MCF-7 cells was suppressed by FE in in a concentration-dependent manner during each incubation period. In MCF-7 cells, concentrations less than 125 µg /ml exhibited no meaningful difference (P = 0.14) in the viability percentage of FE-treated MCF-7 cells. Nevertheless, at concentrations 1-2 mg/ml, approximately a three-fold difference in the viability percentage of FE-treated MCF-7 cells was observed $P < 0.05$. The viability percentage at concentrations (500-1000) µg /ml showed 6-fold decrease (P = 0.004) for 48 hours. The proliferation of FE-treated BT-474 cells remarkably repressed at (3.9-31.2) and (250-2000) concentrations across all time points (24,48 and 72h) (P = 0.0003). The IC50 values (in µg/ml) for the BT-474 cell line were calculated as follows: 724.7 µg/ml at 24 hours, 634.6 µg/ml at 48 hours, and 406.9 µg/ml at 72 hours. For the MCF-7 cell line, the IC50 values were 884.5 µg/ml at 24 hours, 593.4 µg/ml at 48 hours, and 398.3 µg/ml at 72 hours.

Flaxseed extraction effects on BCL2L1 (bcl-xl), BAX, BCL-2 gene expression levels

The expression levels of these genes were evaluated at the IC50 values of BT-474: 724.7 µg/ml at 24 hours, 634.6 µg/ml at 48 hours, and 406.9 µg/ml at 72 hours, and for the MCF-7 cell line: 884.5 µg/ml at 24 hours, 593.4 µg/ml at 48 hours, and 398.3 µg/ml at 72 hours.

BAX mRNA levels in MCF-7 cells significantly increased after 24 h (P = 0.0008), 48 h (P = 0.0002), and 72 hours (P < 0.0001) of FE treatment, in comparison with control group. *BAX* gene expression increased with longer incubation times (fold changes: 6.06 at 24 hours; 6.72 at 48 hours; and 7.41 at 72 hours). Therefore, the *BAX* gene expression was elevated in FE-treated MCF-7 cells, relative to the control cells, across all treatment durations. The mRNA levels of *BCL-2* significantly decrease in all treatment groups at 24 hours (P = 0.0001), 48 hours (P = 0.0001), and 72 hours (P < 0.0001) in comparison with the control group. Additionally, *BCL-2* expression decreased with longer incubation times (fold changes: 0.095 at 24 hours; 0.081at 48 hours, and 0.053 at 72 hours). Thus, the *bcl-2* gene expression was reduced in MCF-7 cells exposed to FE, in comparison with untreated MCF-7 cells. The levels of *bcl-xl* mRNA significantly decrease in all treatment groups at 24 hours ($P < 0.0001$), 48 hours (p-value = 0.0001), and 72 hours ($P < 0.0001$) treatment groups compared with the control group. In addition, gene expression decreased with longer treatment time (fold changes: 0.22 at 24 hours; 0.175 at 48 hours, and 0.161 at 72 hours).

In the BT-474 cells, *BAX* mRNA levels increased at all time points: 24 hours ($P = 0.0031$), 48 hours ($P = 0.0001$), and 72 hours ($P = 0.0008$) of treatments compared with the control group. Additionally, BAX mRNA levels increased with longer incubation times (fold changes: 4.59 at 24 hours; 5.2 at 48 hours; and 5.77 at 72 hours). Therefore, the BAX gene exhibited increased expression in FE-treated BT-474 cells, as in comparison with the control BT-474 cells, across all treatment durations. *BCL-2* mRNA levels decreased significantly at all time points: 24 hours ($P = 0.0434$), 48 hours ($P = 0.0058$), and 72 hours ($P = 0.002$). Besides, the expression of *BCL-2* gene reduced by increasing the time of incubation (fold changes: 0.293 at 24 hours; 0.238 at 48 hours; and 0.185 at 72 hours). Consequently, the *BCL-2* gene expression decreased in BT-474 cells exposed to FE, in comparison with untreated cells. *bcl-xl* mRNA levels decreased at all time points: 24 hours ($P = 0.0007$), 48 hours ($P = 0.0021$), and 72 hours ($P = 0.0011$) in comparison with the control BT-474 cells. Also, it decreased through increasing the time of incubation (fold changes: 0.23 at 24 hours; 0.146 at 48 hours; and 0.128 at 72 hours). Therefore, the *BCL2L1 (bcl-xl)* gene expression was down-regulated in FE-exposed BT-474 cells compared with untreated BT-474 cells. Figure 2 illustrates the relative expression of the *BCL2L1* (*bcl-xl*), *BCL-2* and *BAX* genes.

Discussion

Our study showed that the extract obtained from flaxseed effectively repressed the growth of BT-474 and MCF-7 cells in a manner that depended on the dosage. Furthermore, this extract increased the level of the pro-apoptotic gene BAX, while inhibiting the activity of the anti-apoptotic genes *BCL-2* and *BCL2L1* (*bcl-xl*) in both cell lines. These results indicate that the extract may have the ability to induce apoptosis and minimize the viability of breast cancer cells.

Breast cancer is the second most common cause of cancer-related death for women globally.[1] Due to the heterogeneity in presentation and treatment outcomes,[23] its management is still challenging, making the discovery of novel anti-neoplastic essential. Cancer therapy mainly involves inhibiting cancer cells proliferation or inducing cancer cell death.[24] It is well-documented that some plant extracts, like flaxseeds, can trigger apoptosis in human cancer cells. They are rich in natural polyphenols, mainly lignans, with anti-hormone modulating, and α-linolenic acid (ALA) with anti-inflammatory effects.[25] This study examined the impact of flaxseed extract on the apoptosis and sustainability of MCF-7 and BT-474 breast cancer cells. The findings demonstrated that the extract derived from flaxseed has a dose-dependent cytotoxic impact on MCF-7 and BT-474 cells.

The results exhibit a degree of consistency with many previous studies. Researchers have revealed that ALA prevents the initiation and progression of breast malignancies by generating oxidation products.[26] In a study, incubation of MCF-7 cells with flaxseed extract reduced the viability of cancer cells in a dose-dependent manner, as the flaxseed extract mediated the formation of reactive oxygen species. Furthermore, the fatty acids present in flaxseed have the potential to trigger the mitochondrial pathway of apoptosis specifically in MCF-7 cells .[27] Clinical trials have also proven that consuming 25g of lignans per day reduces tumor development in patients with breast cancer.[28] Feeding flaxseed to athymic mice with established BT-474 tumors, in combination with trastuzumab treatment, reduced tumor size after two weeks. However, in the long term, only improved overall survival.[21] Researchers have indicated that ALA consumption decreased the growth of all BT-47 (luminal), MCF-7, MDA-MB-468 and MDA-MB-231 cell lines in a dose-dependent manner.[22] The concurrent administration of flaxseed oil and trastuzumab led to a decrease in the growth and spread of cancerous cells and an increase in apoptosis, as compared with using trastuzumab alone. Additionally, this combination was as effective as high-dose trastuzumab.[21]

We have shown that the expression of the BAX gene was heightened ($P < 0.05$), while the expression of the BCL-2 gene was decreased ($P < 0.05$) in BT-474 and MCF-7 cells exposed to FE, relative to untreated BT-474 and MCF-7, across all treatment durations. Unregulated apoptosis contributes to the development of breast cancer. The intrinsic apoptotic pathway can be induced through the raised expression ratio of *BAX/BCL-2* genes, thereby promoting cancer cell death.[29]

The results of different studies confirm our findings. Consumption of flaxseed or pure SDG by athymic mice that were injected with human MCF-7 cells decreased mRNA expressions of *Bcl-2,* cyclin *D, IGF-IR, pS2, EGFR*, *ERα, Erβ*.[19] In addition, studies indicated that flaxseed lignans SDG, END, and ENL have substantial anti-neoplastic effects on different human cancer cell lines via modulating signaling pathways that regulate cell proliferation and death.[30] Flaxseed lignans activated the apoptosis in acute myeloid leukemia cells via the mitochondrial pathway in an *in vitro* experiment which also promoted DNA fragmentation.[31]

Although we used proven techniques, such as the MTT test and qRT-PCR, to evaluate the effects of flaxseed extract on apoptosis and gene expression, it is important to note that these approaches do not offer a complete understanding of all the underlying mechanisms involved. Future research should include the integration of supplementary techniques, such as flow cytometry to detect apoptosis, Western blotting to analyze protein expression, and in vivo experiments to confirm our results in an animal model. In addition, our study specifically examined two breast cancer cell lines, BT-474 and MCF-7. Although these cell lines reflect certain subtypes of breast cancer, they do not fully encompass the diverse nature of the condition. By incorporating additional breast cancer cell lines, such as triple-negative and HER2-positive variations, into the study, a deeper understanding of the effects of flaxseed extract may be achieved. Combining flaxseed extract with other anti-cancer drugs is suggested to increase effectiveness and minimize side effects, which may lead to the discovery of powerful therapeutic approaches. Moreover, the process of isolating and purifying the active constituents in flaxseed extract could yield a more profound comprehension of its molecular mechanisms and potentially result in more precise and efficient therapeutic interventions. To the best of our knowledge, our study is the first to examine the effects of FE on *BCL-2* family genes in BT-474 cell lines.

Conclusion

Given the serious side effects and resistance to many anticancer drugs, the development of plant-derived compounds to create more efficient and safer antitumor agents has become of great interest. The study suggests that flaxseed extract has a cytotoxic impact on both molecular subtypes, luminal A and luminal B (BT-474 and MCF-7) of breast cancer, and induces apoptosis. This indicates that flaxseed extract might serve as an effective drug or supplement to prevent and treat breast cancer.

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Authors' Contribution
E.ZM: Study design, data gathering; S.M: Study design, data gathering; M.DT: Data gathering, drafting; S.D: Study design, data gathering; M.DA: Study design, reviewing the manuscript; SM.S: Study design, reviewing the manuscript; All authors have read and approved the final manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Conflict of interest
None declared.

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Table 1. The sequences of genes and control primers

Gene	Primer sequence (5'-3')	Product size
BAX	F:5TGGAGCTGCAGAGGATGATTG R: ATCAGTTCCGGCACCTTGG	178bp
BCL-2	F-GATGACTTCTCTCGTCGCTACCGT R-CGAGTGAGGATGTGCATGAA	118bp
BCL2L1(bcl-xl)	F- TGCATTGTTCCCATAGAGTTCCA R- CCTGAATGACCACCTAGAGCCTT	79 bp
GAPDH	F- GAAGGTGAAGGTCGGAGTC R- GAAGATGGTGATGGGATTTC	106bp

bp: Base pairs

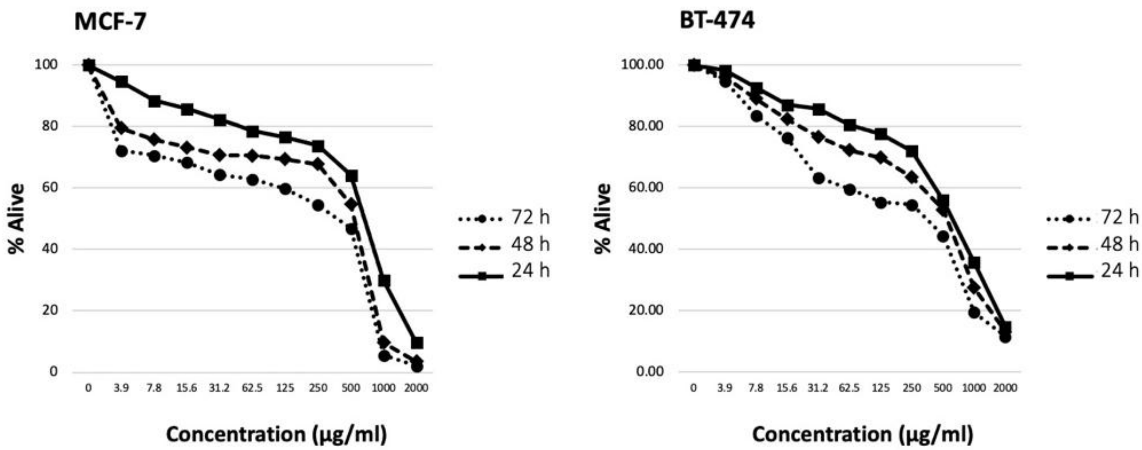

Figure 1. The cytotoxic effect of Flaxseed extract at concentrations (3.90628-2000 µg/ml) on BT-474 and MCF-7 cells. Flaxseed extract inhibited the proliferation and growth of BT-474 and MCF-7 cells in a concentration-dependent manner across all 24, 48, and 72 hours of incubation. The proliferation of MCF-7 cells treated with FE was significantly reduced at 0.5-3.9 µg/ml and 500-2000 µg/ml and the multiplication of FE-treated BT-474 cells remarkably repressed at concentration of 3.9-31.2 µg/ml and 250-2000 µg/ml at all time points (24,48 and 72 hours) ($P < 0.05$).

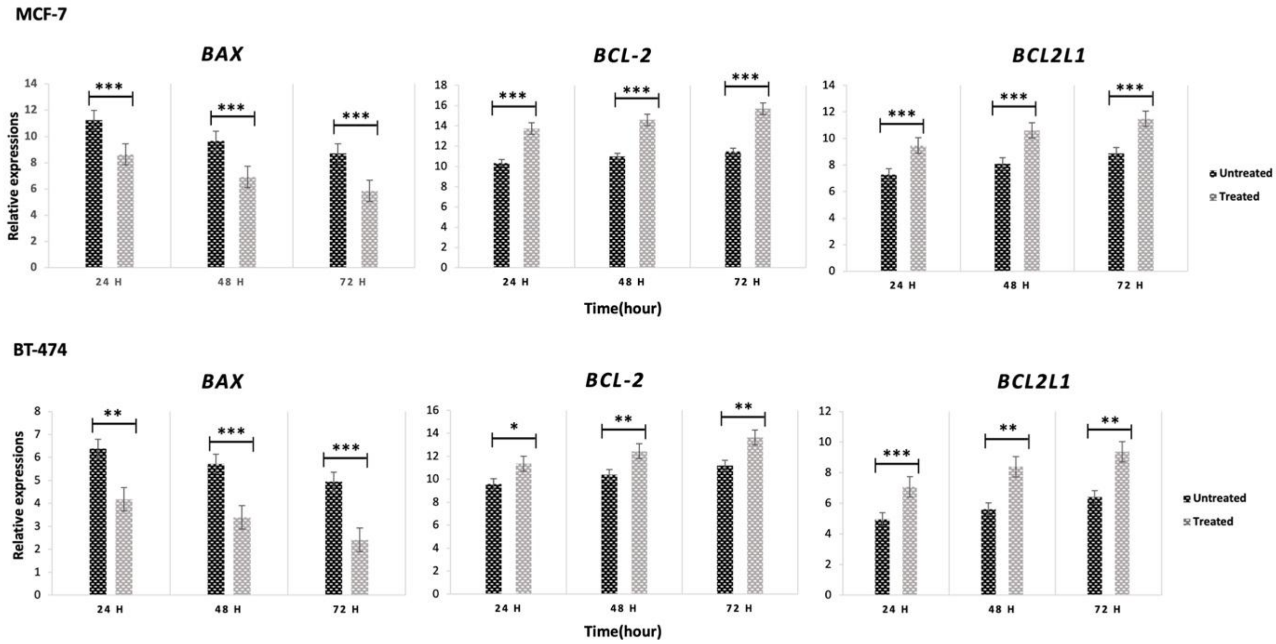

Figure 2. Relative expression of *BCL2L1*, *BCL-2* and *BAX* in BT-474 and MCF-7 cell lines exposed to flaxseed extract. The genes expression profile in treated and control cells was examined using qPCR and Ct method. The expression levels of these gene were evaluated at the IC50 values of BT-474: 406.9 µg/ml at 24 hours, 634.6 µg/ml at 48 hours, and 724.7 µg/ml at 72 hours, and for the MCF-7 cell line: 398.3 µg/ml at 24 hours, 593.4 µg/ml at 48 hours, and 884.5 µg/ml at 72 hours.

*, ** and *** shows significant at ≤ 0.05, ≤0.01 and ≤0.001, respectively.