



Molecular Evaluation of prognostic Markers of Breast Cancer (BC) after Treatment *in vitro*

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ABSTRACT

Breast cancer is a huge burden and poses serious threats to women's health worldwide. Despite significant advances in breast cancer biology and treatment methods. Usually, cancer is related to overexpression or misregulation of important genes which in turn, confer risk of developing breast cancer. Curcumin which is a natural compound found in the spice turmeric has been shown to have a number of potential health benefits, including antioxidant and anti-inflammatory effects. Recently, research has also suggested that curcumin may have potential as a treatment for breast cancer. Studies have shown that curcumin may be able to inhibit the growth of breast cancer cells and induce cell death in some types of breast cancer cells. It may also have the ability to inhibit the formation of new blood vessels through angiogenesis, which is necessary for cancer cells to grow and spread. This study is designed to investigate the effect of curcumin on of HER-2, PS2 and CTD on breast cancer. To achieve this aim we conducted the study on MCF-7 cell lines, and evaluate the cytotoxicity of different concentration of curcumin by MTT assay, RNA extracted from MCF-7 cell lines by Qiagen method and reverse transcriptase to cDNA finally quantification of the expression of genes (HER-2, PS2 and CTD) by Real time PCR (RT-PCR). The results showed that different concentration of curcumin make inactivation for genes related to breast cancer (HER-2, PS2 and CTD) and can stop the breast cancer pathway.

Keywords: Breast cancer, HER-2, PS2, CTD, Curcumin.

1. Introduction

Breast cancer (BC) is a significant burden and a major threat to the health of women everywhere. Locally advanced breast cancer (LABC), which has poor prognosis and a higher chance of local, regional, and distant metastases, continues to be a clinical concern despite substantial breakthroughs in breast cancer biology and therapeutic techniques. Unfortunately, neoadjuvant chemotherapy, the standard treatment for LABC, fails to eradicate tumours in 20 to 30 percent of patients (complete pathological response, or PCR) (Al-Kharashi *et al.*, 2022).

Based on clinical traits, treatment sensitivity, and prognoses, breast cancer can be divided into a wide variety of subtypes. Twenty to twenty-five percent of breast tumours globally have an overexpression of HER-2 (human epidermal growth factor receptor 2). Previous studies have demonstrated the viability of HER-2 as a therapeutic target for the treatment of breast cancer. Breast cancer has been influenced by HER-2 oncogenes since its inception (Yersal, 2014).

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The way that HER-2 is used to treat breast cancer has evolved significantly during the past ten years. Medical therapy innovations, such as the widespread application of HER-2-oriented therapy in early and advanced breast cancer, have transformed breast cancer care and altered the natural history of HER-2-positive breast cancer in addition to advancements in screening, genetic testing, imaging, surgical, and radiation technology. Numerous HER-2 medications have been created, including monoclonal antibodies, antibody-selective conjugates, and small molecule inhibitors (Ahmed *et al.*, 2015).

Targeted therapy has been referred to as one of the most advanced choices for cancer treatment due to its greater anti-cancer activity and decreased adverse effects. The prevalence of HER-2 breast cancer is around 34%. Breast cells that have HER-2 genes that are abnormally amplified "express" HER-2 on their surface, causing unchecked cell division and proliferation. The most advanced method of treating HER-2 positive breast cancer is Herceptin (HER), a monoclonal antibody that has been approved by the FDA. The interaction between HER and HER-2 not only prevents intracellular signaling but also sets off an immune response that results in cell death. HER still faces significant challenges, despite recent advancements. Very little anticancer activity is present (Ko *et al.*, 2017).

A small protein with plenty of cysteine called pS2 is released by around half of all human breast tumours. In the human breast cancer cell line MCF-7, oestrogen significantly activates the pS2 protein, and "cloning and sequence analysis of the pS2 gene" discovered a "oestrogen sensitive element" in the 5'-flanking region of the gene. The pS2 protein is a biomarker for hormone-dependent breast cancers, and research using breast cancer samples in immunohistochemical and radioimmunoassay methods have shown that its expression is associated with a longer overall and disease-free life. The pS2 protein is also present in normal stomach mucosa and tissues that regenerate in gastrointestinal ulcerative diseases. Its physiological function is unknown (Rio *et al.*, 1990)

CTD is a tumour suppressor gene, which means it helps control cell growth and division and may lessen the risk of developing cancer. The CTD gene, also referred to as the CTBP1 gene, produces the C-terminal binding protein protein. This protein controls how genes are expressed, and when it is altered or not working properly, it can cause unchecked cell growth and division, which raises the risk of cancer (Blevins *et al.*, 2017). Mutations in the CTD gene have also been connected to colorectal, gastric, and lung cancer in addition to breast, ovarian, and prostate cancer. It's crucial to remember that having a mutation in the CTD gene does not guarantee that a person will get cancer, but it may increase their risk (Saha *et al.*, 2022).

CTD and PS2 are both genes that have been studied in the context of breast cancer. It is important to note that the role of CTD and PS2 in breast cancer is complex and still being studied. Further research is needed to fully understand the relationship between these genes and breast cancer.

Curcuma Longa Extract (CUR) is a secondary metabolite and bioactive element of turmeric spice derived from the pulverized rhizome of the Curcuma longa plant, which belongs to the ginger family Zingiberaceae. This complex molecule, which possesses a wide range of pharmacological activity such as anti-inflammatory, antibacterial, antioxidant, and anticancer characteristics, has been widely used in Indian traditional medicine to prevent and cure a variety of illnesses. There is strong evidence that CUR plays a positive role in the treatment of breast cancer. By attacking regulatory proteins such "kinases, transcription factors, receptors, enzymes, growth factors, cell cycle" and "apoptosis-related molecules," as well as miRNAs, CUR fights breast cancer. Additionally, it has been shown to control the NF-B, Wnt/-catenin, JAK/STAT, and PI3K/Akt/mTOR signaling pathways (Farghadani & Naidu, 2022).

Numerous biological benefits, such as anti-inflammatory, antibacterial, antioxidant, anticancer, and antidiabetic activities, are present in curcuma longa extract. Because of this, Curcuma Longa Extract is thought to be a promising drug for the treatment of human illnesses such cancer, infectious diseases, neurological ailments, and diabetes. However, the following factors limit the use of curcuma longa extract: inadequate cellular absorption, low water solubility, aqueous instability, and poor bioavailability (Mbese *et al.*, 2019).

2. Material and Methods

Preparation of Curcuma Longa

Curcuma longa (Turmeric) root was selected based on their ethnomedical importance. Healthy disease-free roots and seeds were purchased from local market in Egypt. The plant materials were dried and pulverized. A weight of 40 mg of well air-dried powder of Curcuma longa roots was infused in

distal water (100ml) until complete exhaustion. The infusion was filtered through four-layered muslin cloth. Total concentration of obtained extract was 40 mg/L that was stored at 4°C till further use.

Cell line and Cell culture

Human breast cancer cell line, MCF7, was obtained from central public health laboratories in Egypt (CPHL). The cells were cultivated in T75 tissue culture flasks in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, 100 µg/mL streptomycin, 2 mM/L-glutamine and incubated in a 95% humidified incubator containing 5% CO₂ at 37°C. Now cells ready for treatment with with Curcuma Longa Extract.

MTT Assays

To evaluate the in vitro Cytotoxicity of the Curcuma Longa extract, the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed as previously described. cells were seeded in 96-well plates in DMEM supplemented with 10% fetal bovine serum, and 1% antibiotic antimycotic mixture. cell cultured overnight, the growth medium was removed from each well and the cells washed with 1X phosphate buffered saline (PBS). Different concentrations of Curcuma Long extract starting from 0.4 µg/ml were two-fold serially diluted in DMEM then added to cultured cells in 96-well plate in triplicate and incubated for 24 h post treatment to determine the cytotoxic concentration 50 (CC50). The medium was then discarded, and the cells washed with 1X PBS adding MTT solution (20 µL/well of 5 mg/ml stock solution) and incubated at 37 °C for 4 h till formulation of formazan crystals. Crystals were dissolved using a volume of 200 µL of dimethyl sulfoxide (DMSO) and the absorbance measured at λ max 540 nm using an ELISA microplate reader. Finally, the percentage of cytotoxicity compared to the untreated cells was determined.

$$\% \text{ Cytotoxicity} = \frac{\text{Absorbance of cell without treatment} - \text{Absorbance of cell with treatment}}{\text{Absorbance of cell without treatment}} \times 100$$

The total RNA was then extracted from cells using Qiagen extraction kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Subsequently, 500 ng of the purified RNA were used to synthesize the complementary DNA (cDNA) with random hexamer primers (Thermo Scientific) and (HERA SYBR® green RT-qPCR kit) Reverse Transcriptase (Thermo Scientific) according to the manufacturer's protocol. The quantitative real-time PCR (qRT-PCR) reaction mixture (25 µl) comprises the following: 0.5 µl of cDNA template, 12.5 µl of Maxima SYBR green PCR master mix (Thermo Scientific) and 1 µl of each primer (100 µM forward and reverse primers). Reactions were run in triplicate on Applied Biosystems 7500 real-time PCR system (Applied Biosystems, Foster City, California, USA). The cycling conditions were as follows: 2 min at 50 °C, 2 min at 95 °C, cDNA were amplified by 45 cycles of PCR, with each cycle consisting of 30 s at 94 °C, 30 s at 52 °C, and 30 s at 72 °C.

The primer sequences were as follows:

Primer	Sequence
HER-2-F	5'-AAC TGG TGT ATG CAG ATT GC-3'
HER-2-R	5'-AGC AAG AGT CCC CAT CCT A-3'
PS2 forward	5'-GGAGGAAAGGGCGTGAAG-3'
PS2 reverse	5'-CACAAACCGATGAGGATGGC-3'
CTD-F	5'- GCGAGAAAGCCTCCAGTG -3'
CTD-R	5'- CCACCATTCTCCTCCACCAGT -3'

Ct values were normalized to the values of the control β-actin housekeeping transcripts and log fold change were calculated according to the equation of 2^{-ΔΔct}

3. Results

In this study the effect of Curcuma Longa extract was detected on (MCF7) cell line as models of human Breast cancer cell line. β . Actin was used as housekeeping gene (positive control) , (HER-2, CTD and pS2 Genes).

1-Cytotoxicity of Curcuma Longa extract against MCF-7 Cell Lines Using MTT assay

Cytotoxicity tests are often designed to evaluate damage to cellular membranes, cell viability, cell death, or cell growth. To assess the cytotoxic effect of two different doses of Curcuma Longa Extract against human breast cancer cells (MCF7), the cells were treated with various concentrations of Curcuma Longa Extract (25 to 50 μ M). The MTT test was used to measure cell viability after 24 hours of incubation. The cytotoxicity assay results are shown in (fig .1).

Curcuma Longa Extract 's capacity to suppress the proliferation of MCF-7 cell lines was studied using a cytotoxicity test after the MCF-7 cell lines were exposed with various doses of Curcuma Longa Extract (25-50 μ M). Figure 1 depicts the concentrations and time-dependent effect of Curcuma on the viability of MCF-7 cell lines treated with various doses of Curcuma (25-50 μ M) for 24 and 48 hours. MCF-7 cell lines were tested for vitality after 24 and 48 hours. All amounts inhibited the multiplication of cancer cells (MCF-7) and demonstrated viability (fig .1)

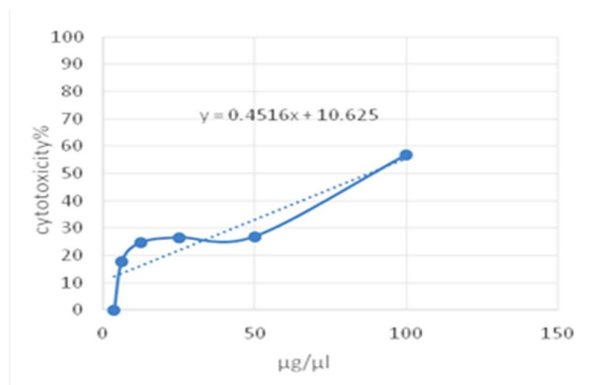


Fig. 1: Effects of different concentrations (25–50 μ M) of curcuma on the viability of MCF-7 cell lines treated for 24 h and 48 h, as measured by MT assay.

Molecular evaluation of the CTD gene after treatment of the breast cancer cell line with 50 μ M curcuma longa extract

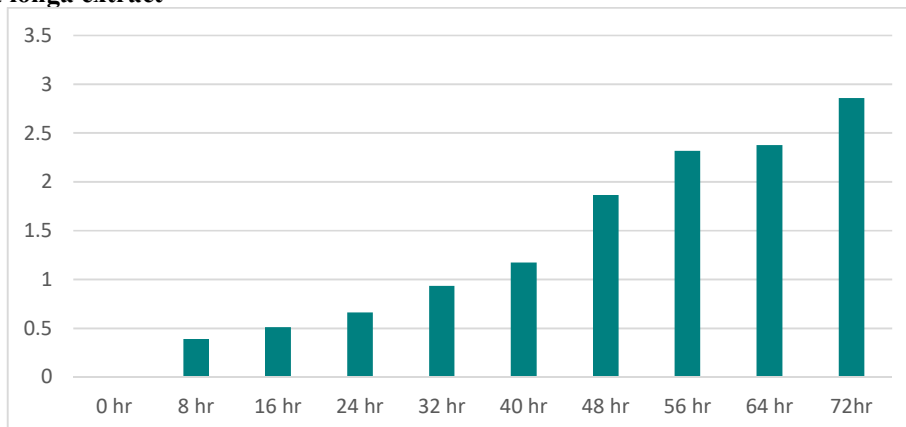


Fig. 2: Illustrate the effect of Curcuma Longa Extract 50 μ M on the gene expression of the CTD Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the Up regulation of the CTD gene was increase

Molecular evaluation of the CTD gene after treatment of the breast cancer cell line with 25 μ M curcuma longa extract

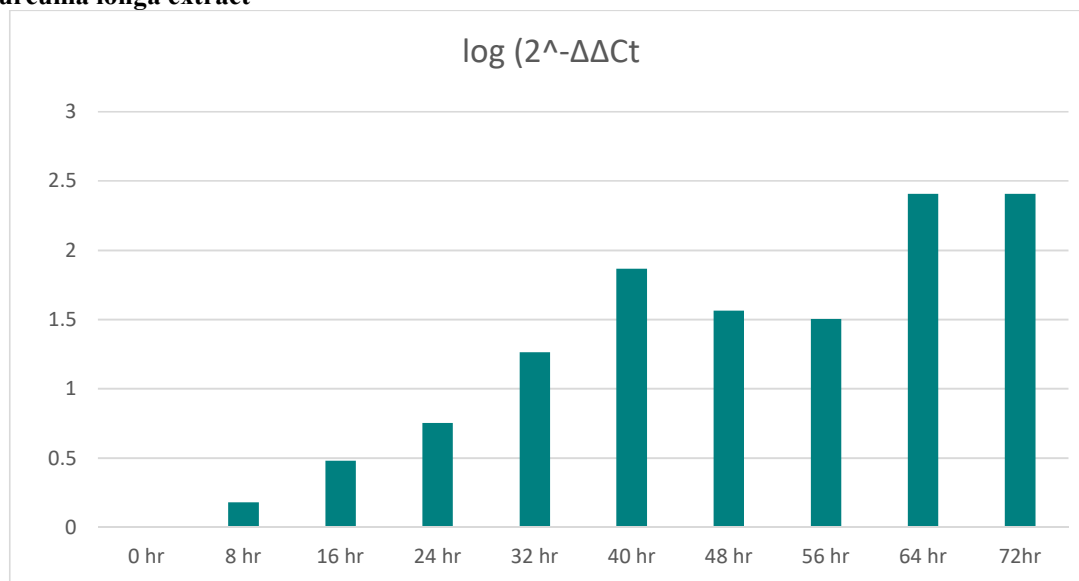


Fig. 3: Illustrate the effect of Curcuma Longa Extract 25 μ M on the gene expression of the CTD Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the Up regulation of the CTD gene was increase

Molecular evaluation of the Her-2 gene after treatment of the breast cancer cell line with 50 μ M curcuma longa extract

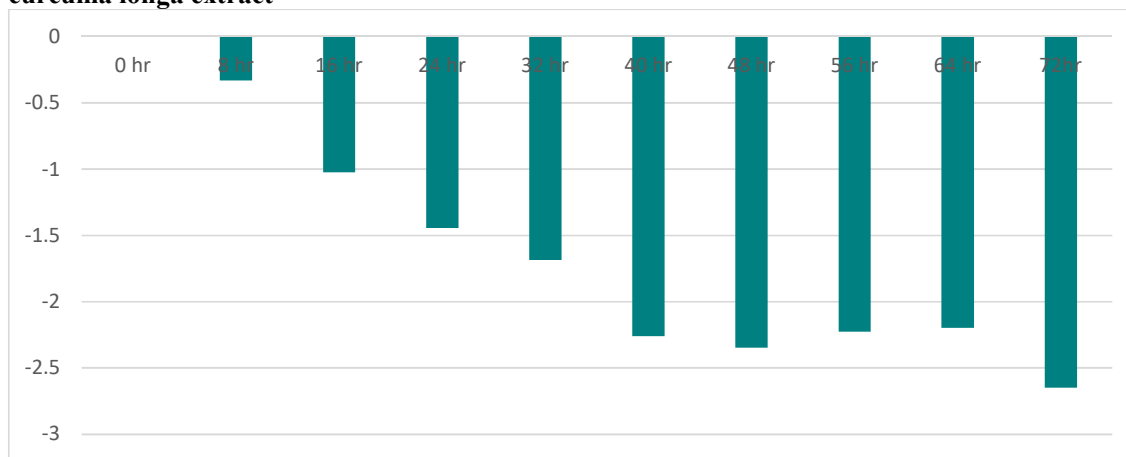


Fig. 4: The effect of Curcuma Longa Extract 50 μ M on the gene expression of the Her-2 Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the Her-2 gene was increase.

Molecular evaluation of the Her-2 gene after treatment of the breast cancer cell line with 25 μ M curcuma longa extract

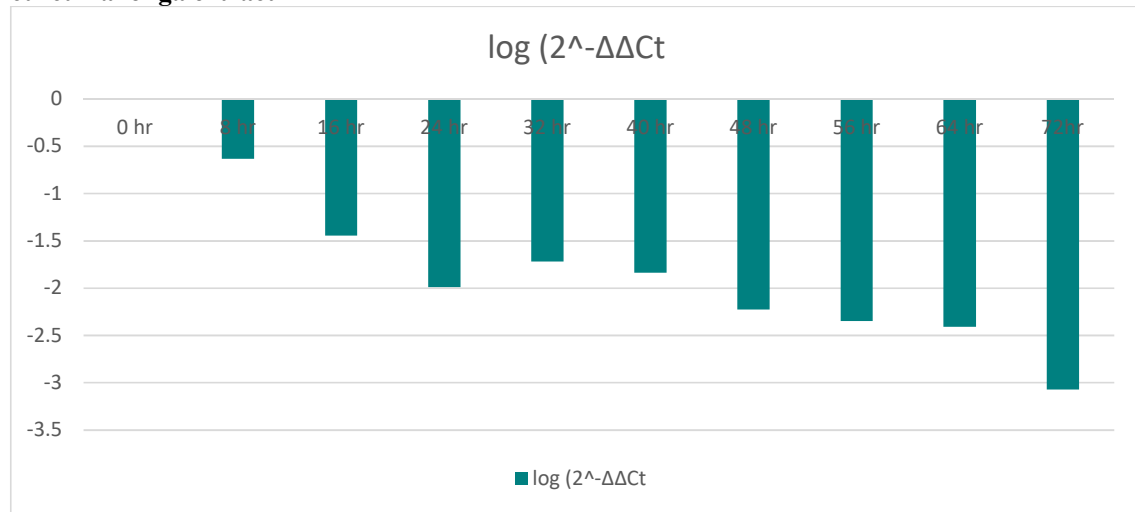


Fig. 5: The effect of Curcuma Longa Extract 25 μ M on the gene expression of the Her-2 Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the Her-2 gene was increase

Molecular evaluation of the PS2 gene after treatment of the breast cancer cell line with 50 μ M curcuma longa extract

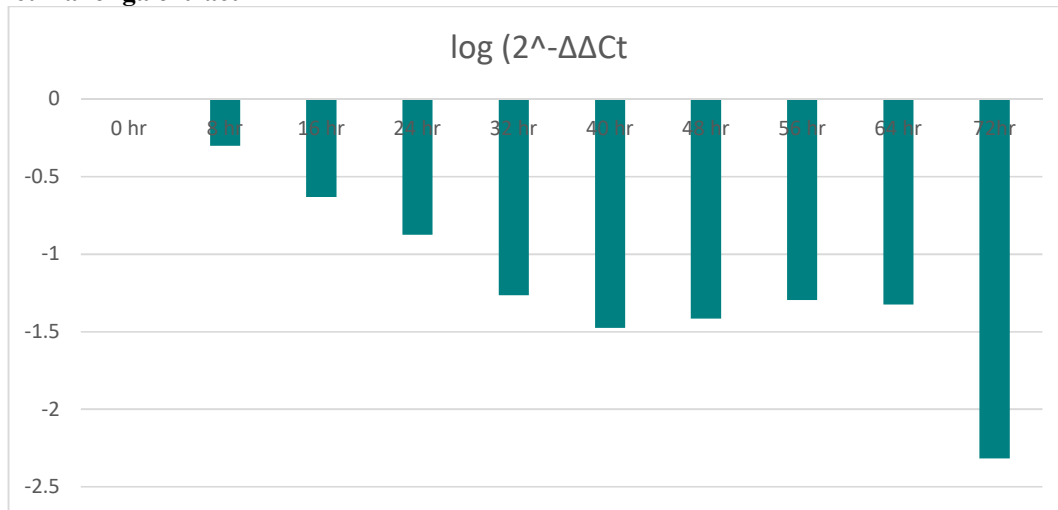


Fig. 6: The effect of Curcuma Longa Extract 50 μ M on the gene expression of the PS2 Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the PS2 gene was increase

Molecular evaluation of the PS2 gene after treatment of the breast cancer cell line with 25 μ M curcuma longa extract

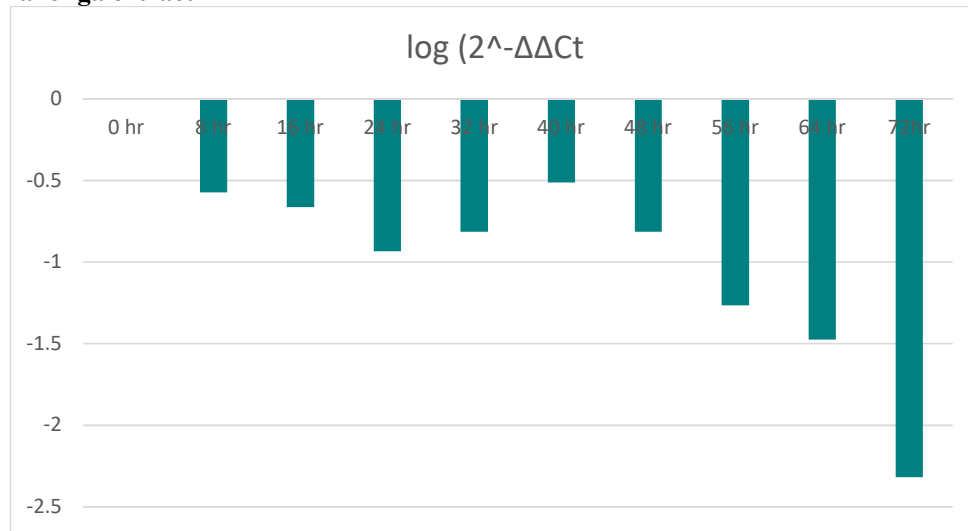


Fig. 7: The effect of Curcuma Longa Extract 25 μ M on the gene expression of the PS2 Gene in MCF-7 Cell Line. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time increase the down regulation of the PS2 gene was increase.

4. Discussion

Breast cancer (BC) is a widespread malignancy that affects people all over the world. BC is one of the top causes of cancer death in women, which has reduced as technology and novel diagnostic and treatment procedures have advanced. BCs are classified histologically as in situ or invasive carcinoma, and both can be ductal or lobular. The major duty after an assessment of invasive breast cancer is to determine which patients should take chemotherapy, which should not, and which should. If adjuvant therapy is chosen, the next difficulty is to determine the best therapy or combination of therapies for a specific patient (Łukasiewicz *et al.*, 2021). Prognostic biomarkers can aid in solving the first difficulty, whereas predictive biomarkers can aid in addressing the second. “ER, PR, HER-2, and the Mib1/Ki-67” proliferation index are the most relevant molecular markers associated with BC and are closely verified in the routine management of all primary, recurrent, and metastatic BC patients. We can begin the route to individualized therapy for every newly diagnosed BC patient by combining prognostic variables with accurate prognostic/predicted biomarkers (Petrelli *et al.*, 2015).

HER-2 belongs to the growth factor type I family and is found on chromosome 17q21.1. HER-2 is identical to ER, a key biomarker in breast cancer. This receptor family is largely engaged in cell-to-cell communication (Iqbal & Iqbal, 2014).

External growth stimuli influence gene transcription by “phosphorylation or DE phosphorylation” of a group of membrane proteins and intracellular signaling mediators. This gene is usually expressed in epithelial cells in a variety of organs, including the breast, prostate, prostate, bladder, pancreas, and the lung, and it is overexpressed in cancer cells. “This biomarker” is ubiquitously expressed in around 20-30% of initial cancers (English *et al.*, 2013). Human BC’s clinical behavior in relation to this gene was first demonstrated in 1987. According to early research, amplification of this gene can predict the period of recurrence and “overall survival (OS)” of BC patients. As a result, it appears that HER-2 can be regarded one of the prognostic biomarkers. Studies on the involvement of HER-2 in BC have produced inconsistent results over time, culminating to the fact that it is no longer recognized an unique and separate symptom of BC prognosis today (Metzger-Filho *et al.*, 2013). In breast cancer, circulating HER-2 receptor protein levels seem to be as beneficial as tumor size or ER and PR expression as a predictive predictor of survival. Cao *et al.*, reported in a research that elevated concentrations of “HER-2/neu receptor protein” were related with a substantial decrease in survival in BC patients. Reix *et al.*, discovered that the HER-2/neu receptor

protein appears to be a helpful regulatory biomarker for early diagnosis of recurrence and prognosis of metastatic BC in another investigation (Kabel, 2017).

In summary, the results indicate that there is a correlation between the different exposure hours and concentrations of curcuma longa extract and the expression of the understudied genes. The data represent a significant difference from control group across different exposure hours to Curcuma Longa Extract as the time and concentration increase the follows was observed: an increasing in the up regulation of the CTD gene, on the other hand, an increasing on the down regulation of Her-2 and PS2 genes.

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