RESEARCH ARTICLE

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# Chalcone-3 Inhibits the Proliferation of Human Breast Cancer MDA-MB-231 Cell Line

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## Abstract

**Objective:** Chalcone-3 has been shown to be cytotoxic and selective against luminal subtype breast cancer cell lines, which are suspected to occur through the mechanism of epidermal growth factor receptors (EGFR) inhibition. However, the cytotoxic effect has never been tested on cell strains from patients with triple negative breast cancer (TNBC), where EGFR expression is known to increase. This study aimed to identify the role of chalcone-3 in one of the downstream targets of EGFR as an antiproliferative agent. **Methods:** Chalcone-3 was examined for its effect on proliferation in human breast cancer MDA-MB-231 cell lines. The percentage of proliferation inhibition was analyzed using methyl-thiazol tetrazolium assay. Flow cytometry was used to analyze the population of cell cycle distribution and the expression of cyclin-D1 and pEGFR. **Results:** Chalcone-3 inhibited the proliferation of MDA-MB-231 cells in a dose and time-dependent manner with an $IC_{50}$ value of 17.98±6.36 µg/mL by inducing cell cycle arrest at the G2/M phase. Flow cytometry assays showed that chalcone-3 significantly reduced the expression of pEGFR and cyclin-D1, contributing to cell cycle arrest. **Conclusion:** Chalcone-3 might have potential as an anti-proliferative drug to treat TNBC.

**Keywords:** Chalcone-3- anti-proliferative drug- triple negative breast cancer- MDA-MB-231

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## Introduction

Breast cancer incidence in Indonesia is the highest of all malignancies, reaching 58,000 cases, followed by cervical and lung cancer. Meanwhile, the death rate from breast cancer in Indonesia has reached nearly 23,000 cases (GLOBOCAN, 2018). Research conducted by Rahmawati et al. (2018) showed that the percentage of breast cancer in Indonesia with Triple Negative Breast Cancer (TNBC) subtype was the second highest after luminal A subtype (25.5%). This subtype shows a poor degree of differentiation and a higher proliferation rate when compared to the luminal type. Progression and recurrence generally occur within 3 to 5 years after being diagnosed, with the distant metastases generally going to vital organs such as the brain and lungs (Dent et al., 2007; Kennecke et al., 2010). The high recurrence rate, and metastasis incidence cause a low survival rate for patients with TNBC (Pogoda et al., 2013; Steward et al., 2014).

In addition to the lack of estrogen receptors (ER), progesterone receptors (PR), and HER2 proteins as targeted therapy, resistance to chemotherapy is also challenging in TNBC treatment (Sharom, 2008; Yamada et al., 2013). Accordingly, new effective treatment strategies are urgently needed. In terms of anticancer pharmacological activity, one of the compounds that is often studied today is the chalcone derivative (Bonakdar et al., 2017; Wang et al., 2016; Dong et al., 2018; Xu et al., 2015; Harmastuti et al., 2012; Zhang et al., 2012; Maioral, et al., 2013; Wankhede et al., 2017). Suma et al. (2019) have synthesized a chalcone derivative with chloro substituent, chalcone 3 or (E) -1- (4-chlorophenyl) -3- (3,4-dimethoxyphenyl) prop-2-en-1-one with high yields (96.69%) and in a short time (65 min.) using the sonication method. The study showed a good anticancer effect on T47D and MCF7 cell lines, with $IC_{50}$ of 0.34 and 0.80 µg / mL, respectively. In vitro and in vivo studies on these compounds have not been conducted yet. However, a docking simulation study involving epidermal growth factor receptors (EGFR) showed an interaction between chalcone-3 and EGFR. The interaction occurs through two hydrogen bonds between the carbonyl oxygen atom and

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the MET769 residue, and between the p-OCH3 oxygen atom and the LYS721 residue. This interaction is thought to cause inactivation of EGFR and inhibit the growth of tumor cells.

EGFR has a very important role in the vital process of tumor cells, including proliferation. EGF activation of the EGFR stimulates many complex intracellular signaling pathways, including RAS/RAF/MEK/ERK and the PI3K/AKT axes (Batzer et al., 1994; Prenzel et al., 2000; Vivanco & Sawyers, 2002). These protein kinases have various cytoplasmic and nuclear targets, which aid in the transcription and translation of cyclin-D1, leading to G1/S cell cycle progression and proliferation (Wee & Wang, 2017; Onodera et al., 2005; Lo et al., 2005). Based on the above research, chalcone-3 is cytotoxic and selective against luminal subtype breast cancer cell lines, which are suspected to occur through the mechanism of EGFR inhibition. However, the cytotoxic effect has never been tested on cell strains from patients with TNBC, where EGFR is frequently overexpressed (Manupati et al., 2017). Therefore, this study aimed to determine the antiproliferative activity of chalcone-3 in MDAMB-231 TNBC cell lines.

## Materials and Methods

*Cells and reagents*

Chalcone-3 or (E)-1-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl) prop-2-en-1-one was synthesized from 4-chloroacetophenone and veratraldehyde, in the Faculty of Chemistry, Universitas Gadjah Mada, Yogyakarta, Indonesia. Chalcone-3 was solubilized in DMSO. Doxorubicin (Kalbe Farma, Indonesia) and gefitinib (AstraZeneca, United Kingdom) as control drugs were purchased from commercial sources in Indonesia. All the cell culture reagents were from Capricorn Scientific, Germany.

*Cells culture*

The human breast cancer MDA-MB-231 cell lines were obtained from Elabscience (EP-CL-0150, USA). MDA-MB-231 cells were grown in DMEM (DMEM-HA, Capricorn Scientific, Germany) containing 10% heat inactivated fetal bovine serum (FBS-HI-12B, Capricorn scientific), 1% penicillin/streptomycin (PS-B, Capricorn Scientific, Germany), and 0.5% amphotericin B (AMP-B, Capricorn Scientific, Germany). Cells were maintained at 37$^{o}$C in humidified air containing 5% CO2. The culture medium of the cells was removed and replaced by fresh medium every 2 days.

*Measurement of cell cytotoxicity*

The methyl-thiazol tetrazolium (MTT) assay was used to determine the cytotoxicity of chalcone-3 toward MDA-MB-231 cell lines. Cells were seeded in a 96-well plate to a final concentration of 10,000 cells/well and incubated for 24 h (37$^{o}$C, 5% CO2). The cells were then treated with serial concentrations of chalcone-3 (6.25, 12.5, 25, 50, 100 and 200 µg/mL) and control drugs (doxorubicin and gefitinib) prepared in growth media (0.781, 1.562, 3.125, 6.25, 12.5, 25, and 50 µg/mL), and incubated for an additional 24 h. Next, the medium was removed and fresh medium was added to each well along with 50 µl of MTT reagent (5 mg/mL). Subsequent to incubation at 37°C for 3 h, 150 µl MTT solvent (ab211091) was added to solubilize the MTT-formazan product and mixed thoroughly. The plates were wrapped in foil and kept overnight at room temperature. The spectrometric absorbance at 590 nm was measured using a microplate absorbance reader iMark (Bio-Rad, USA) and converted into the percentage of viable cells by using the following formula:

$$\% \text{ Viable cells} = \frac{(\text{treated cell absorbance} - \text{medium absorbance})}{(\text{control cell absorbance} - \text{medium absorbance})} \times 100$$

Dose-response curves were generated, and the concentrations of drugs required to inhibit cell viability by 50% ($IC_{50}$) were calculated using probit regression analysis, with SPSS (IBM Corp., Armonk, NY, USA). Three reduplicative wells were used for each treatment. The $IC_{50}$ was calculated for each experiment, and the mean $IC_{50}$s were calculated and presented as ± standard error (SE).

*Determination of cell proliferation*

The $IC_{50}$ evaluation determined from the cytotoxicity assay for each treatment, was used in the cell proliferation assay. Cells were seeded at a density of 10,000 cells/well in 96-well plates and treated with chalcone-3 for the desired concentrations (1/2x$IC_{50}$, $IC_{50}$, 2x$IC_{50}$, 4x$IC_{50}$) and time intervals (24, 48, and 72 h). Following incubation, the cell proliferation was analyzed using MTT assay as described above. The percentages of cell proliferation and proliferation inhibition were calculated using the following equations:

$$\% \text{ proliferation cells} = \frac{(\text{treated cell absorbance} - \text{medium absorbance})}{(\text{control cell absorbance} - \text{medium absorbance})} \times 100$$

$$\% \text{ proliferation inhibition} = 100 - \% \text{ cell proliferation}$$

Three reduplicative wells were used for each treatment and the cell proliferations were calculated and presented as mean ± SE.

*Analysis of cell cycle distribution*

MDA-MB-231 cells were plated at 5 x $10^5$ cells/mL in a six-well plate. After a 24 h incubation (37$^{o}$C, 5% $CO_2$), the cells were treated with chalcone-3 (1/2x$IC_{50}$, $IC_{50}$, 2x$IC_{50}$, 4x$IC_{50}$), doxorubicin, and gefitinib, and then incubated for an additional 24 and 48 h. The cell cycle phase evaluation was performed as described by Huang et al., (2017). Following trypsinization, cells were centrifuged at 3,000 x g for 5 min, at room temperature, and the pellet was resuspended in 0.5 mL of phosphate buffered solution (PBS). Cells were fixed in 70% ethanol overnight at 4°C and then treated with a staining PBS containing 1 mg/mL PI and 10 mg/mL RNase A at 37°C in the dark for 30 min. Cells were analyzed to determine the cell cycle stage using flow cytometry (BD FACSCantoTM II, San Jose, CA, USA) with an excitation wavelength of 488 nm and an emission at 670 nm. Percentages of cell populations distributed in the various phases of the cell cycle (sub-G1, G1, S and G2/M) were calculated.

*Analysis of cyclin-D1 and pEGFR expression*

Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 for 5 min and then blocked with 1% BSA/10% normal goat serum/0.3 M glycine in 0.1% PBS-Tween for 1 h. The cells were then labeled with Cyclin-D1 (ab16663) at 1/50 dilution or pEGFR (ab223499) at 1:400 dilution in 1%BSA-PBS overnight at 4°C, followed by a further incubation at room temperature for 1 h with a goat secondary antibody to Rabbit IgG (Alexa Fluor 488, ab150077) at 1/2,000 dilution in 23% glycerol, PBS, 1% BSA. Following a 45 min incubation (4°C, in the dark) and washing with PBS, the cells were centrifuged (5 min, 500 × g) to wash off excess antibodies and resuspended in 200 µl of PBS for flow cytometric analysis (BD FACSAriaTM III Cell Sorter, NJ, USA). Percentages of cyclin-D1 or pEGFR positive cells were calculated and the data presented as representative of those obtained in three independent experiments.

*Statistical analysis*

Data were presented as mean ± SE of at least three independent experiments. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's or Games-Howell post-hoc multiple comparison test. A value of $P < 0.05$ was considered statistically significantly different.

## Results

*Chalcone-3 inhibited MDA-MB-231 cell proliferation*

MDA-MB-231 cells were subjected to the MTT assay to evaluate the cytotoxicity of chalcone-3 treatment by measuring cell viability. The half-maximal inhibitory concentration ($IC_{50}$) after 24 hours of treatment with chalcone-3, doxorubicin, and gefitinib were about 17.98±6.36, 9.48±6.10, and 11.41±3.24 µg/mL, respectively (Figure 1). These values were used in subsequent experiments at serial concentration of 1/2x$IC_{50}$, $IC_{50}$, 2x$IC_{50}$, 4x$IC_{50}$ and time interval of 24, 48, and 72 h. Thus, the cells were exposed to 9, 18, 36, or 72 µg/mL of chalcone-3 with the desired time intervals. As shown in Figures 2 and 3, chalcone-3 affects cell proliferation even at a half concentration of $IC_{50}$ (9 µg/mL). The percentage of inhibition increased significantly to reach about 45.4% at a concentration of 18 µg/mL after 24 h of exposure. By augmenting chalcone-3 concentration to 72 µg/mL, inhibition percentage doubled to reach almost 83% compared to control cells. Chalcone-3 markedly inhibited cell proliferation at 9-72 µg/mL after 24 h of treatment in a dose-dependent manner. Similarly, a marked increase in the percentage of inhibition was detected at 48 h. The percentage of inhibition was about 11% at a half concentration of $IC_{50}$, and enhanced to a five-to eight-fold increase by doubling the concentration to 18-72 µg/mL, respectively. A significant increase in the percentage of inhibition was also observed as time of exposure progressively increases to 72 hours. Chalcone-3 inhibited cell proliferation about 61% at a concentration of 18 µg/mL, and further increased to 93% inhibition at 72 µg/mL compared to control cells. This inhibitory action was also similar to doxorubicin and gefitinib, where potentiation of proliferation inhibitory was noticeable after 24 h of treatment and exerted the highest effect at 72 h. Therefore, chalcone-3 significantly inhibited cell proliferation in a time and concentration-dependent manner, and appeared to be capable of exerting a proliferation inhibition on MDA-MB-231 cells under the present experimental conditions.

*Chalcone-3 arrests MDA-MB-231 cells at G2/M and Sub-G1 phases of cell cycle*

MDA-MB-231 cells treated with DMSO, control drugs, or different concentrations of chalcone-3 (9, 18, 36, or 72 µg/mL) for 24 or 48 h showed a typical DNA pattern that represented sub-G1, G1, S, and G2/M phases of the cell

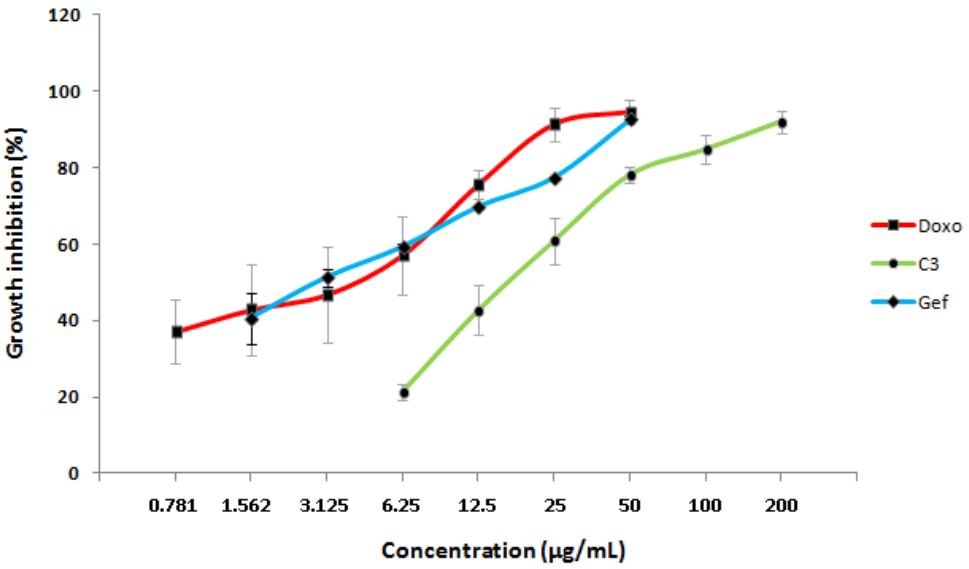


Figure 1. MDA-MB-231 Cells were Treated with Graded Concentrations of chalcone-3, doxorubicin, and gefitinib (0.781–200 µg/mL) for 24 Hours. Probit regression analysis showed that the IC50 of chalcone-3 was 17.98±6.36 µg/mL. Cell viabilities were measured by MTT assay. Data represent the means of three experiments.

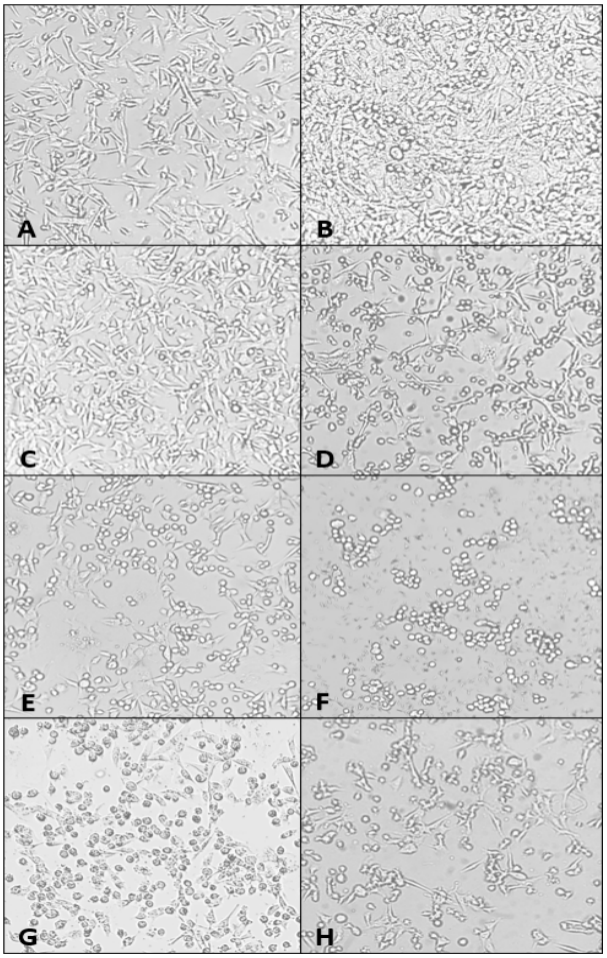


Figure 2. Photomicrograph of MDA-MB-231 Cells Examined under Light-Inverted Microscope, MagniFication x 100). (A). Confluent cells before treatment, (B). Control cells after 24 h, (C-F). Cells treated with 9, 18, 36, or 72 µg/mL of chalcone-3, (G-H). Cells treated with doxorubicin and gefitinib, respectively.

cycle. Tumor cells treated with 9-72 µg/mL chalcone-3 for 24 h showed higher G2/M population (19.8-20.7%) compared to vehicle control (16.4%). The percentages of sub-G1 phase (apoptotic cells) were significantly increased after cells were treated with 36 and 72 µg/mL chalcone-3, up to 7 and 14.4%, respectively, compared with 2% in control. The results of this experiment suggested that chalcone-3 firstly induces G2/M-phase cell cycle arrest even at low concentrations, whereas, chalcone-3 treatment at higher concentrations (36 and 72 µg/mL) induced cell death in MDA-MB-231 cells. Meanwhile, doxorubicin and gefitinib at 24 h cause significant S-phase arrest by increasing its cell population when compared to controls (12, 17.2, and 5.7%, respectively). The number of cells at the G2/M phase was also increased in doxorubicin and gefitinib treatment at 24 h, indicating cell cycle arrest at the G2/M phase. Moreover, the exposure of tumor cells to all treatment groups for 48 h evidently increased the proportion of cells in the Sub-G1 phases, which was accompanied by a significant decrease in cells in the G2/M phase (Figure 4).

*Chalcone-3 downregulates cyclin-D1 expression through the inhibition of EGFR activation*

Chalcone-3 induced a pronounced inhibition of proliferation and G2/M cell cycle arrest. Therefore, we investigated the expression of cyclin-D1, a crucial protein in cell cycle and proliferation induction. We found that chalcone-3 induces low levels of cyclin-D1 protein expression, starting significantly at 18 µg/mL chalcone-3 to almost half expression at 72 µg/mL chalcone-3 exposure (Figure 5). This protein expression was also reduced in doxorubicin and gefitinib treatment when compared to the control. The downstream signal transduction of EGFR is involved in the initiation of cyclin-D1 translation. Thus, EGFR might be involved in

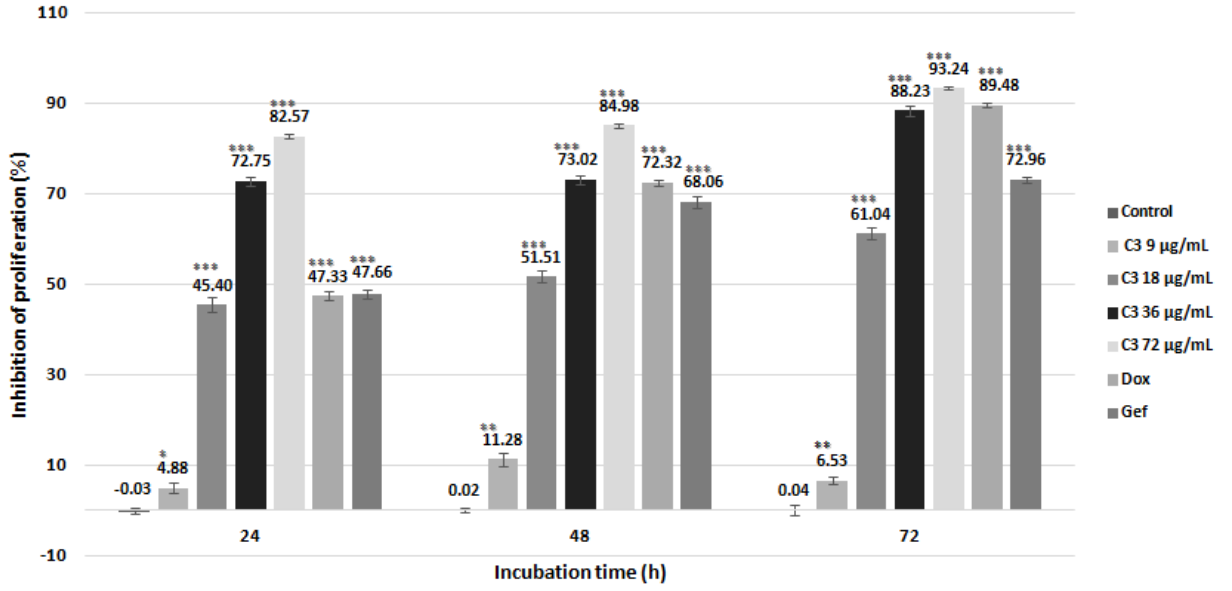


Figure 3. Effects of Chalcone-3 on Cellular Proliferation Inhibition of MDA-MB-231 Cells. The MTT assay was used to define the time-response curves. Data represent the means of three experiments ± standard error. *P<0.05, **P<0.01, ***P<0.001 compared to control cells.

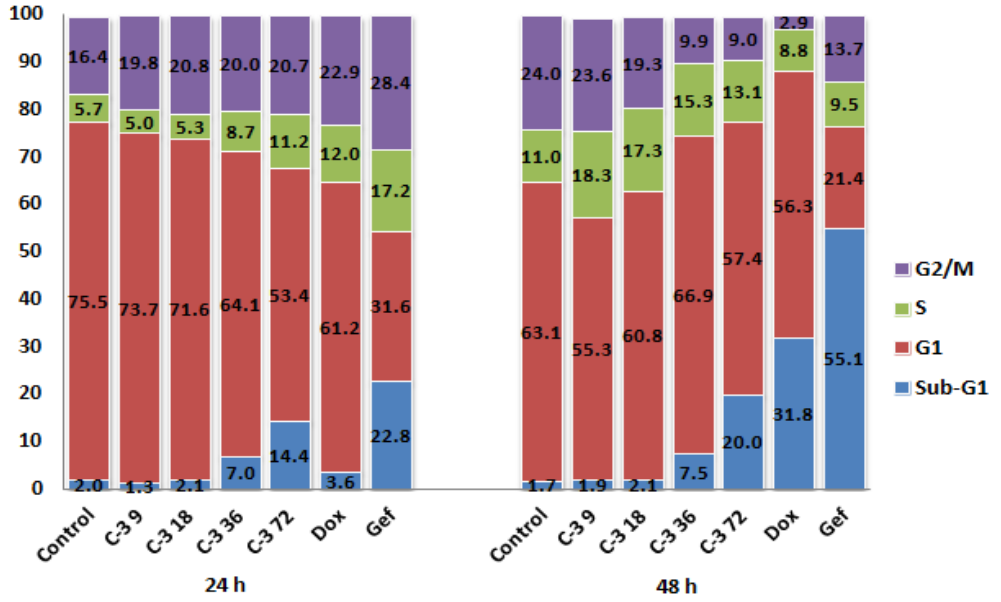


Figure 4. The Effects of Chalcone-3 on Cell Cycle Distribution after 24 h and 48 h Incubation. Bar diagram showing the distribution of the cells in the Sub-G1, G1, S and G2/M phases in MDA-MB-231 cells treated with 24 h and 48 h of chalcone-3 or controls. Treated cells showed higher G2/M population after 24 h and Sub-G1 population after 48 h incubation.

the chalcone-3 induced inhibition of MDA-MB-231 cell proliferation. The activation of this receptor (pEGFR) at the same concentrations was also confirmed by flow cytometry, which was decreased in a dose-dependent manner (Figure 6). All the treatment groups except doxorubicin showed inhibition of EGFR activation, where gefitinib had the most significant result.

## Discussion

Cell proliferation is the increase in the number of cells, through the process of cell division. It is a physiological process and generally increases in pathological conditions such as malignancy (Dang et al., 2003). Therefore, proliferation has emerged as an important mechanism by which chemical compounds may exhibit chemotherapy

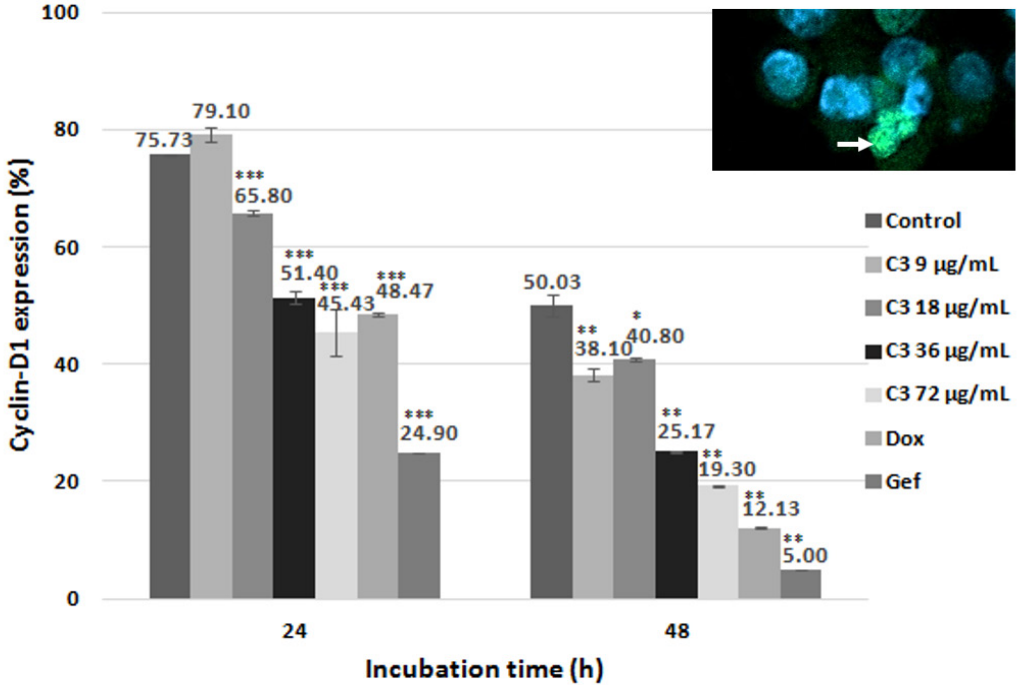


Figure 5. Effects of Chalcone-3 on Cyclin-D1 Expression of MDA-MB-231 Cells. Flow cytometry was used to analyze the percentage (%) of cylin-D1 positive cells. Data represent the means of three experiments ± standard error. *P<0.05, **P<0.01, ***P<0.001 compared to control cells. Cyclin-D1 (arrow) located in cell nucleus as depicted in the inset (Immunofluorescence micrograph).

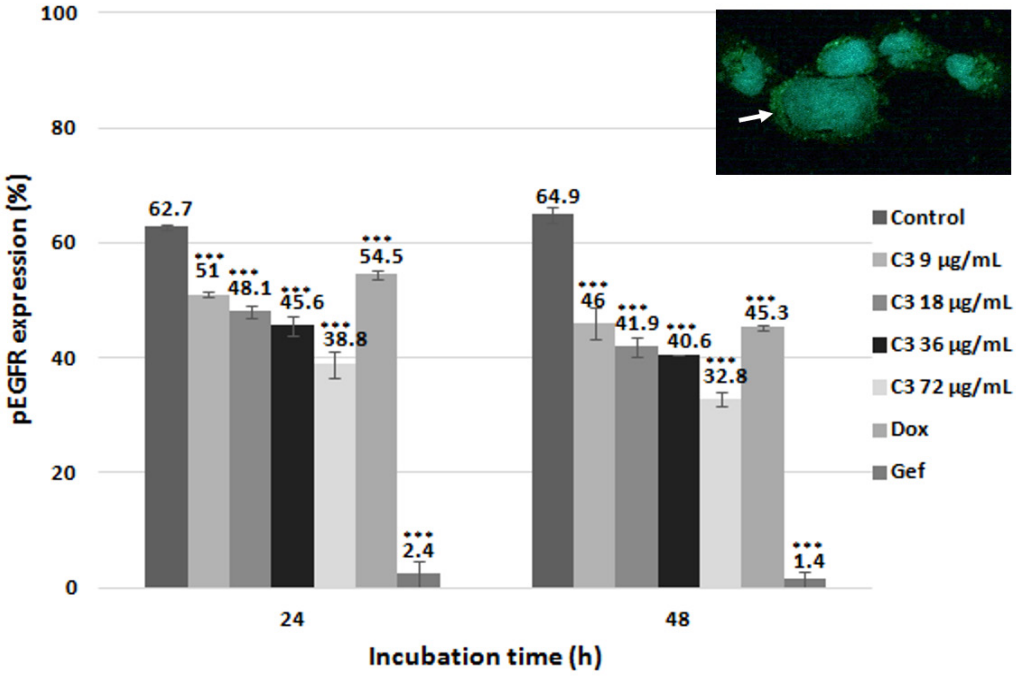


Figure 6. Effects of Chalcone-3 on pEGFR Expression of MDA-MB-231 Cells. Flowcytometry was used to analyze the percentage (%) of pEGFR positive cells. Data represent the means of three experiments ± standard error. *P<0.05, **P<0.01, ***P<0.001 compared to control cells. pEGFR (arrow) located in cell membrane as depicted in the inset (Immunofluorescence micrograph).

potential. In the present study, synthetic chalcone-3 exerted potent anti-proliferative effects on MDA-MB-231 cells in a dose and time-dependent manner with an $IC_{50}$ value of 17.98±6.36 µg/mL. Our previous study has documented the antiproliferative activity of chalcone-3 in other malignant human cell lines, such as MCF-7, T47D, HeLa, and WiDr cells, with the $IC_{50}$ values of 0.8, 0.34, 4.78, and 5.98 µg/mL, respectively (Suma et al., 2019). The most common proteins to be altered in chalcone antiproliferative studies are the cell cycle regulating proteins (p21, p27, Rb, Chk1 kinase, cdc2, cylin-B1, D, and E, as well as their complementary CDKs) and tubulins (α, α1c, and β tubulin).

The exposure of MDA-MB-231 cells to growth-suppressive concentrations of chalcone-3 in this study resulted in a significant increase in the proportion of cells in the G2/M and Sub-G1 phases. These findings are consistent with a recent report that chalcone compounds can induce cell cycle arrest and G2/M blockade in MDAMB-231 cells. As Hsu et al., (2006) reported in their previous study, chalcone (1,3-diphenyl-2-propenone) significantly decreased the expression of cyclin-B1, cyclin-A and Cdc2 protein, contributing to cell cycle arrest in the G2/M phase. Another natural chalcone (cardamonin) reported by Kong et al., (2020) was shown to enhance the expression and nuclear translocation of FOXO3a, inducing the expression of FOXO3a and its target genes, including p21 and p27. Furthermore, this process led to the reduction of cyclin-D1 and G2/M arrest. MDA-MB-231 cells treated with synthetic chalcone (ZK-CH-11d) for 24 h were also shown to cause G2/M cell cycle arrest by significantly downregulating phosphorylated cyclin-B1, cyclin-D1, cdc2, Rb and altering the expression of α, α1c tubulin, and β tubulin with a maximum of 72 h after treatment (Michalkova et al., 2022). The same result was reported by Elkhalifa et al., (2020), when using a nitrogen-based chalcone analog (E)-3-(4-(Bis(2-chloroethyl) amino) phenyl)-1-(3-methoxyphenyl) prop-2-en-1-one to treat MBA-MB-231 cells for 48 h. A chalcone-based small molecule, (E)-3-(2-bromo-3,4,5-trimethoxyphenyl)-1-(2,4-dihydroxyphenyl) prop-2-en-1-one (BDP), efficiently induced the degradation of EGFR, Akt, c-Raf, and Cdk4 in MDAMB-231 cells in a dose- and time-dependent manner. These consequently led to cell cycle arrest at the G2/M phases (Oh and Seo, 2017). Taken together, our study and previous studies suggest that chalcone may blockade the cell cycle progression at the G2/M phase due to several mechanisms, involving cell cycle regulatory proteins such as cyclins and CDKs. Doxorubicin and gefitinib were used in this study as a comparison to chalcone-3. Our results suggested that cell cycle arrest is predominantly induced at the G2/M checkpoint in MDA-MB-231 cells treated with doxorubicin or gefitinib for 24 h, although the effect was more evident in gefitinib. Similar results were also reported in previous studies, where MDA-MB-231 cells accumulated largely in the G2/M phase due to reduced expression of CDK1, CDK2, and cyclin-B1 (Oncul and Ercan, 2017; Wei et al., 2020). A significant increase in a sub-G1 fraction was observed in the cells treated with gefitinib for 24 h compared with control (22.8 and 2%, respectively), and maximum at 48 h (55.1 and 1.7%, respectively). Similar results were reported by a previous study, where gefitinib may increase p21 expression, a potent CDK inhibitor, which is necessary for orderly

progression through the S phase and G2/M (Okubo et al., 2004).

Cyclin-D1 as cell cycle regulatory protein, is initiated by the downstream signaling pathway of EGFR activation. Phosphorylation of EGFR-specific tyrosine residues leads to activation of various intracellular signaling pathways, including the PI3K/AKT, RAS/RAF/MEK/ERK, PLCγ/PKC, and JAK/STAT pathways. EGFR activation via the PI3K/AKT pathway leads to the formation of GRB2 and PI3K complexes. Phosphorylated PI3K converts PIP2 to PIP3 and further activates AKT, which have inhibitory phosphorylation targets, namely FoxO and GSK-3β. These proteins can inhibit cyclin-D1 expression and activity (Schmidt et al., 2002; Diehl et al., 1998). Another AKT substrate target is mTOR, where its activation inhibits 4E-BP. This protein plays a role in inhibiting the initiation of the cyclin-D1 translation process (Altomare et al., 2004; Jiang et al., 2003; Maennling et al., 2019). Meanwhile, in RAS/RAF/MEK/ERK signaling pathway, phosphorylation of the receptor tyrosine kinase leads to the formation of the GRB2-SOS complex. This causes a conformational change in SOS and converts GDP to GTP for RAS activation. pRAS activates RAF (MAPKKK), and pRAF further activates MEK (MAPKK). Furthermore, pMEK activates ERK1/2 (MAPK), which targeted substrates of proteins that play a role in the Cyclin-D1 transcript, such as c-MYC, c-JUN, and c-FOS (Liao et al., 2007; Sears, 2004; Zhang and Liu, 2002; Murphy et al., 2002). Another ERK1/2 target substrate is MNK, which activates eIF4E, and initiates the cyclin-D1 translation process (Maennling et al., 2019; Hou et al., 2012; Averous et al., 2008). The interaction between EGFR and PLCγ causes the hydrolysis of PIP2 to inositol 1,4,5-triphosphate (IP3). Furthermore, IP3 increases the concentration of intracellular calcium and DAG, and further co-activates PKC. This protein can activate the Ras/Raf/Erk and Akt pathways, and induce cell proliferation through both pathways (Sung et al., 2007; Schmidt et al., 2002; Diehl et al., 1998; Altomare et al., 2007; Jiang et al., 2003; Maennling et al., 2019). In addition to the three pathways above, EGFR and Janus Kinase (JAK) interaction will activate STAT3. Furthermore, pSTAT3 will undergo homodimerization and translocation to the nucleus to mediate cyclin-D1 expression (Park et al., 1996; Leslie et al., 2006).

Cyclin-D1 forms the cyclin-D1-CDK4/6 complex, and phosphorylates the Rb protein. Inactivation of Rb causes the release of Rb binding to E2F, which is a cyclin-E transcription factor, resulting in cell cycle progression from the G1 phase to the S phase (Cobrinik, 2005). Cyclin-D1 expression also increases in the G2/M phase (Yang et al., 2006). Thus, EGFR inactivation of the four signaling pathways above can suppress the cell proliferation process by inhibiting cyclin-D1 transcription and translation, and further cell cycle progression. Present studies showed that chalcone-3 affects the inhibition of cyclin-D1 expression in a dose and time-dependent manner. These results are consistent with the cell cycle arrest at the G2/M phase. The interaction between chalcone-3 and EGFR occurs through two hydrogen bonds between the carbonyl oxygen atom and residue MET769, and between the oxygen atom of p-OCH3 and residue LYS721, located in the tyrosine kinase domain of this receptor. This interaction is thought to cause EGFR inactivation, thereby inhibiting the various processes above and the growth of breast cancer cells. The results of this study indicated an inactivation of EGFR, which is marked by a decrease in pEGFR expression after treatment with serial concentrations of chalcone-3.

In conclusion, results of the present study provided clear evidence that chalcone-3 inhibits the cell proliferation through cell cycle arrest at the G2/M phase of human MDAMB-231. It may be mediated by the inactivation of EGFR and the subsequent signaling target, cyclin-D1. Thus, our findings provide a mechanistic framework for further exploration of the use of chalcone-3 as a promising chemotherapeutic agent for treatment of human breast cancer.

## Author Contribution Statement

All authors conceived and designed the study. The first author conducted the experiment, analyzed the data, and wrote the paper. All authors contributed to manuscript revisions, and approved the final version of the manuscript and agreed to be held accountable for the content therein.

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*General*

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*Ethical Declaration*

This study was approved by Medical and Health Research Ethics Committee (MHREC) of Faculty of Medicine, Public Health and Nursing (FKKMK), Universitas Gadjah Mada – Dr. Sardjito General Hospital, with a code number of KE/FK/1050/EC/2021.

*Availability of Data*

Data are available from the corresponding author, upon reasonable request.

*Conflicts of Interest*

The authors declare that they have no competing interests.

## References

Altomare D, Wang H, Skele K, et al (2004). AKT and mTOR phosphorylation is frequently detected in ovarian cancer and can be targeted to disrupt ovarian tumor cell growth. *Oncogene*, **23**, 5853–57.

Averous J, Fonseca B, Proud C (2008). Regulation of Cyclin-D1 expression by mTORC1 signaling requires eukaryotic

initiation factor 4E-binding protein 1. *Oncogene*, **27**, 1106–13.
Batzer AG, Rotin D, Urena JM, Skolnik EY, Schlessinger J (1994). Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. *Mol Cell Biol*, **14**, 5192-201.
Bonakdar APS, Vafaei F, Farokhpour M, Esfahani MHN, Massah AR (2017). Synthesis and anticancer activity assay of novel chalcone-sulfonamide derivatives. *Iran J Pharm Res*, **16**, 565-8.
Bortolotto LF, Barbosa FR, Silva G, et al (2017). Cytotoxicity of trans-chalcone and licochalcone A against breast cancer cells is due to apoptosis induction and cell cycle arrest. *Biomed Pharmacother*, **85**, 425-33.
Cobrinik D (2005). Pocket proteins and cell cycle control. *Oncogene*, **24**, 2796-809.
Dang C, Gilewski TA, Surbone A, Morton L (2003). Cell proliferation. In 'Holland-Frei Cancer Medicine', Eds Kufe DW, Pollock RE, Weichselbaum RR, et al. BC Decker Inc, Hamilton.
Dent R, Trudeau M, Pritchard KI, et al (2007). Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*, **13**, 4429-34.
Diehl JA, Cheng M, Roussel MF, Sherr CJ (1998). Glycogen synthase kinase-3 beta regulates Cyclin-D11 proteolysis and subcellular localization. *Genes Dev*, **12**, 3499–511.
Dong N, Liu X, Zhao T, et al (2018). Apoptosis-inducing effects and growth inhibitory of a novel chalcone, in human hepatic cancer cells and lung cancer cells. *Biomed Pharmacother*, **105**, 195-203.
Elkhalifa D, Siddique AB, Qusa M, et al (2020). Design, synthesis, and validation of novel nitrogen-based chalcone analogs against triple negative breast cancer. *Eur J Med Chem*, **187**, 111954.
GLOBOCAN (2018). Indonesia. The Global Cancer Observatory-IARC.
Harmastuti N, Herowati R, Susilowati D, Pranowo HD, Mubarika S (2012). Synthesis and cytotoxic activity of chalcone derivatives on human breast cancer cell lines. *Indones J Chem*, **12**, 261–7.
Hou J, Lam F, Proud C, Wang S (2012). Targeting Mnks for cancer therapy. *Oncotarget*, **3**, 118-31.
Hsu YL, Kuo PL, Tzeng WW, Lin CC (2006). Chalcone inhibits the proliferation of human breast cancer cell by blocking cell cycle progression and inducing apoptosis. *Food Chem Toxicol*, **44**, 704-13.
Huang L, Li A, Liao G, et al (2017). Curcumol triggers apoptosis of p53 mutant triple-negative human breast cancer MDA-MB 231 cells via activation of p73 and PUMA. *Oncol Lett*, **14**, 1080-88.
Jiang H, Coleman J, Miskimins R, Miskimins WK (2003). Expression of constitutively active 4EBP-1 enhances p27Kip1 expression and inhibits proliferation of MCF7 breast cancer cells. *Cancer Cell Int*, **3**, 1-10.
Kennecke H, Yerushalmi R, Woods R (2010). Metastatic behavior of breast cancer Ssubtypes. *J Clin Oncol*, **28**, 3271-7.
Kong W, Li C, Qi Q, Shen J, Chang K (2020). Cardamonin induces G2/M arrest and apoptosis via activation of the JNK-FOXO3a pathway in breast cancer cells. *Cell Biol Int*, **44**, 177-88.
Leslie K, Lang C, Devgan G, et al (2006). Cyclin-D1 is transcriptionally regulated by and required for transformation by activated signal transducer and activator of transcription 3. *Cancer Res*, **66**, 2544-52.
Liao DJ, Thakur A, Wu J, Biliran H, Sarkar FH (2007). Perspectives on c-Myc, Cyclin-D1, and their interaction in cancer formation, progression, and response to chemotherapy. *Crit Rev Oncog*, **13**, 93-158.
Lin S, Makino K, Xia W, et al (2001). Nuclear localization of EGF receptor and its potential new role as a transcription factor. *Nat Cell Biol*, **3**, 802–8.
Lo HW, Hsu SC, Hung MC (2005). EGFR signaling pathway in breast cancer: From traditional signal transduction to direct nuclear translocalization. *Breast Cancer Res Treat*, **95**, 211-8.
Maennling AE, Tur M, Niebert M (2019). Molecular targeting therapy against EGFR family in breast cancer: progress and future potentials. *Cancers*, **11**, 1826.
Maioral MF, Gaspar PC, Souza GRR, et al (2013). Apoptotic events induced by synthetic naphthylchalcones in human acute leukemia cell lines. *Biochimie*, **95**, 866-74.
Manupati K, Dhoke NR, Debnath T, et al (2017). Inhibiting epidermal growth factor receptor signalling potentiates mesenchymal–epithelial transition of breast cancer stem cells and their responsiveness to anticancer drugs. *FEBS J*, **284**, 1830-54.
Michalkova R, Kello M, Kudlickova Z, et al (2022). Programmed cell death alterations mediated by synthetic indole chalcone resulted in cell cycle arrest, DNA damage, apoptosis and signaling pathway modulations in breast cancer model. *Pharmaceutics*, **14**, 503.
Murphy LO, Smith S, Chen RH, Fingar DC, Blenis J (2002). Molecular interpretation of ERK signal duration by immediate early gene products. *Nat Cell Biol*, **4**, 556-64.
Oh YJ, Seo YH (2017). A novel chalcone-based molecule, BDP inhibits MDA-MB-231 triple-negative breast cancer cell growth by suppressing Hsp90 function. *Oncol Rep*, **38**, 2343-50.
Okubo S, Kurebayashi J, Otsuki T, et al (2004). Additive antitumour effect of the epidermal growth factor receptor tyrosine kinase inhibitor gefitinib (Iressa, ZD1839) and the antioestrogen fulvestrant (Faslodex, ICI 182,780) in breast cancer cells. *Br J Cancer*, **90**, 236-44.
Oncul S, Ercan A (2017). Discrimination of the effects of doxorubicin on two different breast cancer cell lines on account of multidrug resistance and apoptosis. *Indian J Pharm Sci*, **79**, 599-607.
Onodera Y, Hashimoto S, Hashimoto A, et al (2005). Expression of AMAP1, an ArfGAP, provides novel targets to inhibit breast cancer invasive activities. *EMBO J*, **24**, 963-73.
Park OK, Schaefer TS, Nathans D (1996). In vitro activation of Stat3 by epidermal growth factor receptor kinase. *Proc Natl Acad Sci U S A*, **93**, 13704-8.
Pogoda K, Niwinska A, Murawska M, Pienkowski T (2013). Analysis of pattern, time and risk factors influencing recurrence in triple-negative breast cancer patients. *Med Oncol*, **30**, 388.
Prenzel N, Zwick E, Leserer M, Ullrich A (2000). Tyrosine kinase signalling in breast cancer: Epidermal growth factor receptor-convergence point for signal integration and diversification. *Breast Cancer Res*, **2**, 184-190.
Rahmawati Y, Setyawati Y, Widodo I, Ghozali A, Purnomosari D (2018). Molecular subtypes of Indonesian breast carcinomas: lack of association with patient age and tumor size. *Asian Pac J Cancer Prev*, **199**, 161-6.
Schmidt M, Fernandez de Mattos S, van der Horst A, et al (2002). Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of Cyclin-D1. *Mol Cell Biol*, **22**, 7842–52.
Sears RC (2004). The life cycle of c-myc from synthesis to degradation. *Cell Cycle*, **3**, 1133-7.
Sharom FJ (2008). ABC multidrug transporters: structure, function and role in chemoresistance. *Pharmacogenomics*,

**9**, 105-27.
Steward L, Conant L, Gao F, Margenthaler JA (2014). Predictive factors and patterns of recurrence in patients with triple negative breast cancer. *Ann Surg Oncol*, **21**, 2165-71.
Suma AAT, Wahyuningsih TD, Mustofa (2019). Anticancer activities and molecular docking studies. *Rayasan J Chem*, **12**, 502-10.
Sung SM, Jung DS, Kwon CH, et al (2007). Hypoxia/reoxygenation stimulates proliferation through PKC-dependent activation of ERK and Akt in mouse neural progenitor cells. *Neurochem Res*, **32**, 1932-39.
VanArsdale T, Boshoff C, Arndt KT, Abraham RT (2015). Molecular pathways: targeting the cyclin d–cdk4/6 axis for cancer treatment. *Clin Cancer Res*, **21**, 2905-10.
Vivanco I, Sawyers CL (2002). The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer*, **2**, 489-501.
Wang Y, Hedblom A, Koerner SK, et al (2016). Novel synthetic chalcones induce apoptosis in the A549 non-small cell lung cancer cells harboring a KRAS mutation. *Bioorg Med Chem Lett*, **26**, 5703-6.
Wankhede S, Kumar N, Simon L, et al (2017). Evaluation of in vitro and in vivo anticancer potential of two 5-acetamido chalcones against breast cancer. *EXCLI J*, **16**, 1150-63.
Wee P, Wang Z (2017). Epidermal growth factor receptor cell proliferation signaling pathways. *Cancers*, **9**, 1-45.
Wei T, Xiaojun X, Peilong C (2020). Magnoflorine improves sensitivity to doxorubicin (DOX) of breast cancer cells via inducing apoptosis and autophagy through AKT/mTOR and p38 signaling pathways. *Biomed Pharmacother*, **121**, 109139.
Xu S, Chen M, Chen W, et al (2015). Chemopreventive effect of chalcone derivative, L2H17, in colon cancer development. *BMC Cancer*, **15**, 870.
Yamada A, Ishikawa T, Ota I, et al (2013). High expression of ATP-binding cassette transporter ABCC11 in breast tumors is associated with aggressive subtypes and low disease-free survival. *Breast Cancer Res Treat*, **137**, 773-82.
Yang K, Hitomi M, Stacey DW (2006). Variations in cyclin-D1 levels through the cell cycle determine the proliferative fate of a cell. *Cell Div*, **1**, 1-8.
Zhang W, Liu HT (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res*, **12**, 9-18.
Zhang Y, Srinivasan B, Xing C, Lu J (2012). A new chalcone derivative (e)-3-(4-methoxyphenyl)-2-methyl-1-(3,4,5-trimethoxyphenyl) prop-2-en-1-one suppresses prostate cancer involving p53-mediated cell cycle arrests and apoptosis. *Anticancer Res*, **32**, 3689-98.