Research Article

Curcumin as an Anti-Proliferative Agent in Breast Cancer through RASSF1A, Bax, and Caspase-3 Protein

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ABSTRACT

Introduction: Curcumin is a polyphenol that has pharmacological activity that can inhibit tumor cell growth and induce apoptosis through various mechanisms. However, the specific mechanism of curcumin cytotoxicity remains controversial because of many anti- and pro-apoptotic signaling pathways in various cell types. This study aims to examine the relationship among curcumin on RASSF1A, Bax protein levels, and caspase-3 activity in supporting the apoptotic mechanism in CSA03 breast cancer cells. **Method:** Curcumin administration to cancer cells is based on differences in dosage with 24-hour incubation. Cytotoxicity after curcumin administration was determined using MTS. RASSF1A and Bax protein levels were tested through ELISA. Caspase-3 activity was used to determine apoptosis and was tested using flow cytometry. **Results:** The results indicated that curcumin had a cytotoxicity effect of 40.85 μg/mL. At concentrations of 40 μg/mL and 50 μg/mL, curcumin increases levels of protein RASSF1A (Δ = 26.53% and 47.35%, respectively), Bax (Δ = 48.79% and 386.15%), and caspase-3 (Δ = 1,678.51% and 1,871.889%) significantly. **Conclusions:** Curcumin exhibits anti-proliferative activity and apoptotic (Caspase-3) effects through activation of RASSF1A and Bax.

Keywords: Bax, caspase-3, curcumin, CSA03, RASSF1A

Introduction

The frequency of breast cancer tends to increase. Data from the National Cancer Institute (NCI)'s Surveillance Epidemiology and End Results (SEER) program indicate that the incidence rate of breast cancer in the United States was 12.4% for 2005–2009, and in 2018 there were 454,865 new cases of invasive breast cancer [1]. In Indonesia, breast cancer in 2010 ranked first in terms of malignancy, accounting for 18.58% of cancer cases, most often at the age of 35–54 years [2]. Recently, resistance to hormone therapy or chemotherapy has been reported since the beginning or during the

treatment. Many strategies for selecting the therapy based on molecular mechanisms are currently being implemented to overcome the resistance in breast cancer treatment [3].

One of the molecular mechanisms of breast cancer is epigenetic changes in the form of hypermethylation [4]. The tumor suppressor gene in breast cancer that often undergoes hypermethylation is the RASSF1A gene that plays a role in cell cycle processes, mitosis, and apoptosis [5]. RASSF1A can regulate the activity of the Bax protein to release cytochrome-c so that it activates caspase-3, which is the executor and is responsible for apoptosis [6]. Hypermethylation is reversible, and it is possible to re-express hypermethylated genes in cancer with demethylation drugs, so that the tumor suppressor genes that were previously inactive can be regulated and their function be improved. The administration of demethylation drugs in the tumor cell lines causes the reactivation of the tumor suppressor function, but demethylation drugs have side effects that limited the dose and duration of the treatment and have potential to form mutagenic lesions [4], [7], [8]. Therefore, the use of new compounds from natural substances that can affect epigenetic changes and have low toxicity to inhibit the development of cancer cells is an important field of cancer treatment research.

One of the natural compounds that have an epigenome target is curcumin. Curcumin is the polyphenol that can efficiently induce apoptosis [9], [10]. However, the specific mechanism of curcumin cytotoxicity via the RASSF1A pathway is still controversial in various cell types [11]. One study demonstrated that curcumin can increase Bax expression via the P53 pathway.

The previous study demonstrated that the administration of curcumin treatment to MCF-7 and MDA-MB-468 cells efficiently enhances the suppression of the growth of the breast cancer cells *in vitro* through reactivation of RASSF1A, upregulation of Bax, and induction of apoptosis [12]. To be able to implement the results of these studies in Indonesia, it is necessary to research cell lines originating from Indonesia, because differences in the microenvironment can affect research results. Experimental research was carried out on the luminal

A breast cancer cells from Indonesian patients using CSA03 cells. The present study was aimed at investigating the cytotoxic effect and apoptosis-induced effects of curcumin via the RASSF1A pathway CSA03 cell line *in vitro*. The results of this study can be used as the basis of literature for further research.

Methods
Materials
The CSA03 breast cancer cells were obtained from the integrated laboratory of the Faculty of Medicine, University of Indonesia which was operated on at Dr. Cipto Mangunkusumo Jakarta. Dubelco's minimum essential medium (DMEM) (Gibco, New York, USA) was supplemented with 10% fetal bovine serum (Biowest, Riverside, USA), 5% amphotericin, and 5% penicillin-streptomycin (Biosciences, San Jose, USA). Curcumin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The MTS kit was obtained from Promega (Madison, USA). The RASSF1A kit was obtained from MyBioSource, and the Bax kit was obtained from Abcam. The caspase-3 activity was determined using PE-conjugated the monoclonal active the caspase-3 antibody apoptosis kit (BD PharMingen, Heidelberg, Germany).

Morphology of Cells
The cell morphology examination was carried out on a paraffin block of the cancer patient using hematoxylin-eosin staining.

The Cell Culture
The CSA03 cells were cultured in a Tissue Flask containing complete culture media. After the cells were harvested, the cells were counted using a hemocytometer. There were 1×10^6 cells/well, and curcumin that had been dissolved in DMSO was added. After the harvesting of CSA03 cells, cell blocks were made, and hematoxylin-eosin staining was carried out to see morphological features of the cells.

The Cytotoxicity Examination
The cytotoxicity examination used the colorimetric principle with the MTS assay. As many as 5×10^3 cells of CSA03 per well were plated in 96-well plates and treated with curcumin at concentrations of 20, 30, 40, 60, 80

μg/mL. 0.1% DMSO was used as a control. It was then incubated for 24 hours. After adding 20 mg/mL 3-(4, 5 dimethylthiazol-2-yl)-5-(3-carboximetoniphenol)-2-(4-sulphodenil)-2-H-tetrazolium) (MTS) to each well, it was incubated again for 2 hours at temperature 37 °C. Furthermore, the optical density was determined using a microplate reader that was set at 490 nm. IC50 was calculated based on

$$\% \text{ inhibition} = \frac{\Delta \text{ Control uptake} - \Delta \text{ Sample uptake}}{\Delta \text{ Control uptake}} \times 100 \%.$$

Furthermore, the data from the cytotoxicity examination were used as the basis for determining the concentration of curcumin to be exposed to breast cell cultures. The determination of the curcumin concentration was carried out around the IC50 value.

Caspase-3 Examination

Caspase-3 examination was carried out using a immunofluorescence technique (apoptosis Kit Catalog No. 550914 from BD PharMingen). The cells that had been treated were then given the steps on the datasheet and put into the flow chamber. The electronic system converted the detected light signals into electronic signals that could be processed by a computer. Cells that did not indicate an autofluorescent pattern were grouped, compared against the whole cell, and their proportion was then determined as a percentage.

RASSF1A and the Bax Protein Assay

RASSF1A and Bax protein examination was conducted using the ELISA technique (RASSF1 ELISA Kit Catalog No. MBS2706790 from MyBioSource and Bax Catalog No. ab133048 from Abcam). The results of RASSF1A and Bax protein levels obtained from the ELISA reader were optical density (OD) values converted into RASSF1A protein levels (ng/mL) and Bax protein levels (pg/mL) using standards.

Data Analysis

Data were processed statistically using the Statistical Program for Social Science (SPSS) version 20.0 software for Windows. Hypothesis testing was performed using a non-parametric numerical comparative hypothesis test flow. To assess the difference between >2 groups of unpaired concentrations, the Kruskal–Wallis non-parametric test was used, and to assess the significant difference in the two groups, a Mann–Whitney posthoc analysis was made. The correlation test between the two variables was conducted using the Spearman non-parametric test.

The average growth rate (delta) was calculated using the following formula:

Result and Discussion
Results
Cell Morphology

The morphological picture of CSA03 cells consists of cells with pleomorphic nuclei, fine or coarse granular chromatin, partially irregularly shaped nucleolus, a small vacuole, and an eosinophilic cytoplasm (Figure 1). Figure 2 illustrates the CSA03 cells in Tissue Flask that are ready to be harvested. The results of hematoxylin-eosin staining on CSA03 cells that had been made block cells indicate the same nuclei image as the nuclei image on the paraffin block; the cell morphology examination was carried out on the paraffin block of the cancer patient using hematoxylin-eosin staining (Figure 3).
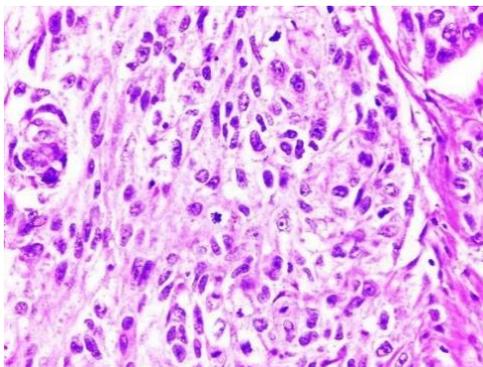

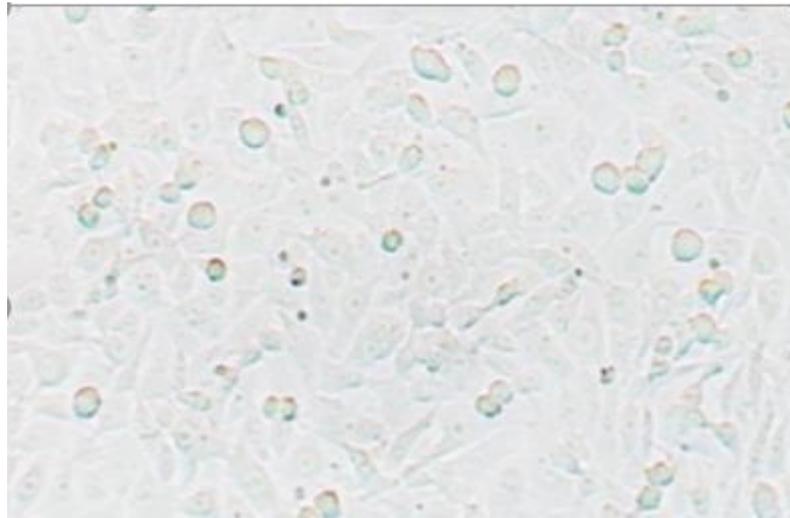*Figure 1. The morphological feature of CSA03 (HE, 400×)*

Figure 2. Microscopic feature of CSA03 Cells in the Tissue Flask (400×)

$$\text{Growth rate (Delta)} = \frac{\text{Data after exposure} - \text{Data without exposure}}{\text{Data without exposure}} \times 100\%$$
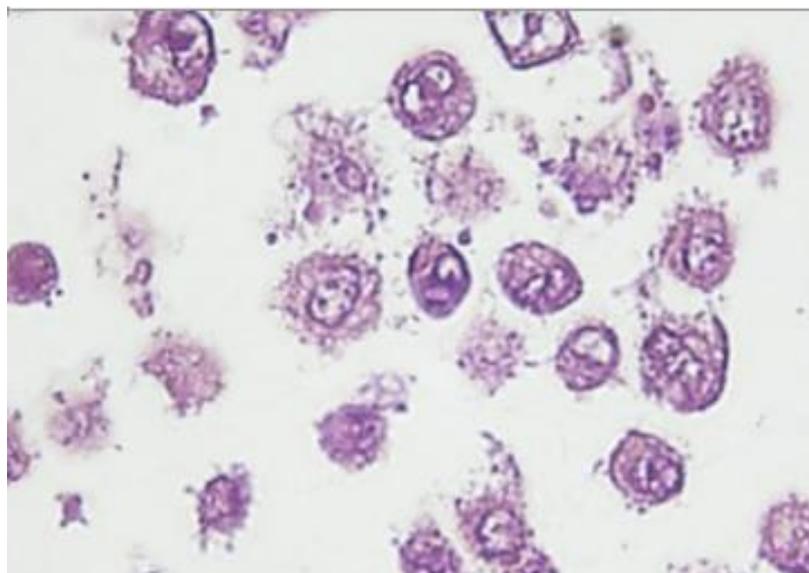

Figure 3. Microscopic feature of CSA03 made of cell blocks (HE, 1,000×)

Cytotoxicity Examination

In this study, the curcumin concentrations used were 0 μg/mL (without exposure), 40 μg/mL, and 50 μg/mL. The IC50 of curcumin on CSA03 cells was 40.85 μg/mL (Figure 4). These results indicated that curcumin has a potent anti-proliferative effect on breast cancer cells.
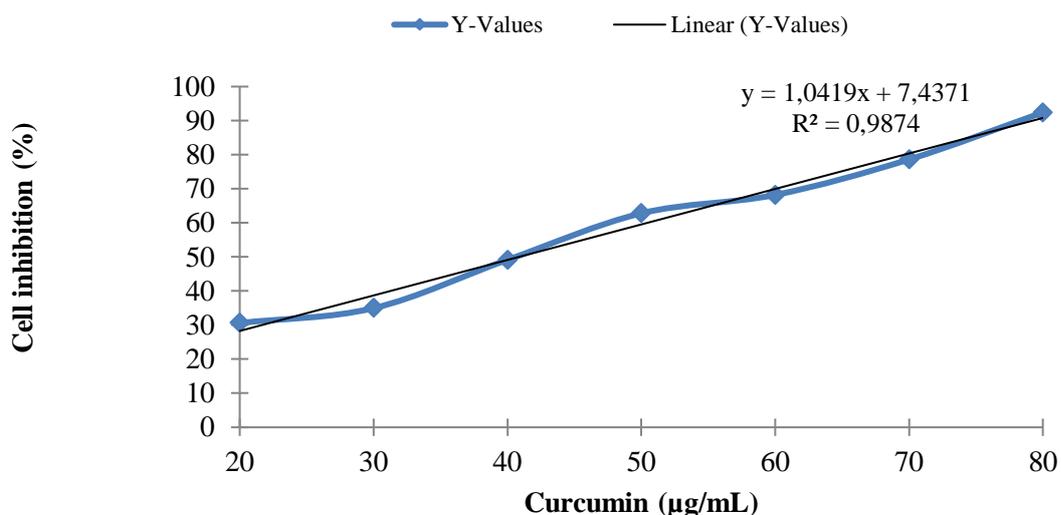
$$y = 1{,}0419x + 7{,}4371$$
$$R^2 = 0{,}9874$$

Figure 4. Cell inhibition of curcumin on CSA03 cells

Curcumin Upregulated the Protein Expression of RASSF1A on CSA03 cells

To investigate whether suppression of proliferation of breast cancer cells by curcumin is due to the downregulation of molecules involved in cell proliferation, we tested the protein levels of RASSF1A in cells. As illustrated in Figure 5, at the previously demonstrated pharmacological effective (anti-proliferation and apoptosis) concentrations, curcumin could significantly increase protein levels of RASSF1A on CSA03 cells. We found that curcumin treatment increased the protein expression of RASSF1A in a dose-dependent manner. At concentrations of 40 µg/mL and 50 µg/mL, curcumin increased levels of protein RASSF1A (Δ = 26.53% and 47.35%, respectively).
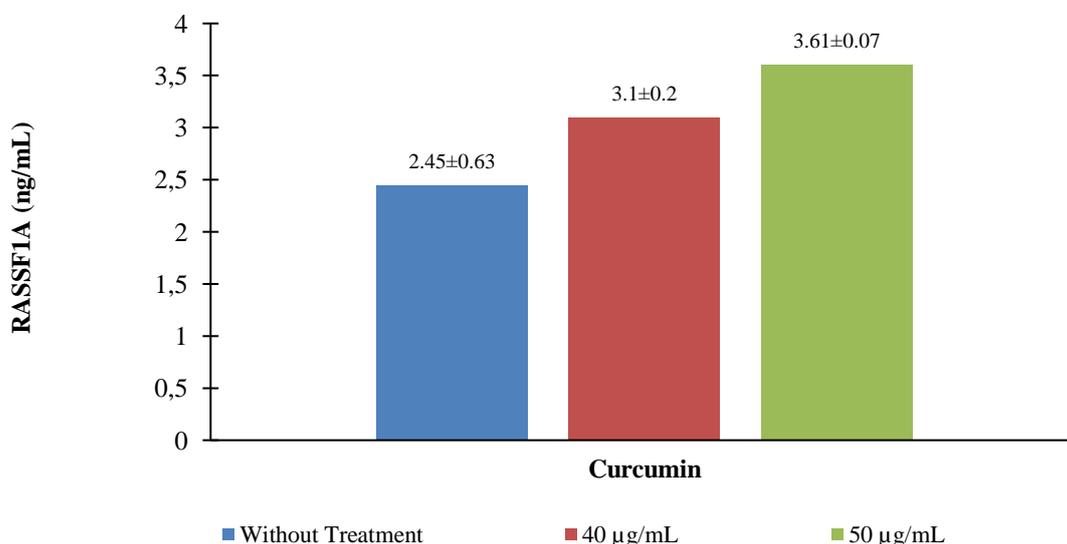

Figure 5. Curcumin upregulated RASSF1A of protein on CSA03 cells (statis¬tical significance: p < 0.05). ELISA assay of curcumin-induced apoptosis of cells for 24 h (RASSF1A protein level ± standard deviation, ng/mL Y axis); untreated, 40 µg/mL, and 50 µg/mL (dose of curcumin, X axis). Curcumin upregulated RASSF1A of protein on CSA03 cells in a dose-dependent manner

Curcumin Upregulated the Protein of Bax on CSA03 cells

We further investigated mechanisms underlying curcumin-induced apoptosis of breast cancer cells. As illustrated in Figure 6, Bax levels were significantly upregulated by curcumin in both cells in a dose-dependent manner. Curcumin treatment increases the protein expression of Bax in a dose-dependent manner. At concentrations of 40 μg/mL and 50 μg/mL, curcumin increases levels of protein Bax (Δ = 48.79% and 386.15%, respectively).
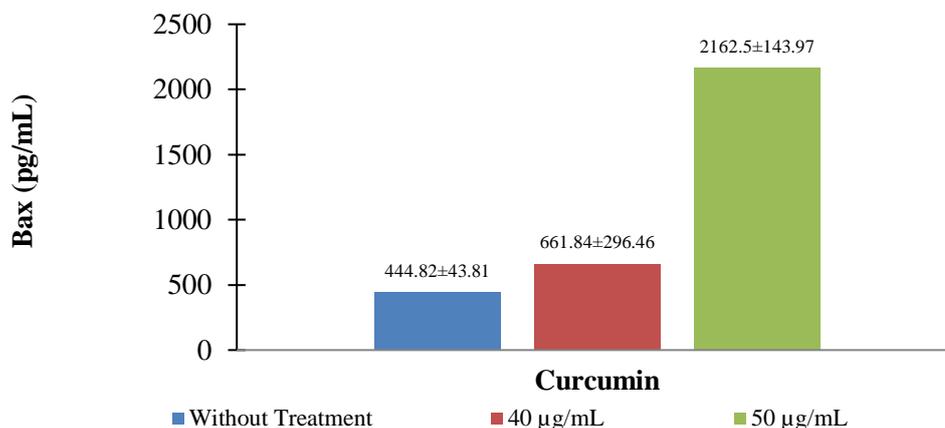

Figure 6. Curcumin upregulated Bax of protein on CSA03 cells (statistical significance: p < 0.05). ELISA assay of curcumin-induced apoptosis of cells for 24 h (Bax protein level, pg/mL, Y axis); untreated, 40 μg/mL, and 50 μg/mL (dose of curcumin, X axis)

Curcumin Induces Apoptosis (Caspase-3) on CSA03 cells

In this study, the curcumin concentrations used were 0 μg/mL (without exposure), a low concentration below the IC50 (40 μg/mL), and a high concentration above the IC50 (50 μg/mL). The cells were treated of curcumin for 24 h. Our results indicated that curcumin in a dose-dependent manner induced apoptosis. As illustrated in Figure 7, the percentage of caspase-3 with a curcumin concentration of 40 μg/mL increased to 1678.51% in CSA03 cells. Under the treatment with a curcumin concentration of 50 μg/mL, the level of caspase-3 increased to 1871.889% in CSA03 cells.
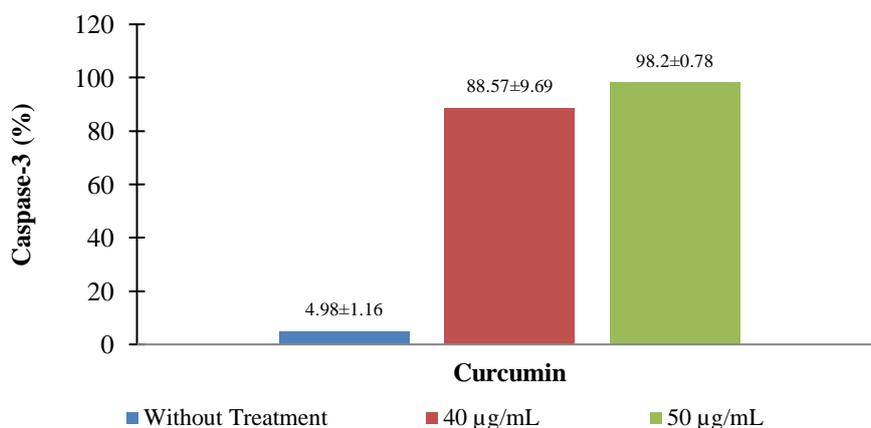

Figure 7. Curcumin upregulated Caspase-3 on CSA03 cells (statistical significance: p < 0.05)

Discussion

The effectiveness of curcumin as a chemopreventive agent has been investigated using a cell culture. The types of breast cancer cells commonly used for research on anti-proliferative drugs are cancer cells that have similar molecular features with those human breast cancer cells so that they are relevant for the development of anti-proliferative therapies for these types of subtypes. Therefore, experimental research on CSA03 cells is relevant to the situation in Indonesia, to determine the relationship between curcumin and levels of the RASSF1A protein, Bax protein, and caspase-3 activity in supporting the apoptotic mechanism.

In this study, CSA03 cells originating from Indonesia were used. An MTS assay was performed to investigate the cytotoxicity effect of curcumin on CSA03 cells. The MTS assay in this study indicated that the IC50 of curcumin on CSA03 cells was 40.85 µg/mL. This result was lower than that on MCF-7 breast cancer cells (75.73 µg/mL), which had the same molecular picture as that of CSA03 cells [12]. This indicates that CSA03 cells are quite sensitive. At low concentrations, curcumin was able to inhibit growth by 50% in CSA03 cells. This occurs because in several Southeast Asian countries, including Indonesia, turmeric is often used as a cooking spice, so that lower concentrations have shown a cytotoxicity level of 50%. In the study of Hafner et al. on growth rates, it was reported that modification of genes or the microenvironment often results in changes in cell division [13], [14]. One of the cytotoxicity mechanisms of curcumin is the oxidative stress pathway. Curcumin in high doses can activate mitochondrial enzymes that lead to the production of ROS. The induction of ROS by curcumin can occur through its interaction with thioredoxin reductase, thereby altering the activity of NADPH oxidase, which in turn can leads to the production of ROS. Several studies have demonstrated that curcumin can induce ROS [15, 16].

In the study of Lv et al. [17], it was reported that curcumin administration would increase Bax on MCF-7 cells in a concentration-dependent manner. In that study, curcumin was only given at one time, 48 hours, so it is not known whether the effect of curcumin is time-course.

However, the results of this study prove that the effect of curcumin as pro-apoptotic breast cancer cells is concentration-dependent. Furthermore, based on the results of the immunohistochemical examination, CSA03 cells have a molecular picture of luminal subtype A. Based on the secondary data results of the immunohistochemical examination, CSA03 cells have a molecular picture of luminal subtype A. CSA03 cells have low ER-positive, HER2-negative, low Ki67-positive so that the receptors on the cell surface for cancer growth and cell proliferation are also low, but cancer growth occurs with sufficiently large mass (>5 cm). In these circumstances, it should be suspected that a series of mechanisms occur and involve many genes in cells for cancer growth.

In cancer, there is a susceptibility to genetic variant loci that influence the regulation of gene expression, including variants in genes involved in DNA repair, cell cycle control, apoptosis, tumor suppressor ubiquitination, and mitotic kinases. The DNA methylation factor is also suspected to be one of causes of tumor growth. DNA methylation is one of the most common epigenetic modifications. These modifications do not alter the main sequence of DNA but are critical factors for normal development, gene expression patterns, and genomic stability. The RASSF1A gene is epigenetically inactivated in various solid tumors. In several studies reported, RASSF1A hypermethylation was detected in 48%–70% of breast cancer patients [18], [19].

The results of this study indicated that giving curcumin to CSA03 breast cancer cells increased levels of RASSF1A protein (Figure 5). This is in agreement with the study by Ung et al., that DNA hypermethylation correlates with ER-positive, although it is also ER negative [20]. On this basis, it should be suspected there has been an increase in DNA hypermethylation in CSA03 cells. In DNA hypermethylation, a DNMT catalytic mechanism occurs that involves the formation of covalent bonds between cysteine residues on the active site of the enzyme and carbon 6 (C6) cytosine DNA. This bond increases the flow of electrons to carbon 5 (C5) so that it readily accepts the methyl base cytosine group facilitated by the DNA methyltransferase (DNMT) enzyme, known as

5Methylcytosine (5 mC) [21]. Curcumin can also act as a competitive inhibitor with the C6 atom of the cytosine ring in the catalytic space through the C3 keto-enol curcumin portion. This covalent bond will inhibit DNMT1 activity without joining the DNA [22].

The increase in the RASSF1A protein was also followed by an increase in the levels of Bax and caspase-3 proteins in CSA03 breast cancer cells after 24 hours of curcumin administration, both at concentrations of 40 μg/mL and 50 μg/mL (Figures 6 and 7). This is according to the research of Law et al. [6], who demonstrated that RASSF1A can regulate the activity of the Bax protein. The results of this study indicated an increase in the percentage of Bax protein levels delta, the highest occurred in the administration of curcumin 50 μg/mL. In the study of Lv et al. [17], it was reported that curcumin administration would increase Bax on MCF-7 cells in a concentration-dependent manner. Curcumin also increases caspase-3 protein levels, especially in the administration of curcumin of 50 μg/mL. This increase was higher than the increase in RASSF1A protein levels. This can occur because curcumin can restore the function of protein genes of other tumor suppressors that are also hypermethylated. Together with the RASSF1A gene, these tumor suppressor proteins can activate Bax levels and apoptotic functions that end in caspase-3 being an executor and responsible for apoptosis.

Conclusion

The administration of the curcumin to CSA03 cells efficiently decreased the growth of breast cancer cells in vitro through the reactivation of RASSF1A as cancer the tumor suppressor gene and upregulated the Bax protein as the induction of apoptosis (caspase-3).

Acknowledgements

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