Recevied : 11 January 2022 Revised : 8 September 2022 Accepted : 11 January 2022
DOI: 10.29244/primatology.1.01 EISSN: 2964-5441

REVIEW ARTICLE

INAJP
INDONESIAN JOURNAL OF PRIMATOLOGY

Apoptosis of Human Breast Cancer Cells (MCF-7) Induced by *Psidium guajava* Simplisia Extract

Iin Indriawati[1*], Silmi Mariya[1], Cindy[2], Vincent Andrianto[2], Diah Iskandriati[1]

[1]*Primate Research Center, IPB University, Indonesia*
[2]*Faculty of Technobiology, Universitas Katolik Indonesia Atma Jaya, Jalan Jenderal Sudirman 51, Jakarta 12930*

Abstract

Cancer is one of the leading causes of death in the world. In 2014, the death rate caused by this cancer reached 585,720 cases. The most common cancers suffered by women include breast cancer (29%), lung cancer (13%), colon cancer (8%), uterine cancer (6%), and thyroid cancer (6%) (Siegel *et al.* 2014). Therefore, many studies have been conducted to find the best treatment for fighting this cancer. Breast cancer is caused by abnormal cells in the breast that occur continuously. Breast cancer can be affected by several mechanisms, such as angiogenesis (Schneider & Miller 2005), metastasis (Weigelt *et al.* 2005), and apoptosis (Yang *et al.* 2006). Apoptosis is a normal cell death process that occurs after several times of replication. This process of apoptosis has a critical role in normal cells and cancer cells. Cell death due to apoptosis is regulated by an interplay between pro-apoptotic and anti-apoptotic proteins. These proteins are the result of gene expression, for example, the Bax gene that expresses the Bax protein and the Bcl-2 gene that expresses the Bcl-2 protein (Martin & Dowsett 2013). Various kinds of treatments have been developed to inhibit the growth of cancer cells, such as radiation therapy, chemotherapy, hormone therapy, and surgery. However, this treatment only works in the early stages of cancer, and later, cells become resistant to these various treatments. In addition, multiple medications can also kill other normal cells. Therefore, research on the treatment of cancer has been developed using bioactive compounds found in natural ingredients, such as fruits (Abrahim *et al.* 2012). One of the plants that have been widely developed for cancer treatment is guava (Psidium guajava). This plant has been known to have many purposes, such as antibacterial, antidiarrheal, anti-acne, and anti-inflammatory (Ryu *et al.* 2012). This study aims to examine the effect of guava extract on the induction of apoptosis in MCF-7 breast cancer cells (ATCC HTB 22), which can later be used as candidates for anticancer drugs.

Key words: Apoptosis, Bax, Bcl-2, MCF-7, *Psidium guajava*

1. Introduction

Cancer is one of the leading causes of death in the world. In 2014, the death rate caused by this cancer reached 585,720 cases. The most common cancers suffered by women include breast cancer (29%), lung cancer (13%), colon cancer (8%), uterine cancer (6%), and thyroid cancer (6%) (Siegel *et al*. 2014). Therefore, many studies have been conducted to find the best treatment for fighting this cancer. Breast cancer is caused by abnormal cells in the breast that occur continuously. Breast cancer can be affected by several mechanisms, such as angiogenesis (Schneider & Miller 2005), metastasis (Weigelt *et al*. 2005), and apoptosis (Yang *et al*. 2006).

Apoptosis is a normal cell death process that occurs after several times of replication. This process of apoptosis has a critical role in normal cells and cancer cells. Cell death due to apoptosis is regulated by an interplay between pro-apoptotic and anti-apoptotic proteins. These proteins are the result of gene expression, for example, the Bax gene that expresses the Bax protein and the Bcl-2 gene that expresses the Bcl-2 protein (Martin & Dowsett 2013).

Various kinds of treatments have been developed to inhibit the growth of cancer cells, such as radiation therapy, chemotherapy, hormone therapy, and surgery. However, this treatment only works in the early stages of cancer, and later, cells become resistant to these various treatments. In addition, multiple medications can also kill other normal cells. Therefore, research on the treatment of cancer has been developed using bioactive compounds found in natural ingredients, such as fruits (Abrahim *et al*. 2012). One of the plants that have been widely developed for cancer treatment is guava (*Psidium guajava*). This plant has been known to have many purposes, such as antibacterial, antidiarrheal, anti-acne, and anti-inflammatory (Ryu *et al*. 2012).

*Coresponding author
Email Address: indriawati.iin@gmail.com

This study aims to examine the effect of guava extract on the induction of apoptosis in MCF-7 breast cancer cells (ATCC HTB 22), which can later be used as candidates for anticancer drugs.

2. Materials and Methods

2.1. Guava Extract Preparation

Guava simplicia extract was obtained from the Research Institute for Medicinal and Aromatic Plants (Balittro) Bogor in powder form after going through the stages of cutting, drying, and crushing. This extract was then dissolved with DMSO (Sigma USA) and RPMI medium (Gibco, USA) for use in the Methyl Tetrazolium (MTT) assay (Sigma USA).

2.2. Cell Viability Test

MCF-7 cells were thawed or thawed after cryopreservation, then grown on RPMI1640 medium supplemented with 10% FBS and 1% Fpenicillin-streptomycin and incubated at 37°C and 5% CO_2. The confluent cells in the flask were then subcultured. The cell media was discarded, and then 5 mL of sterile 1X PBS was added to clean the flask from the rest of the media, then PBS was discarded. A 0.125% trypsin was added to the flask as much as 5 mL and incubated at 37°C with 95% O_2 and 5% CO_2 for 5 minutes, and the medium was added to the flask as much as 1 mL to stop the trypsin reaction. The released cells were transferred into a 15 mL centrifuge tube and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded, and cells were added as much as 5 mL of media, resuspended until homogeneous, and then counted using a hemacytometer.

Cytotoxicity tests against simplicia, acetone extract, and ethanol extract were tested against MCF-7 breast cancer cells. Cells were grown as many as 5000 cells/well on 96 wells tissue culture plates, incubated for 24 hours at 37°C with 95% O_2 and 5% CO_2. Samples were made in several concentrations (800, 400, 200, 100, 50, 25, 12.5 and 6.25 ppm). The samples were then added to cells incubated for 24 hours, as much as 100 µL/well for each concentration, with three replications. Cells to which the sample was added were incubated again for 48 hours at 37°C with 95% O_2 and 5% CO_2. After 48 hours, the sample was added with MTT solution (Thiazolyl Blue Tetrazolium Bromide 5 mg/mL) as much as 10 µL/well, which was then incubated again for 4 hours at 37°C with 95% O_2 and 5% CO_2. The media was removed, and 100 µL/well of ethanol p.a was added. Analisis of absorption were performed using a spectrophotometer at 595 nm. Percent inhibition can be calculated by the following formula:

$$\% \text{ Inhibition} = \frac{Abs.\ Control - Abs.\ Samples}{Abs.\ Control} \times 100\%$$

2.3. Analysis of *Bax* and *Bcl-2* Gene Expression by RT-PCR

As much as 10^4 cells/well MCF-7 cells were grown in 12 wells tissue culture plate incubated for 24 hours at 37°C with 95% O_2 and 5% CO_2. After the cells reached about 50% confluency, simplicia extract was added at the concentrations of 100, 50, and 25 ppm. Doxorubicin was included as a positive control and cells without treatment was used as negative control. The cells were then incubated for 48 hours. The live cells were harvested using 0.125% trypsin, stained with 0.1% trypan blue and the number of live cells was counted by hemacytometer. The live cell population was extracted to obtain mRNA for gene expression analysis. Extraction of mRNA was carried out using the RNeasy Kit (Qiagen, Germany) following the procedure specified by the manufacturer. The concentration of isolated mRNA was calculated using the NanoDrop 2000 UV-Vis Spectrophotometer.

The mRNA expression of *Bcl-2* and *Bax* genes was measured using an RT-PCR machine (IQ5 Multicolor Real Time PCR Detection System, Biorad). Each reaction contained 2.5 µl of RNA template, 1 µl of primer for each gene, 12.5 µl of 2×SYBR Green RT-PCR reaction mixture (BioRad), 0.5 µl of iScript One-Step RT-PCR (BioRad), and 2.5 µl of nuclease-free water. The reaction was performed under conditions: 50 °C for 10 minutes for reverse transcriptase activation, and 95°C for 5 minutes for reverse transcriptase inactivation. The reaction was repeated for 40 cycles at 95°C for 10 seconds for DNA denaturation, 52°C for 20 seconds for primer attachment, and 72°C for 10 seconds for DNA elongation. The baseline and threshold are set automatically from the RT-PCR machine software. All target genes were normalized against GAPDH and expressed in fold change. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) as follows:

- Ct normalization of target genes with reference genes for treated (test) and control samples:
  ΔCt (test) = Ct (target, test) – Ct (ref, test)
  ΔCt (control) = Ct (target, control) – Ct (ref, control)
- Normalization of Ct of the sample with the treatment of the control:
  ΔCt = Ct (test) – Ct(control)
- Calculating gene expression ratios:
  $2^{-\Delta\Delta Ct}$ = normalized gene expression ratio

2.4. Hoechst Stain Test #33342

MCF-7 cells were cultured using an eight wells chamber slide (Labtech, USA) for 18-20 hours at 37 °C, 95% O_2, and 5% CO_2. The cell media was removed and then added guava extract at concentrations of 100 ppm, 50 ppm, and 25 ppm, and doxorubicin 3 ppm, then incubated for 48 hours. Cells were washed with PBS twice and fixed using 2% glutaraldehyde for one hour at 37 °C. Cells were washed with PBS twice, then Hoechst #33342 (Invitrogen, USA) 10 mg/L dye was added and incubated for one hour at 37 °C. Cells were washed with PBS twice, then observed using a fluorescent microscope at a wavelength of 365-460 nm.

3. Result

The results of the MTT assay of simplicia, acetone extract and ethanol extract against MCF-7 breast cancer cells are presented in Figure 1

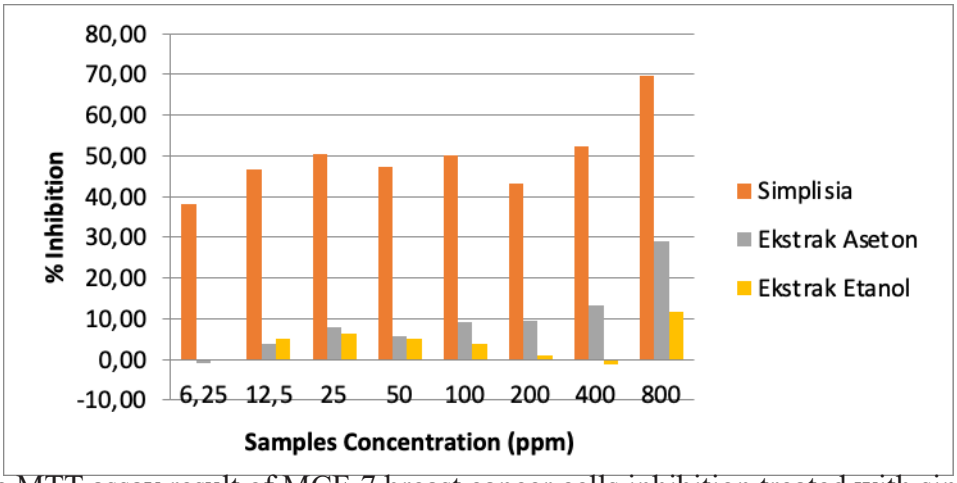

Figure 1. Graphic MTT assay result of MCF-7 breast cancer cells inhibition treated with simplicia, acetone and ethanol extract.

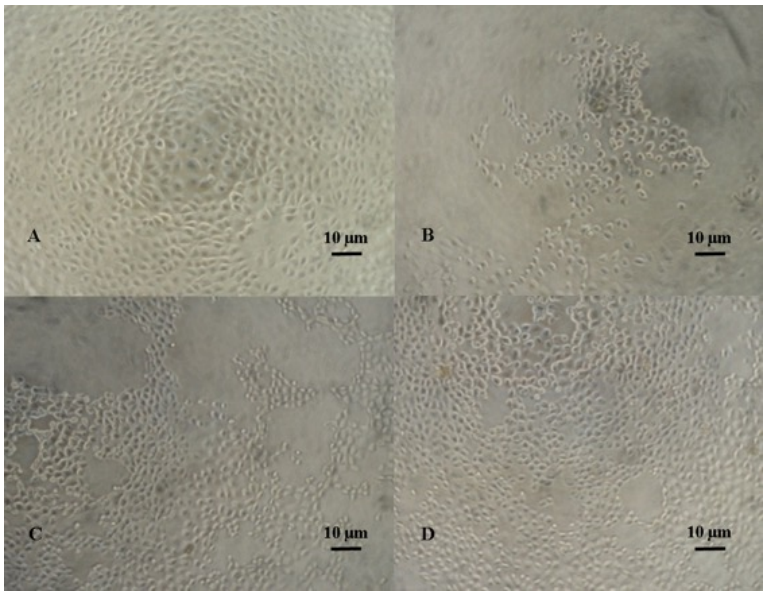

Figure 2. MCF-7 breast cancer cells. A. MCF-7 control cells; B, C, D MCF-7 cell treated with 800 ppm samples concentration (B simplicia, C acetone extarct, D ethanol extract).

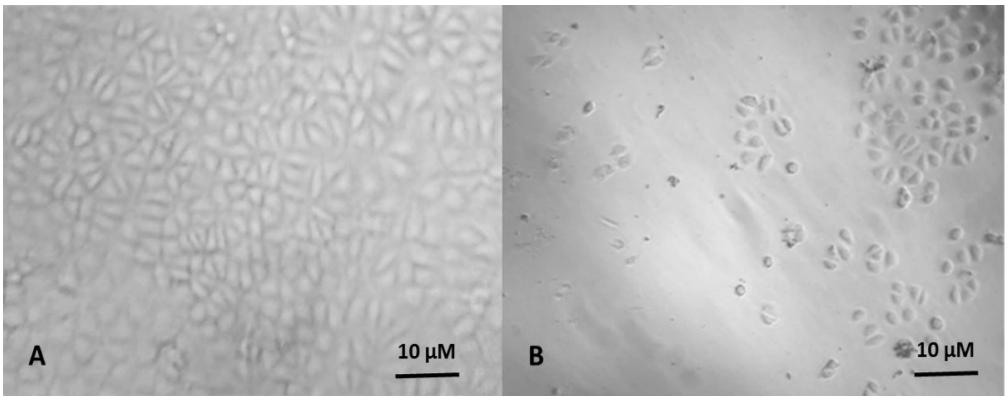

Figure 3. A. MCF-7 breast cancer cells without inhibition. B. MCF-7 breast cancer cells inhibition after exposed with 50 ppm guava simplicia extract.

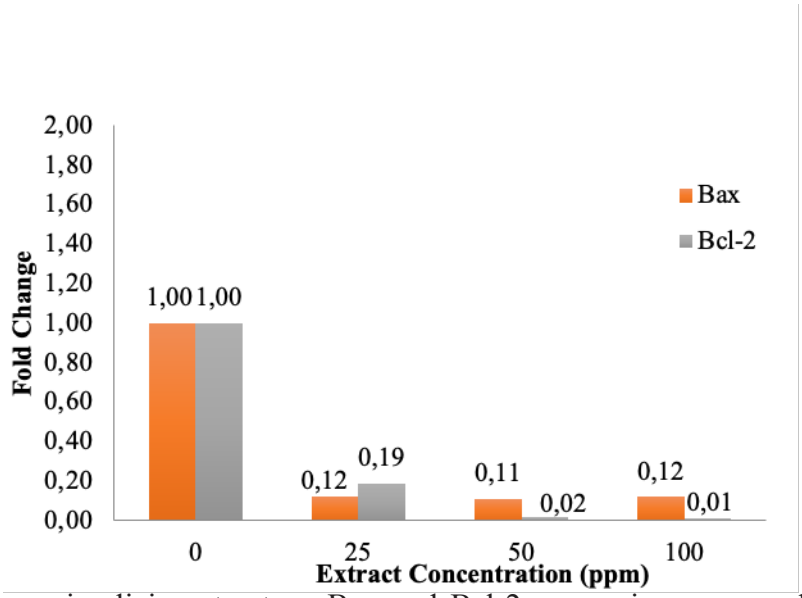

Figure 4. The effect of guava simplicia extract on Bax and Bcl-2 expression measured using RT-qPCR normalized to GAPDH gene as reference gene.

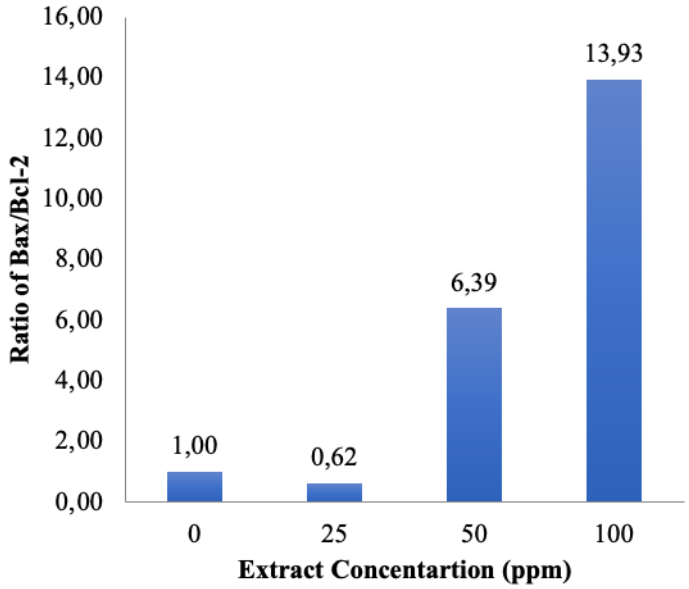

Figure 5. The effect of guava simplicia extract on the ratio of Bax and Bcl-2 expression measured using RT-qPCR normalized to GAPDH gene as reference gene.

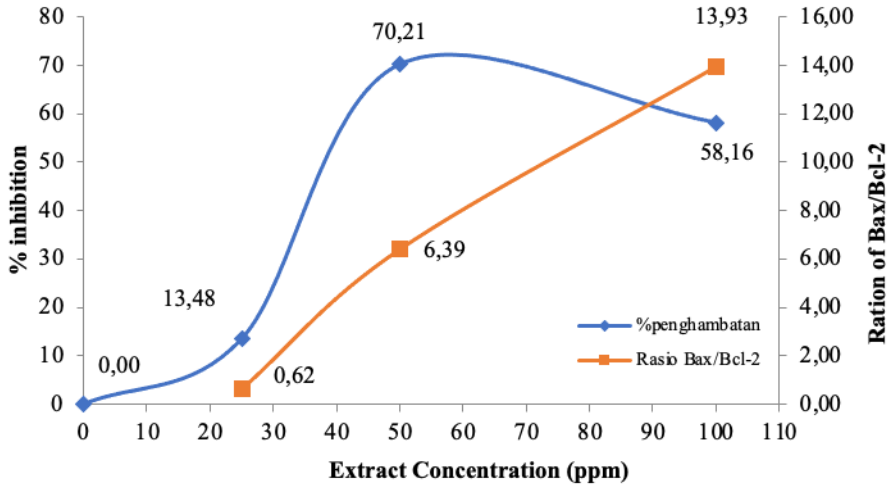

Figure 6. The effect of guava simplicia extract on the MCF-7 cells proliferation and the ratio of Bax and Bcl-2 expression.

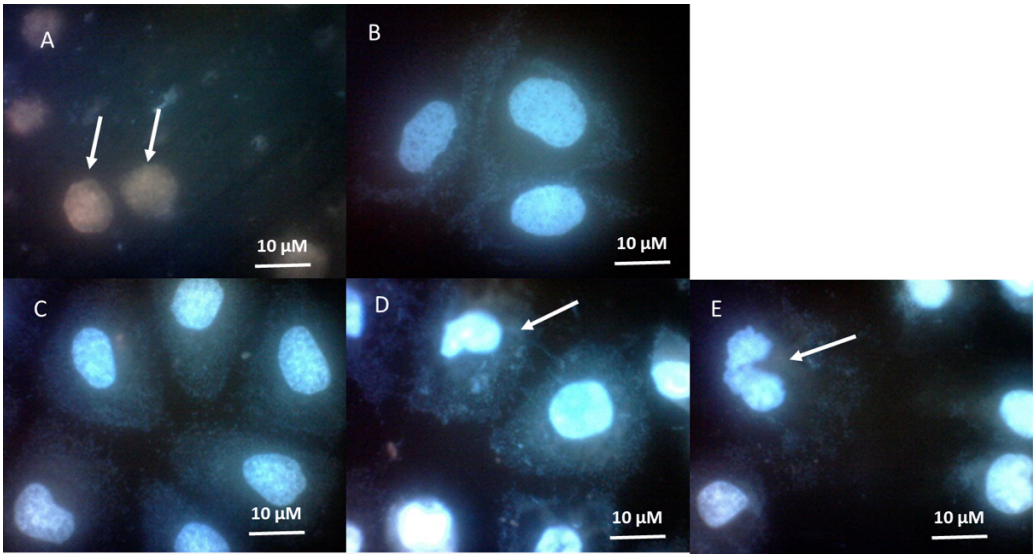

Figure 7. Hoechst stain 33342 to analyse the apoptosis process in MCF-7 cells; A. Cells treated with Doxorubisin 3 ppm; B. Cells without extract treatment (negative control); C. Cells treated with 25 ppm of guaca simplicia extract showed no DNA fragmentation; D-E. Cells treated with 25 and 50 ppm of guaca simplicia extract showed the DNA (indicated by white arrow

4. Discussion

The Figure 1 shows that both three samples tested against MCF-7 breast cancer cells had the inhibition activity. However, the percentage of inhibition were differs from one sample to another. The concentration of 800 ppm of simplicia was able to inhibit the growth of MCF-7 cells by 69.53%. The smallest concentration of simplicia, which is 6.25 ppm, gave an inhibition of 38.00%, this value is still greater than that of the acetone and ethanol extract samples at a concentration of 800 ppm, which only inhibited 29.00% and 11.07%, respectively. Both extracts contained only half of the active compounds, so they provided less inhibition. In another study, guava acetone extract gave 80% inhibition of NB4 cells at a concentration of 3000 ppm (Bontempo *et al*., 2012). Therefore, guava acetone extract must be highly concentrated to produce significant inhibition.

The results of the MTT assay on MCF-7 breast cancer cells showed that simplicia was able to provide considerable inhibition at each concentration when compared to the two extracts. Acetone extract gave more significant inhibition than ethanol extract because the acetone extract contained carotene compound at the concentration of 40 mg/100 g dry raw material, while the ethanol extract contained flavonoids only 0.31 mg/100 g dry material (Vanitha *et al.*, 2012).

The MCF-7 breast cancer cell control shown in figure 2A was growth excellent, the distribution is evenly distributed, and it occupies the entire surface of the well. Figures B, C and D show the inhibition of the growth of MCF-7 breast cancer cells, with the largest inhibition in Figure B followed by C and D. Figure 2B shown the MCF-7 breast cancer cells that have treated with simplicia samples at concentration of 800 ppm indicated that the morphological cells was very different from figure 2A, in which Figure 2B only a few MCF-7 cells partially fill the surface of the well. This means that the simplicia that added to the cells has an inhibition activity on the growth of MCF-7 breast cancer cells by 69.53%. Meanwhile, in Figures 2C and 2D, the MCF-7 breast cancer cells have treated with 70% acetone extract and 70% ethanol extract at 800 ppm concentration shown that the inhibition is not much different with the percentage of inhibition were 29.00% and 11.67%, respectively. This lower inhibition possibly occurred because the acetone extract contained only polar compounds while the ethanol extract contained only non-polar compounds, therefore the inhibition was not maximal. Simplicia samples showed the greatest inhibition of MCF-7 breast cancer cells.

The MCF-7 cells, which were given the addition of simplicia extract at concentrations of 100 ppm, 50 ppm, and 25 ppm, were incubated for 48 hours and then observed for inhibition. The results of observations under inverted microscope showed that there was cell death at 50 ppm extract exposure (Fig. 3). Morphological observations of dead cells showed by cell shrinkage and cell shape became rounded because it was no longer attached to the bottom of the flask.

The MCF-7 cell viability test results were obtained through hemocytometer calculation after incubation for 48 hours exposed to simplicia extract at concentrations of 100 ppm, 50 ppm, 25 ppm, and doxorubicin (Fig.3). MCF-7 cells that were not given simplicia extract served as the negative control, while doxorubicin treatment functioned as the positive control.

Based on the results of the proliferation test, it was suspected that there were other cell death mechanisms besides apoptosis which caused quite high inhibition to occur by MCF-7 cells on exposure to 50 ppm simplicia extract. Mechanisms of cell death other than apoptosis include necroptosis, pyroptosis, autophagy cell death, and caspase-free cell death (Tait *et al*., 2014). In addition, van Breeman and Pajkovic (2008) stated that guava is a fruit that is a source of lycopene. Lycopene is one of the carotenoid compounds synthesized by plants and other photosynthetic microorganisms. These hydrocarbon compounds have been known to have antioxidant activity, trigger apoptosis, induce cell cycle inhibition in the G1 phase, and have anti-metastatic properties. Therefore, the presence of this compound in guava simplicia extract could inhibit the proliferation of MCF-7 cells.

Ct (*Cycle threshold*) values from RT-qPCR were analyzed by Livak method to obtain the fold change and Bax/Bcl-2 ratio. The fold change value indicates how many times a target gene is expressed in the test sample relative to the control sample and has been normalized to the reference gene (Livak & Schmittgen 2001). The target genes in this study were Bax and Bcl-2 genes, and the test samples are samples that are treated with the extract at concentration of 100 ppm, 50 ppm, and 25 ppm. The control sample was a sample that not exposed with extract, meanwhile the reference gene was the gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The GAPDH gene was used as a reference gene because this gene stably expressed and widely expressed in tissues and cells, therefore called as the housekeeping gene (Barber *et al*. 2005).

Figure 4 explained that the Bax mRNA expression on MCF-7 cells treated with three concentration of guava simplicia extract test were relatively unchanged from one another. Therefore, there was no change in the expression of the Bax gene encoding the pro-apoptotic protein. On the other hand, expression of Bcl-2 mRNA, which decreased significantly at 50 ppm and 100 ppm extract treatment, indicated that there were suppressed expression in the Bcl-2 gene that encodes anti-apoptotic protein. In physiological condition, ratio between the two proteins of pro-apoptotic (Bax) and anti-apoptotic (Bcl-2) must be considered in the balance (Wong 2011).

The value of the Bax/Bcl-2 ratio of 100 ppm extract exposure was higher than that of 50 ppm extract, although the percentage of inhibition shown in 100 ppm extract was lower than that of 50 ppm extract. What might happen in this condition is that the high concentration of simplicia extract triggers the proliferation of MCF-7 cells so that the percentage of inhibition produced is low even though the ratio of pro-apoptotic to anti-apoptotic proteins is high. As stated by Hanahan & Weinberg (2011), the mechanism for converting normal cells into cancer cells can occur by various mechanisms, such as mutations by receptors for proliferative signals, dysfunctional mitosis-inhibiting proteins, and triggering overexpression of the telomerase enzyme.

Doxorubicin, with a concentration of 3 ppm used as a positive control, showed inhibition of cell proliferation by 100%. Furthermore, Doxorubicin administration killed all MCF-7 cells, so RT-qPCR analysis could not be carried out at a later stage. Doxorubicin is a drug that is widely used in chemotherapy treatment. This drug works by inserting into DNA and inhibiting the action of topoisomerase I and II enzymes which function to separate double-stranded DNA. This irreversible DNA strand causes DNA to be unable to replicate, cells become damaged, and undergoes death (Tacar *et al*. 2012).

Based on the data in Figure 5 showed that the ratio of Bax to Bcl-2 were increased according to the extract concentration (Figure 5). This increased ratio compared to the control indicated that the amount of

pro-apoptotic protein was higher than that of anti-apoptotic protein in the 100 ppm and 50 ppm extract treatments. According to Salakou *et al*. (2007), this ratio is the final result that determines the balance between pro-apoptotic and anti-apoptotic proteins in cells. The increase in the ratio of Bax/Bcl-2 causes apoptosis to occur through caspase-3 as the final executor of apoptosis. Apoptosis that occurs through caspase-3 activity is characterized by chromosomal condensation, prominent cell membranes, and DNA fragmentation. Activation of this caspase can occur in three ways, including through the mitochondrial pathway involving the Bax and Bcl-2 proteins, the endoplasmic reticulum, and cell death receptors (Fas, Fas-L).

The ratio of Bax/Bcl-2 at concentrations of 100 ppm and 50 ppm indicated that in both treatments, apoptosis occurred, which was quite significant when compared to control cells and exposure to a concentration of 25 ppm. Research conducted by Bontempo *et al.* (2012) stated that *P. guajava* extract could induce apoptosis, activate caspases, and overexpress p16, p21, Fas, TNF, and the pro-apoptotic protein BAD. Apoptosis that occurs is caused by an increase in the ratio of Bax/Bcl-2 and activation of caspases. Administration of simplicia extract at 100 ppm and 50 ppm concentrations caused downregulation of the Bcl-2 gene expression which resulted the mitochondrial membrane to be less stable and triggered the release of cytochrome c. Therefore, an increase in the Bax/Bcl-2 ratio stimulated MCF-7 cells to perform apoptosis.

The results of the viability test compared with the ratio of the Bax to Bcl-2 genes showed the highest inhibition by the 50 ppm concentration of extract treatment, but the highest ratio was indicated by the 100 ppm extract treatment (Fig. 6). The expression of Bax and Bcl-2 genes was analyzed through RT-qPCR, resulting in fold change data (Fig.4) and the ratio of Bax to Bcl-2 genes (Fig.5). The inhibition of cell proliferation that occurred with the 50 ppm extract was the highest, although the Bax/Bcl-2 ratio was lower than the 100 ppm extract. The Bax/Bcl-2 ratio shows the ratio between pro-apoptotic and anti-apoptotic proteins that regulate the level of cells' ability to perform apoptosis based on specific apoptotic stimuli (Purwani *et al*. 2012).

During apoptosis the DNA becomes condensed, but this process does not occur during necrosis. Nuclear condensation can therefore be used to distinguish apoptotic cells from healthy cells or necrotic cells. The results of Hoechst staining in Figure 7 showed that early apoptotic DNA fragmentation in cells after exposed with guava extract at concentrations of 50 ppm and 100 ppm. DNA fragmentation was not found in the negative control and also in cells treated with 25 ppm guava extract.

Acknowledgements

This research was funded and facilitated by the Primate Research Center, IPB University (PSSP, LPPM-IPB)

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