INTERNATIONAL JOURNAL OF MULTIDISCIPLINARY RESEARCH AND ANALYSIS
ISSN(print): 2643-9840, ISSN(online): 2643-9875
Volume 06 Issue 03 March 2023
DOI: 10.47191/ijmra/v6-i3-25, Impact Factor: 7.022
Page No. 1056-1061

The Effect of Pare Ethanol Extract on Bax and Bcl-2 Protein Expressions (In vitro study on T47D Breast Cancer Cell Culture)

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ABSTRACT: Breast cancer is a genetic disease due to the accumulation of genetic abnormalities in tissues. There are cell proteins that can reduce the growth of cancer cells. Bax and Bcl-2 are proteins that accelerate the process of apoptosis of cancer cells and maintain the permeability of mitochondrial cell membranes and cytotoxic activation of caspase so as to prevent the spread and development of cancer cells. The purpose of this study was to determine the effect of pare ethanol extract on the expression of Bax and Bcl-2 in T47D cell cultures. This research uses experimental in vitro with the Post Test Only Control Group Design method. The subjects of this study were T47D cells divided into 4 treatments with three replications on the variables. The treatment consisted of negative control, positive control, pare ethanol extract 1/2 IC50 (134.7 g/mL), 1 IC50 (269.4 g/mL) and 2 IC50 (538.8 g/mL). On the 10th day, an examination of Bax and Bcl-2 expressions was carried out. The data were analyzed using the Anova One Way Test. One Way Anova test result showed the results of Bax protein expression and BCL-2 expression in all groups there was a significant difference ($p<0.05$). The administration of pare ethanol extract at dose of 2 IC50, 1 IC50, and 1/2 IC50 showed a significantly affect to increase of Bax protein expression in T47D breast cancer cells.

KEYWORDS: Pare Ethanol Extract, Bax Expression, Bcl-2 Expression

I. INTRODUCTION

Cancer is caused by the presence of abnormal genes which are characterized by continuous proliferation signals.(1) Breast cancer is a genetic disease caused by the accumulation of genetic disorders in the tissue. In breast cancer patients, gene mutations are found.(2) There are cell proteins that can reduce the growth of cancer cells. BAX and BCL-2 are proteins that accelerate the apoptotic process of cancer cells and maintain the permeability of mitochondrial cell membranes and activate cytotoxic caspase so as to prevent the spread and development of cancer cells. (3) pare ethanol extract has cytotoxic and carcinogenic potential. The results of previous studies showed that 70% ethanol extract of pare (Momordica charantia L.) in HeLa cells obtained an IC50 value of 51.56 µg/ml which could inhibit the growth of cancer cells.(4) Therefore it is necessary to study further the benefits of ethanol extract of pare in triggering expression of BAX and BCL-2 in an effort to inhibit growth and induce apoptosis of T47D breast cancer cells.

Based on the results of Riskesdas in 2018, the prevalence rate of cancer in Indonesia is 1.8% (per mile), or it is estimated that of Indonesia's population of 263,991,379 people, approximately 475,185 people suffer from cancer. According to World Health Organization (WHO) in 2018 breast cancer in women is the largest cancer in Indonesia. Cancer is difficult to cure in the world of medicine, pharmacists as well as medicine continue to seek and develop innovative drugs that can be used to treat cancer. The treatment of cancer in general is divided into two types, namely natural medicinal ingredients such as medicinal plants and synthetic drugs. (5) In 2020, there are 65,856 cases of breast cancer in Indonesia with 22,430 deaths from this cancer. This disease is also the second leading cause of death due to cancer in Indonesia in 2020 after lung cancer.(6)

Alternatives treatment of chemotherapy and synthetic drugs can be used with traditional medicines such as herbal plants. Many of these traditional medicines are taken from plant parts such as rhizomes, roots, stems, leaves, flowers, or the whole of a plant. The use of plants can be used as an alternative cure for a disease, especially cancer.(7) Plants used as medicine still require further research on their contents and clinical trials. Active compounds from herbal plants can be an alternative in cancer treatment, this herbal treatment has the advantage of not having side effects.(8)

Based on data on the incidence rate of breast cancer and the very high number of deaths from this cancer, many experts are conducting research and developing breast cancer treatment in order to obtain efficient, effective and affordable treatment

results. Research in 2018 showed that pare has an anti-cancer substance called lecithin. This substance is able to fight cancer cells and has preventive properties for people who do not have cancer.(9) In addition, according to previous research, pare has potential activity against breast cancer cells MCF-7 and MDA-MB-231, colon cancer cells. HCT-116, pancreatic cancer, liver cancer, PC3 and LNCaP prostate cancer cells, and skin cancer.(4) Other researchers also stated that the compound 3β, 7β-dihydroxy-25-metoxicukurbita-5,23-din-19-al (DMC) isolated from pare has cytotoxic activity against MCF-7 cells and MDA-MB-231 cells with IC50 values of 14.3 and 17.6 µM respectively and can suppress MCF-7 cell proliferation by inducing apoptosis.(10) Based on the description above, it is necessary to conduct research on the effect of pare ethanol extract on BCL and BCL-2 expression in vitro in T47D breast cancer cell culture.

II. MATERIAL AND METHOD

The research method uses an in vitro experimental study with Post Test Only Control Group Design. This study used T47D cells as research subjects and divided into 4 groups: control group, P1 T47D treated with pare ethanol extract at a dose of ½ IC50, P2 T47D at a dose of 1 IC50, and P3 T47D at a dose of 2 IC50. Pare ethanol extract used in this study was extracted by maceration method using 90% alcohol for 72 hours.

RESULT

In this study, researchers found that pare ethanol extract was able to increase BAX protein expression and decrease BCL-2 expression in T47D breast cancer cells depending on the dose given (Table 1).

Table 1. Results of Average Analysis, Normality Test, Homogeneity Test on BCL-2 Expression and BAX Expression

Variable	Group				
	KN N=6	**P1 N=6**	**P2 N=6**	**P3 N=6**	**Sig.(p)**
SEE Express					
Mean	18.52	16.07	27.46	7.78	
Std. deviation	7.53	2.12	5.43	2.21	
Shapiro Wilk	0.580*	0.462*	0.100*	0.821*	
Low Test					0.102**
One Way Annova					0.008***
BCL-2 expression					
Mean	15.11	16.93	20.93	5.97	
Std. deviation	1.82	3.64	2.23	1.23	
Shapiro Wilk	0.497*	0.065*	0.460*	0.412*	
Levene Test					0.139**
One Way Anova					0.000***
Information: * Normal p>0.05 **Homogeneous p>0.05 ***Significant p<0.05					

Effect of Pare Ethanol Extract on BAX Protein Expression

Based on the Table 2 and Figure 1, shows that the highest average BAX protein expression was in the negative control group (KN), followed by the second treatment group (P2), the third treatment group (P3) and the first treatment group (P1). The BAX expression data of the four groups were all normally distributed, as indicated by Shapiro Wilk's results with a value of p>0.05 and also having a homogeneous variant of the data as indicated by the results of the Levene's Test with a value of p=0.139 (p>0.05). The distribution and variance of the data on BAX expression levels were normal and homogeneous, so a parametric statistical analysis was carried out with the One Way Anova test yielding a value of p = 0.000 (p <0.05) so that there was a significant

difference in the average BAX expression between the four groups. The significant results of the One Way Anova test were followed by a Post Hoc test to see which group had the most influence.

Table 2. Differences in BAX Protein Expression between the 2 groups using pos hoc test

Group	*p-Value*
KN vs P1	0.925
KN vs P2	0.193
KN vs P3	0.103
P1 vs P2	0.082
P1 vs P3	0.239
P2 vs P3	0.005*

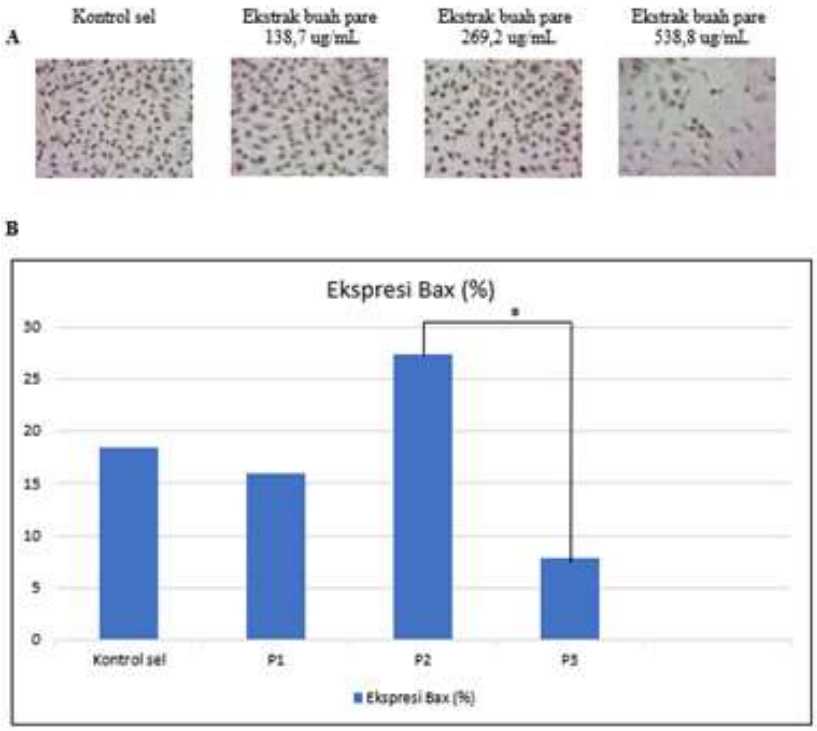

Figure 1. The effect of pare ethanol extract on the expression of protein caspase-9 in T47D cells for 24 hours of treatment. (A) Images of BAX expression observed using the ICC method, (B) Graph of the percentage of BAX expression after being treated with pare ethanol extract on T47D cells for 24 hours. Protein expression profiles are presented from the mean ± standard error (SE) of 3 experiments.

Based on the Table 2 and Figure 1, it was found that there was a significant difference in the mean P2 and P3 (0.005), while there was no significant difference in control cells and P1 (0.925), there was also no significant difference in control cells and P2 (0.193). At P1 and P2, a value of 0.082 ($p < 0.05$) was obtained so that there was no significant difference between the two groups. Post Hoc test results on BAX protein expression data showed that pare ethanol extract increased the percentage of BAX protein expression in T47D breast cancer cells.

The Effect of Pare Ethanol Extract on BCL-2 Protein Expression

In this study, 3 doses were selected for the BCL-2 expression test according to IC results$_{50}$ namely ½ IC50, 1 IC50, 2 IC50. Based on the research results shown in table 1. The average BCL-2 expression in the P3 group was the lowest, followed by the average BCL-2 expression in the P1 group and then P2. All four groups' BCL-2 expression data were normally distributed, Shapiro Wilk test result obtained a value of $p > 0.05$ and also has a homogeneous data variant as indicated by the results of the Levene's Test with a value of $p = 0.139$ ($p > 0.05$). The distribution and variance of BCL-2 expression level data were normal and homogeneous, so a parametric statistical analysis was performed by One Way Anova test resulted in a value of $p = 0.000$ ($p < 0.05$) so that it was stated

that there was a significant difference in the average BCL-2 expression between the four groups. One Way Anova test result showed a significant followed by post hoc test to see which groups have the most influence.

Table 3. Differences in BCL-2 Protein Expression between 2 groups using the Post Hoc test

Group	*p-Value*
KN vs P1	0.797
KN vs P2	0.071
KN vs P3	0.008*
P1 vs P2	0.253
P1 vs P3	0.002*
P2 vs P3	0.000*

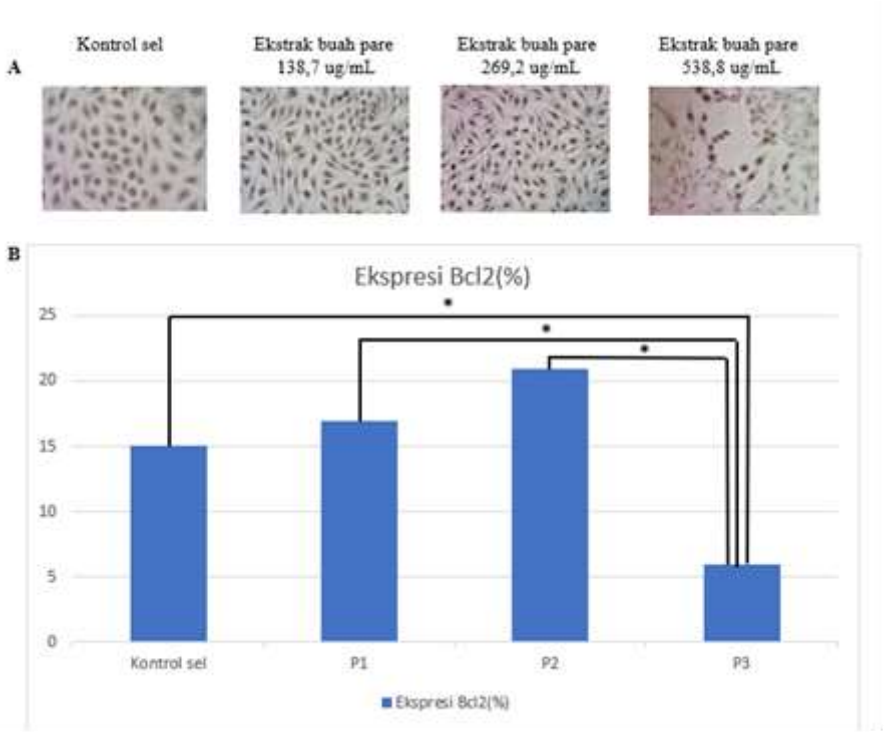

Figure 2. Effect of pare ethanol extract on BCL-2 protein expression in T47D cells for 24 hours of treatment. (A) BCL-2 expression observed using the ICC method, (B) Graph of the percentage of BCL-2 expression after being treated with pare ethanol extract on T47D cells for 24 hours. Protein expression profiles are presented from the mean ± standard error (SE) of 3 experiments.

Based on the Table 3 and Figure 2, it was found that the average control cell and P3 (0.008) had a significant difference, P1 and P3 (0.002) also had a significant difference, P2 and P3 (0.000) had a significant difference. Post Hoc test results on BCL-2 protein expression data showed that administration of pare ethanol extract reduced the percentage of BCL-2 protein expression in T47D breast cancer cells.

III. DISCUSSION

Breast cancer cells are the most common cancer cell population in the world with a therapeutic resistance rate of up to 78%. Induction of apoptosis is a strategy to kill cancer cells, including breast cancer cells. (11) Recent research has revealed that T47D cancer cells have the ability to protect themselves from the apoptotic program by increasing the expression of anti-apoptotic proteins such as BCL-2, suppressing the expression of pro-apoptotic proteins such as BAX.

The concentration of each dose of pare ethanol extract used in this study was 7.8125; 15,625; 31.25; 62.5; 125; 250; 500; and 1000 ug/mL for 24 hours of therapy. The results showed that pare ethanol extract was cytotoxic (Figure 5.1). The cell growth pattern will decrease with increasing dose. Cytotoxic test results on T47D cells obtained IC values$_{50}$ of 269.4 ug/mL. This value will be used to study the effect of pare ethanol extract on BAX and BCL-2 protein expression at doses of 2 IC50, 1 IC50, and ½ IC50. This cytotoxic result illustrates that pare ethanol extract has the property of reducing the growth of T47D cells. The mechanism of

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T47D cell growth inhibition can occur through the induction of apoptosis. Based on the results of the cytotoxic test, the ethanol extract of pare ethanol had a relatively high IC50 value against T47D cells.

The potencial of pare ethanol extract in stimulating cell apoptosis is probably due to the it flavonoids and momordin. Flavonoids can stimulate apoptosis through several mechanisms, including inhibition of DNA topoisomerase I/II activity, modulus signalling pathways, decreased BCL-2 and Bcl-XL gene expression, increased BAX and Bak gene expression and endonuclei activation. (11) BCL-2 protein is an antiapoptotic protein, while BAX protein is proapoptotic. These proteins play a role in the regulation of apoptosis by regulating Cyt c (cytochrome) release. Previous studies have suggested that BCL-2 expression prevents Cyt c release from mitochondria. BAX will induce the release of Cyt c. In the cytosol Cyt C will form a complex with Apaf-1 (Apoptotif Protease Activating Factor-1), ATP and Procaspase-9. This complex is called the apoptosome, which activates caspase-9. Caspase-9 activates caspase 6 and caspase-7 to execute apoptosis.(11)

The cytotoxic effect of pare is due to the content of secondary metabolites such as flavonoids, saponins, alkaloids, and tannins in pare which can inhibit cell proliferation. Previous research also reported that pare ethanol extract can inhibit cell growth through the mechanism of inducing apoptosis, cell cycle, and metastasis.(12) IC results$_{50}$ in this study tended to be weakly cytotoxic with IC values$_{50}$ > 100 ug/mL, this can be influenced by the content of active compounds in pare ethanol extract which are polar, making it difficult to penetrate the non-polar T47D cell membrane. This study showed that the expression of Bax in the P2 group experienced a significant increase compared to the control cell group. This shows that pare ethanol extract has an effect on the apoptotic process of T47D breast cancer cells through increasing the expression of BAX. This is presumably due to the influence of secondary metabolites of pare ethanol extract such as flavonoids which can play a role in the process of apoptosis through induction of Bax.

Based on the result, a pare ethanol extract result showed an increase in Bax expression but showed a decrease in BCL-2 expression. This indicated that each concentration showed a different response to BAX and BCL-2 expression because each concentration had a different response in apoptosis. However, compared between three concentrations at concentrations of 138.7 µg/ml and 538.8 µg/ml there was a decrease in BCL-2 expression and an increase in BAX when compared to other concentrations, this means that a smaller concentration can increase apoptosis.(13) This research is in line with Safitri *et al* (2020) showed that the expression of BCL-2 in cells without treatment was 13.024% of the visual field area, while the expression of BCL-2 with extracts and fractions with concentrations of 63.035 µg/mL and 43.498 µg/mL were 9.119% and 9.299% respectively. These results illustrate a decrease in BCL-2 expression after sample treatment.(2) Another studies also showed that the active substance in green apples can reduce BCL-2 expression. The release of cytochrome C by mitochondria is due to inhibition of BCL-2 expression which then induces the caspase pathway.(14)

This study shows that the mechanism of apoptosis from the pare ethanol extract is through the mitochondrial pathway by increasing the BAX/BCL-2 ratio. In this study, the percentage of BAX and BCL-2 after treatment with pare ethanol extract from BAX 95% became 80%, then BCL-2 95% became 70%. This percentage showed that it be able to disrupt the mitochondrial membrane potential resulting in the release of Cyt c into the cytosol. The affects in the expression of caspase-9, caspase-6 and caspase-7 still needs further research.

IV. CONCLUSION

The administration of pare ethanol extract at doses of 2 IC50, 1 IC50, and ½ IC50 can increase the BAX protein expression and decreased the BCL-2 protein expression in T47D breast cancer cells.

ACKNOWLEDGEMENT

The authors declare no conflict of interests and no involvement of sponsors or funding for this research.

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