Antioxidant Activity of Ethanol Extract of Okra (*Abelmoschus esculentus* (L.) Moench) and Its Effect on the Expression of p53 in Breast Cancer Rat Model

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Keywords: Okra (*Abelmoschus esculentus*), benzo(α)pyrene -induced Breast Cancer, DPPH Test, p53.

Abstract: Breast cancer is a type of cancer with a high incidence in Indonesia, ranking second highest after cervical cancer. The search for effective therapeutic agents for breast cancer, including the investigations of natural agents, still needs to be done. The study was conducted to evaluate the anticancer effect of seed pods extracts of okra (*Abelmoschus esculentus* L., Moech) which is traditionally used in the treatment of various diseases. The objectives of the study were to determine the antioxidant activity of okra seed pods extracts and evaluate the effect of the extract on tumor severity in the benzo(α)pyrene induced rat breast cancer model. The experiment was carried out using a completely randomized design, with 5 treatments and 6 replications. These treatments were: 2 control groups (K0 = normal group; K1 = breast cancer model rat due to benzo(α)pyrene injection), and 3 extract groups (breast cancer model rats were given okra seed pods extract dose of 150, 300, and 450 mg /kg body weight, respectively). The results showed that okra fruit ethanol extract has a strong antioxidant activity with IC50 value = 68.79, but the extract has not been able to trap free radicals due to the induction of benzo(α)pyrene in test animals. Immunohistochemical examination showed that there was no significant difference and effect between ethanol extract of okra fruit and wild type p53 expression in test animals induced with benzo(α)pyrene cancer growth compounds.

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1 INTRODUCTION

The threat of cancer is increasing along with the occurrence of lifestyle changes such as smoking, consumption of fast food, increased pollution, and ozone layer depletion. Indonesia is a country with a high cancer burden. The results of early detection with the Acetic Acid Visual IV (IVA) method to detect cervical cancer and clinical breast examination (SADANIS) to detect breast cancer until 2017 obtained the results of early detection reaching 1,925,943 or 5.2%. Cancer is a disease caused by changes in body cells into abnormal cells and grows out of control.

Cancer treatments such as surgery and chemotherapy are very expensive, so many traditional treatments are used as a substitute for medical treatment, which is herbal treatment by using plant extracts. One of the plants known to treat cancer is the okra plant (*Abelmoschus esculentus* L. (Moench)). Okra is an important vegetable that is widespread in Africa, Asia, Southern Europe, and America. Okra has a role as a source of carbohydrates, minerals, and vitamins such as potassium, sodium, magnesium, and calcium (Khomsug et al. 2010). Okra is a plant that is rich in flavonoid components as an anti-cancer, maintain cardiovascular condition, reduces blood sugar levels and has a series of other properties. Leaves, flowers, and fruit are parts that contain high flavonoids (Liu et al. 2017; Van Dam et al. 2013)

The anti-cancer effect of the newly discovered lectin from the isolation of okra, was investigated in human breast cancer cells and fibroblast cells where lectin from okra induced a significant inhibition of cell growth (63%) in MCF7 cells (Monte et al. 2014).

Research into the anti-cancer mechanism of okra fruit extracts needs to be done because of the high incidence and limited information on the anticancer mechanism of okra fruit ethanol extract. The anticancer mechanism referred to is one of them through increased expression of p53 protein, thus, the

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presence of p53 will inhibit the function of apoptosis (Cancer Research Center, 2011). In addition, there is a relationship between anticancer and antioxidants because antioxidants can trap free radicals which are one of the agents that cause breast cancer in humans. Therefore, this study aims to determine the antioxidant activity of ethanol extract of okra fruit (*Abelmoshcus esculentus* L. (Moench)) and its effect on the expression of p53 protein in breast cancer induced by benzo(α)pyrene so it can become a source of information for those who need to be developed into an alternative cancer treatment, especially breast cancer with raw materials derived from plants.

2 MATERIALS AND METHOD

This research has been conducted at the Pharmacy Research Laboratory, Organic Chemistry Laboratory, Laboratory of Structure and Animal Development, Faculty of Mathematics and Natural Sciences (FMIPA) and Anatomy Pathology Laboratory, Faculty of Medicine, University of North Sumatra.

2.1 Research Design

This study used a Completely Randomized Design (CRD) with 5 treatments and 6 replications so that the number of samples used was 30 samples, 30 female rats (*Rattus sp*) Wistar strains.

2.2 Extract Preparation

The okra fruit was obtained from the Growth Center Laboratory of KOPWIL 1, North Sumatra. After being collected from the field, the okra fruit that has been washed clean is dried in an oven at 40ºC until it meets the requirements of general moisture content. Simplisia that is dried and then made into powder until smooth and sieved with a B_{30} sieve. Making ethanol extract of okra fruit is done by maceration, ie okra fruit powder is put into a brown bottle and ethanol is added until submerged and then stirred and left for 1 night. Take the filtrate and re-soak the residue with ethanol until a clear filtrate is obtained. The filtrate obtained was separated with a rotary evaporator so that a thick extract was obtained.

2.3 Animal Preparation

The experimental animals used were rats (*Rattus sp.*) of Wistar strains, female, healthy, aged 8-11 weeks, with a weight of 200-250 g. Thirty animals obtained from the North Sumatra Animal Disease Investigation Center Medan. The animals were placed in plastic cages with lids made of ram wire, feed in the form of pellets and drinking water were given *ad libitum*. The environment of the cage was arranged with adequate ventilation and sufficient light where the light time was 14 hours and the dark time was 10 hours. Before experimenting, the mice were adapted in a cage for 7 days. Rat health is monitored every day.

2.4 Treatment Administration

Breast cancer induction was carried out by injecting a solution of benzo(α)pyrene to the mammary tissue of the rat. A single dose of 50 mg/kg body weight was dissolved in olive oil and injected subcutaneously. The emergence of tumor mass in rat breast was observed by palpation for 4 months, then continued with the administration of the ethanol extract of okra (*Abelmoschus esculentus* L., Moech) seed pods for 15 days. The animals were divided into 5 groups:

a. A blank control group (K_0): no treatment.
b. A positive control group (K_1): single dose of benzo(α)pyrene
c. Extract I group (P_1): a single dose of benzo(α)pyrene + 150 mg/kg body weight extract.
d. Extract II group (P_2): a single dose of benzo(α)pyrene + 300 mg/kg body weight extract.
e. Extract III group (P_3): a single dose of benzo(α)pyrene + 450 mg/kg body weight extract.

2.5 Immunohistochemistry

The immunohistochemical method used in this study is an indirect method with a brief procedure: deparaffination, rehydration, and then immerse the tissue section in peroxidase blocking solution at room temperature for 10 minutes. The slides immersed in a 25°C prediluted blocking serum for 10 minutes, then incubated in a 25°C anti-p53 wild type primary antibody for 10 minutes. After washed in phosphate buffer saline (PBS) for 5 minutes, the slides incubated in a secondary antibody solution at 25°C for 10 minutes. The slides washed with PBS for 5 minutes then incubated in a freshly made diamino benzidine (DAB) solution at 25°C for 10 minutes, and then counterstained with haematoxylin. Observations was conducted by calculating the percentage of cells with p53 expressions and the

color intensity and the expression area. Observations were made using a light microscope with a magnification of 400x (CCRC, 2015).

$$\% \text{ Cell expression} = \frac{\text{number of brown cells}}{\text{total cell count}} \times 100\%$$

Immunoreactive scores = intensity scores x broad scores

(Tan and Putti, 2005)

2.6 Antioxidant Activity Test

The antioxidant activity test of okra extract was carried out by DPPH free radical trapping method. A 0.5 mM (200 ppm) solution of DPPH prepared, and its maximum absorption wavelength was measured using a UV-Vis spectrophotometer (400nm-800nm) and obtain a maximum wavelengths of 516 nm. Ethanol extract of okra fruit prepared in concentration of 10, 20, 30, 40 and 50 ppm, incubated for 30 minutes, and measured their absorbance. The DPPH free radical trapping was calculated using the formula (Tristantini et al. 2016).

$$(\%) \text{ damping} = \frac{\text{abs. control} - \text{abs. sample}}{\text{abs. control}} \times 100\%$$

3 RESULTS AND DISCUSSION

3.1 Antioxidant Activity

The antioxidant activity test of ethanol extract of okra fruit *(Abelmoschus esculentus* L. Moench), showed a decrease in absorbance of DPPH. This is due to the activity of trapping by the test solution that is okra fruit ethanol extract. In this process, an interaction occurs between the extract solution of okra fruit with DPPH. Ethanol extract of okra fruit donated 1 hydrogen atom to DPPH so that DPPH changed into its reduction form (Molyneux, 2004).

Table 1 shows the value of IC_{50} determined through the equation of a linear regression line with the concentration of the sample as the X-axis and damping activity as the Y-axis (Utami, 2017).

Table 1: Value IC_{50} of the ethanol extract sample of okra fruit.

Replication	Line equation	value y	IC_{50} x
1	y= 0,3682x + 0,8547	50	138.11
2	y= 2,0602x – 20,563	50	34.25
3	y = 1,7585 – 9,8027	50	34
total			206.37
mean			68.79

From the results of data analysis, obtained IC_{50} of 68,79 (strong category). This value is inversely related to the antioxidant activity where the higher the value of antioxidant activity, the lower the IC_{50} value (Molyneux, 2004). The antioxidant activity of the ethanol extract of okra fruit can also be noted through the change in color of the test sample which is dark purple when added DPPH will turn yellowish color if the extract has the damping as seen in the picture below:

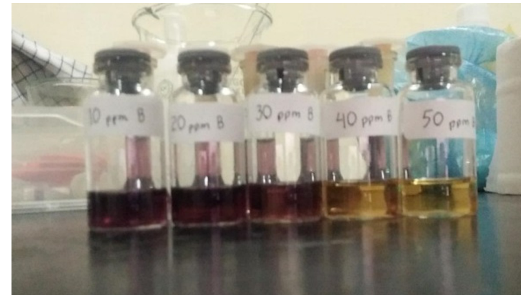

Figure 1: The color change of the test sample.

The presence of antioxidants in plant extracts can neutralize DPPH radicals by giving electrons to DPPH, resulting in a change in color from purple to yellow or the intensity of the purple color of the solution to be reduced (Molyneux, 2004). This discoloration causes a decrease in DPPH absorbance (DPPH purple reduction/ DPPH trapping activity).

3.2 p53 Protein Expression

Quantitative immunohistochemical observations show that the benzo(α)pyrene control group (K1) shows almost no wild p53 expression compared to the normal control group (K0).

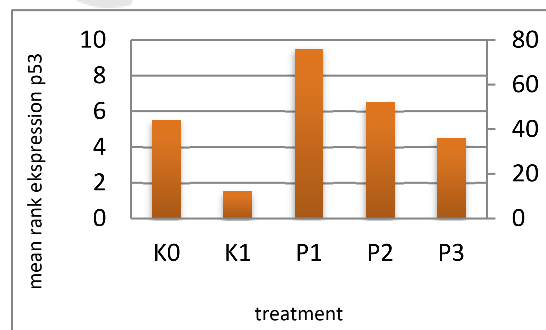

Figure 2: Diagram of increasing wild-type p53 expression; K_0 = normal control, K_1 = benzo(α)pyrene control, P_1 = benzo(α)pyrene + okra 150 mg/kg BW, P_2 = benzo(α)pyrene + okra 300 mg/kg BW, P_3 = benzo(α)pyrene + okra 450 mg/kg BW.

In Figure 2, the lowest percentage of wild p53 expression occurs in the benzo(α)pyrene group (K_1), with a mean rank of 1.50 this is due to benzo(α)pyrene induction can damage the DNA structure so that many p53 wild type genes mutate into mutant p53. When DNA damage occurs, p53 holds cells from entering the next phase and gives DNA time to make repairs, or if the damage is severe enough, p53 will initiate a cell death program (apoptosis). The highest p53 expression occurred in group P1, with a mean rank of 9.50. This increase in p53 expression will spur apoptosis through 2 mechanisms, namely by increasing Bax protein and decreasing Bcl-2 protein expression (King, 2000).

In contrast to group P_1, groups P_2 and P_3 actually experienced a decrease in wild type expression of p53 with a mean rank of 6,50 to 4,50. This data explains that the higher the dose of ethanol extract given okra can actually reduce the expression of p53 even if the extract dose continues to be increased it could trigger tumors. It is suspected that there are other substances in the extract that are interested when doing maceration with ethanol solvent. Further research is needed to carry out phytochemical screening and ascertain the active substance and its relationship with p53 expression.

In addition to quantitative analysis, qualitative analysis is also performed to see the expression of p53 by determining the immunoreactive score of p53 against the p53 antibody staining. Look at the picture below:

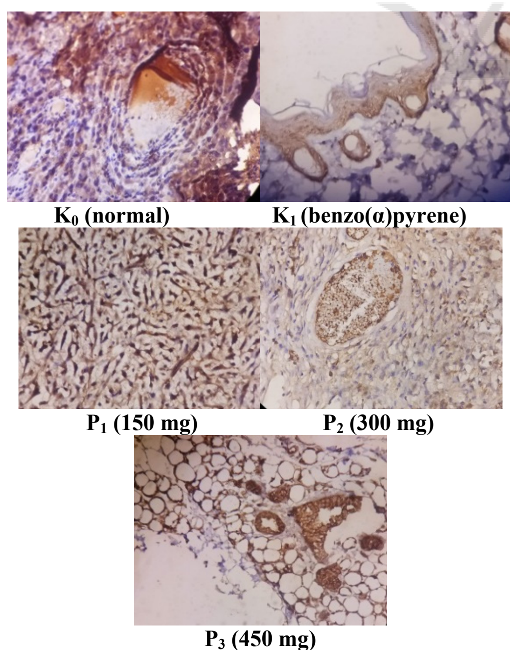

Figure 3: Observation of p53 expression using a light microscope with a magnification of 400 x.

From the picture above we can see the difference in color intensity ranging from dark brown, medium brown, light brown and blue. Likewise with the colored areas ranging from 0 -> 10%, 10-50% and> 50% by the scoring rules so that the immunoreactive data obtained in the table below:

Table 2: Immunoreactive score of p53 expression.

Group	Intensity	Large	Immunoreactive	Information
K^0	+3	2	6	overexpression
K^1	+1	1	1	not expressed
P1	+2	3	6	overexpression
P2	+2	2	4	overexpression
P3	+3	2	6	overexpression

Information:

K0 = normal control
K1 = benzo(α)pyrene control
P1 = benzo(α)pyrene + okra 150 mg /kg BW
P2 = benzo(α)pyrene + okra 300 mg / kg BW
P3 = benzo(α)pyrene + okra 450 mg / kg BW

Immunohistochemical observations showed the presence of wild type p53 protein accumulation. This accumulation is likely caused by physiological responses to DNA damage or impaired cell proliferation in tumor cells (Louis, 1994). The existence of p53 activation by ethanol extract of okra fruit (*Abelmoshcus esculantus*) through p53 stabilization will affect the cell cycle process so that cells will not experience division and cells will die due to chromosome condensation that causes apoptosis, so based on this research it is suspected that okra fruit extract has activity antimitosis and proapoptosis in tumor cells. Drugs that have an antimitotic effect are also thought to have an antitelomerase effect that can inhibit cell division and rapid development such as cancer cells and result in cell death (apoptosis).

Based on the analysis of SPSS data, the Kolmogorov-Smornov test and the Levene Statistics test show that the data is abnormally distributed and not homogeneous because the significance value is 0.00 <0.05 so that the Kruskal-Wallis test is carried out to determine whether there is an effect of treatment on the research variables. The results of data analysis showed there was significant effect of ethanol extract of Okra (*Abelmoschus esculantus* L. Moench) on p53 expression and immunoreactive scores in breast cancer induced by benzo(α)pyrene.

4 CONCLUSIONS

Based on the results of the study, it can be concluded that the ethanol extract of okra fruit has a strong antioxidant activity with an IC_{50} value of 68.79, the extract has been able to trap free radicals due to the induction of benzo(α)pyrene in test animals. In addition, there was significant effect of ethanol extract of okra fruit on wild type p53 expression in breast cancer rat model.

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