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RESEARCH ARTICLE

Effect of *Cayratia trifolia* Ethanol Extract on Bcl-2 expression and Apoptotic Cells in Dmba-induced Breast Cancer of Sprague Dawley Rats

I Ketut Sudiana[1*], Willy Sandika[1], Dyah Fauziah[1], Djoko Santoso[2], Desak Gede Agung Suprabawati[3], Muhammad Ilyas Yusuf [4], Ferry Efendi[5]

1. *Pathology Department, Faculty of Medicine, Universitas Airlangga.*
2. *Internal Medicine Department, Faculty of Medicine, Universitas Airlangga.*
3. *Surgery Department, Faculty of Medicine, Universitas Airlangga.*
4. *Doctoral Program, Faculty of Medicine, Universitas Airlangga.*
5. *Faculty of Nursing, Airlangga University, Surabaya, Indonesia.*

***Corresponding Author:** I Ketut Sudiana

Abstract

The study aimed to clarify the effect of the administration of ethanol extract of *Cayratia trifolia* plants to breast cancer. This study was an experimental study using female animal model of Sprague Dawley rats, consisting of three groups, K0, the normal experimental animals, K1, the experimental animals with breast cancer receiving CMC 0.5%, and KP, experimental animals with breast cancer receiving ethanol extract of *Cayratia trifolia* in a dose of 300 mg/kgBW at 0.5% CMC. The material to induce breast carcinoma formation was DMBA of 20 mg/kg in corn oil. The extraction of *Cayratia trifolia* plants used ethanol. The variables were the number of cells expressing Bcl-2 observed with immunohistochemical methods, and the number of cells undergoing apoptosis, which was observed using tunnel assay method. Statistical analysis was performed using ANOVA test. The number of cells expressing Bcl-2 and those undergoing apoptosis in breast cancer tissue between groups of animals receiving and not receiving ethanol extract of *Cayratia trifolia* showed significant difference ($P<0.05$). Oral administration of *Cayratia trifolia* ethanol extract has an effect on the decrease in the number of cells expressing Bcl2 and an increase in the number of cancer cells undergoing apoptosis.

Keywords: *Breast carcinoma; Proto-oncogene; Tumor suppressor gene; Cayratia trifolia.*

Introduction

Cancer is a disease characterized by uncontrolled cell division. Based on the hospital information system, the highest incidence of cancer patients in Indonesia in women is breast cancer and cervical cancer, with a percentage of 28.7% and 12.8% respectively [1], where cervical cancer ranks second after breast cancer [1, 2].

Currently the methods used to treat breast cancer are surgery, administration of radiotherapy, and chemotherapy. However, these methods have not provided satisfactory results. They often give adverse side effects for the patients. Therefore, chemicals that can be used as anticancer and have low side effects need to be formulated. Some researchers report

chemicals that can be used as anti-cancer, one of which is piceid. Piceid is reported to be a chemical that acts to inhibit DNA synthesis [3]. Other chemicals are kaempferol, a chemical used as an anti-angiogenesis in ovarian cancer [4], and resveratrol, which in vitro can inhibit various tumor cell lines, but does not inhibit normal cells [5].

One of plant containing piceid, kaempferol, and resveratrol, is a *Cayratia trifolia*, which has an integration of (1) stilbenes (piceid, resveratrol, viniferin, and ampelopsin), (2) kaempferol, and (3) quercetin [6].

However, the effect of providing *Cayratia trifolia* plant extract to breast cancer is unclear. Therefore, this study aims to reveal the effect of extracts of *Cayratia trifolia* plants on cancer cells. Disrupted control of cell division cycles in cancer cells is due to the presence of multiple gene defects, the proto-oncogenes, such as Bcl-2 and tumor suppressor genes, including the p53 [7]. Disability of the two groups of genes results in uncontrolled cell division, leading to abnormal cell accumulation.

Materials and Methods

This study was an experimental study. Based on cancer incidence [1], [2], this study focused on breast cancer. Because it was not possible to be conducted in humans, we conducted a study using experimental animal models of Sprague Dawley rats with breast cancer. This study was granted ethical approval by the Animal Care and Use Committee (ACUC) in the Faculty of Veterinary Medicine, Universitas Airlangga No. 359-KE.

The measured variables were the number of cells expressing Bcl-2 and cancer cells that experienced apoptosis in breast carcinoma. Twenty-five Sprague Dawley rats were divided into two groups by random allocation, the normal group of 5 rats (K0) and DMBA-induced groups of 20 rats (K). Materials used in this study were the ethanol extract of *Cayratia trifolia*; corn oil; 7,12 dimethyl benz (a) anthracene (DMBA); carboxymethyl cellulose (CMC: 0.5 %); antibodies to Bcl-2; and apoptec tunnel Assay Kit.

The 7.12 dimethyl benz (a) anthracene (DMBA), which was suspended in corn oil and administered orally, was used to induce breast cancer formation in mice. *Cayratia trifolia* was given as an extract by extracting from the stems and leaves of the plant using ethanol method.

The obtained extract was suspended in carboxymethyl cellulose (CMC: 0.5%) and given using intra-gastric sonde. DMBA was given in a dose of 20 mg/kgBW [8], [9] which was suspended in corn oil. To induce breast cancer formation, this study used DMBA of 20 mg/kgBW/2.0 ml in corn oil, and was given using the gastric sonde 2 times a week for 10 weeks. Breast cancer began to appear in 11th week. Of the 20 experimental animals receiving DMBA suspension, only 17 had breast cancer. Of these, one rat was sacrificed, then the breast cancer was taken for haematoxylin-eosin (H & E) staining with paraffin method to ensure that the tumor was breast cancer. After determining that the animal breasts were cancerous, 16 other rats in K-group were divided randomly into two groups, the K1 and KP groups, so that in this study there were three groups of experimental animals, the K0 groups of 5 rats, and K1 and KP groups with 8 rats each.

The K0 group comprised normal Sprague Dawley rats, the K1 group consisted of Sprague Dawley rats who had breast cancer and receiving 0.5% CMC suspension of 2.00 ml using intragastric sonde every day for 4 weeks, while KP group was Sprague Dawley rats who experienced breast cancer receiving ethanol extract of *Cayratia trifolia* in a dose of 300 mg/kgBW which was suspended in CMC 0.5 % every day using intragastric sonde of 2.00 ml for 4 weeks. After treatment for 4 weeks, a week later all groups of experimental animals were sacrificed.

The anesthetic material used was ether. Then, the breast cancer tissue was taken and immediately put into a 10% formalin buffer solution for 24 hours. Thereafter, tissue processing was done through several stages, ie. dehydration using ethanol, starting from 70%, 80%, 95%, and 100% ethanol, clearing using xylol three times, impregnating with paraffin (56-58 degrees C), then embedding in paraffin blocks. The paraffin block was then sliced with a microtome (Leyca, Model: RM2125RTS) in a thickness of incision between 4-6 microns.

The incision was then placed in a water bath (Leyca, Model HI1210) at a temperature of 45-55 degrees C, which was then taken with glass slides (Biogear Microscope premium polysilane Coated slides). The tissue that had been attached to the glass slide was placed on the hot plate for 30 minutes. To determine the effect of *Cayratia trifolia* ethanol extract on cancer cell death, we observed two variables, the Bcl-2 and cell death (apoptosis).

To determine the expression of Bcl-2, indirect staining of immunohistochemistry method (biotin-streptavidin amplified/BSA method) was carried out using anti-Bcl-2 from R & D abiotechne (Cat.No: MAB8272). To determine the apoptotic cancer cells, staining was performed using tunnel assay method with

reagents from BioVision MAB827Apo-BrdU-IHC In Situ Fragmentation Assay Kitt (Cat.No: K403-50). The assessment was carried out using Olymphus type microscope CX31 (Model CX31RBSF) with 400 x magnification by evaluating 10 visual fields. Each visual field had an area of 15625□² using graticule. Documentation using the Olympus Camedia camera (Model C-4000ZOOM).

Results

During the study, in K1 group, two rats died, so that in group K1, which originally consisted of eight rats, only remaining six rats. In K0 and KP groups no rats died, so in the K0 group there were still five rats, and in the KP group eight rats. Mean sample in K0, K1 and KP groups was tested with homogeneity and normality test. The results showed that the data analyzed were homogeneous and normally distributed, so that the test was followed with ANOVA test (Table 1).

Table 1: Anova comparative test of Bcl-2 expression on breast tissue epithelium between groups (K0, K1, and KP)

K0 (n=5)		K1 (n=6)		Sig
Mean	SD	Mean	SD	0.002
0.0000	0.0000	22.4000	16.90538	
		KP (n=8)		0.287
		6.1000	3.39327	
K1 (n=6)		KP (n=8)		0.007
Mean	SD	Mean	SD	
22.4000	16.90538	6.1000	3.39327	

Table 1 shows that the epithelium of Sparague Dawley breast tissue that expressed Bcl-2 in K0 and K1 groups showed a significant difference ($p<0.05$). K0 and KP groups did not show significant difference ($p> 0.05$), while K1 and KP groups had significant difference ($p<0.05$)

Table 2: Anova comparative test of the number of apoptotic breast tissue epithelium between groups (K0, K1, and KP)

K0 (n=5)		K1 (n=6)		Sig
Mean	SD	Mean	SD	0.353
0.1600	0.11402	5.4333	1.57184	
		KP (n=8)		0.004
		17.5625	13.70786	
KI (n=6)		KP (n=8)		0.025
Mean	SD	Mean	SD	
5.4333	1.57184	17.5625	13.70786	

Table 2 shows that the epithelium of the breast tissue of Sparague Dawley rats that experienced apoptosis in K0 and K1 groups showed no significant difference ($p> 0.05$). K0 and KP groups showed significant difference ($p<0.05$), and K1 and KP groups also showed significant difference ($p<0.05$)

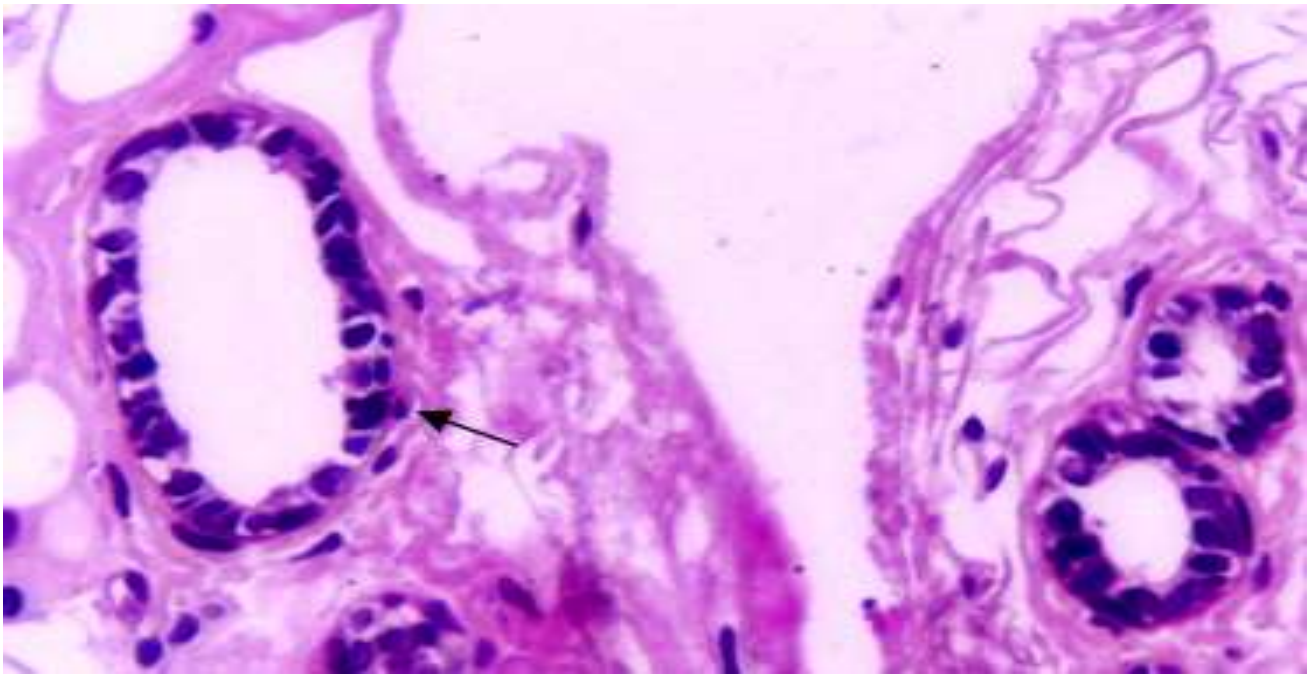

Figure 1: Normal breast tissue incision of the experimental Sprague Dawley rats with Haematoxylin-Eosin (H & E) staining, magnification 400x, light microscope. Arrow shows the epithelium of breast gland in one layer with the basement membrane, looks intact

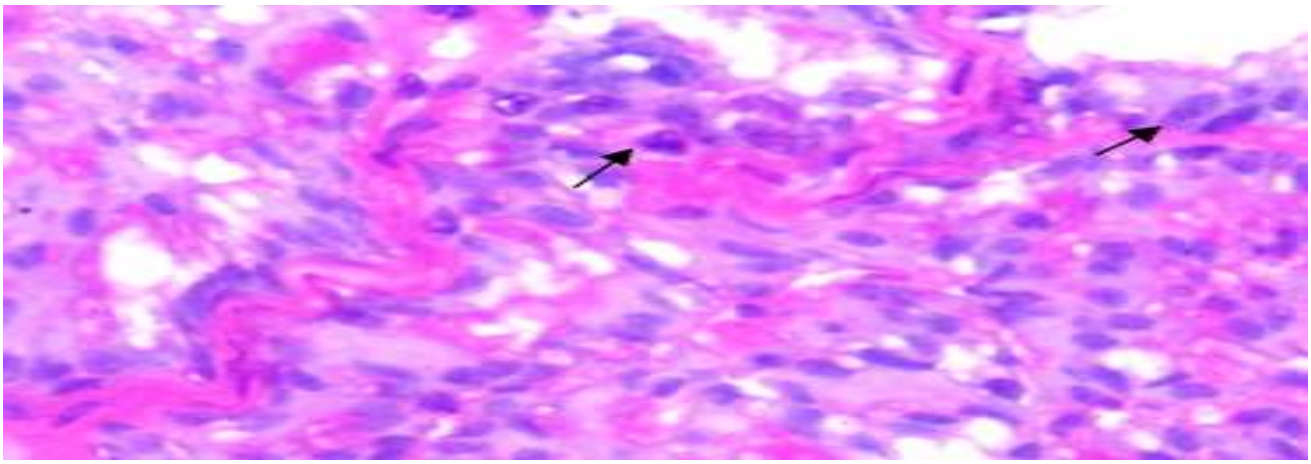

Figure 2: Breast cancer tissue incision of the Sprague Dawley rats after DMBA induction, with Haematoxylin-Eosin (H & E) staining, magnification 400x light microscope. Arrows show that the epithelium of the breast gland appears irregular, with varying size and shape of the nucleus, and basement membrane not intact

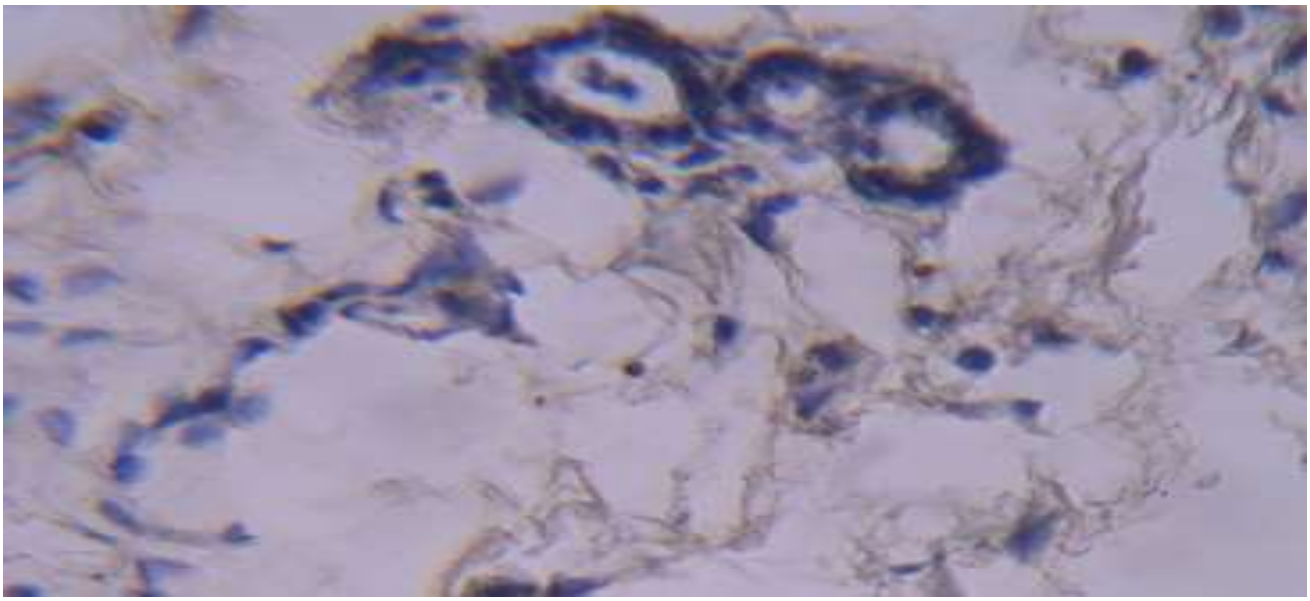

Figure 3: Normal breast tissue incision from the Sprague Dawley rats with indirect immunohistochemistry staining (Streptavidin-Biotin amplified/BSA method) using monoclonal antibodies (anti-Bcl-2), magnification a 400x light microscope, no cells showing positive reaction to anti-Bcl-2

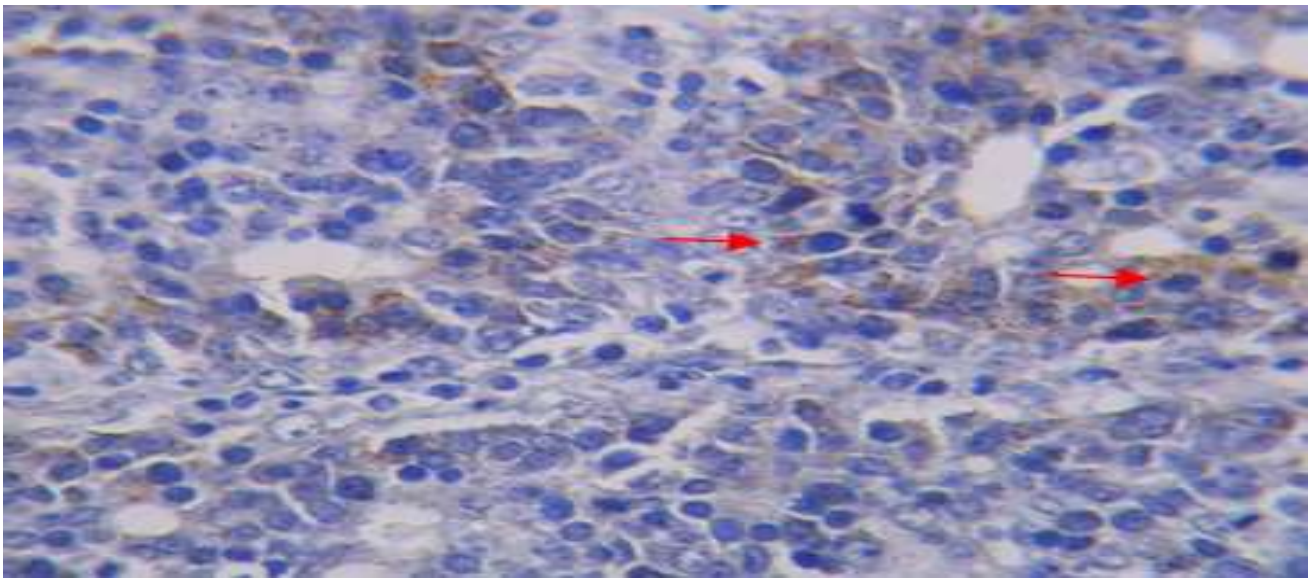

Figure 4: Breast cancer tissue incision in Sprague Dawley rats that did not receive *Cayratia trifolia* ethanol extract with indirect immunohistochemistry (Biotin streptavidin amplified/BSA method) staining using monoclonal antibodies (anti-Bcl-2), magnification 400x with light microscope. Cells expressing cytoplasmic Bcl-2 are brown, those not expressing cytoplasmic Bcl-2 not in brown color (arrows indicate cells expressing Bcl-2)

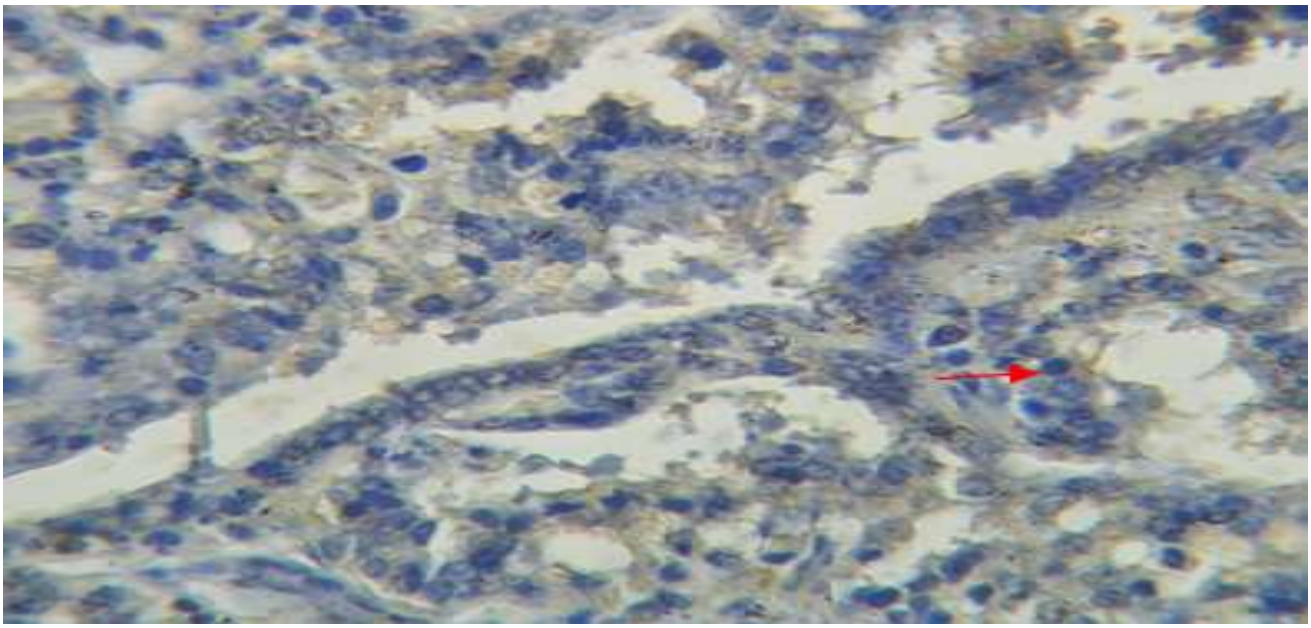

Figure 5: Breast cancer tissue incision of Sprague Dawley rats receiving ethanol extract of *Cayratia trifolia*, with immunohistochemistry (Biotin streptavidin amplified/BSA method) staining using monoclonal antibodies (anti-Bcl-2) with magnification of 400x light microscope. Cells expressing cytoplasmic Bcl-2 are brown, those not expressing cytoplasmic Bcl-2 not in brown color (arrows indicate cells expressing Bcl-2)

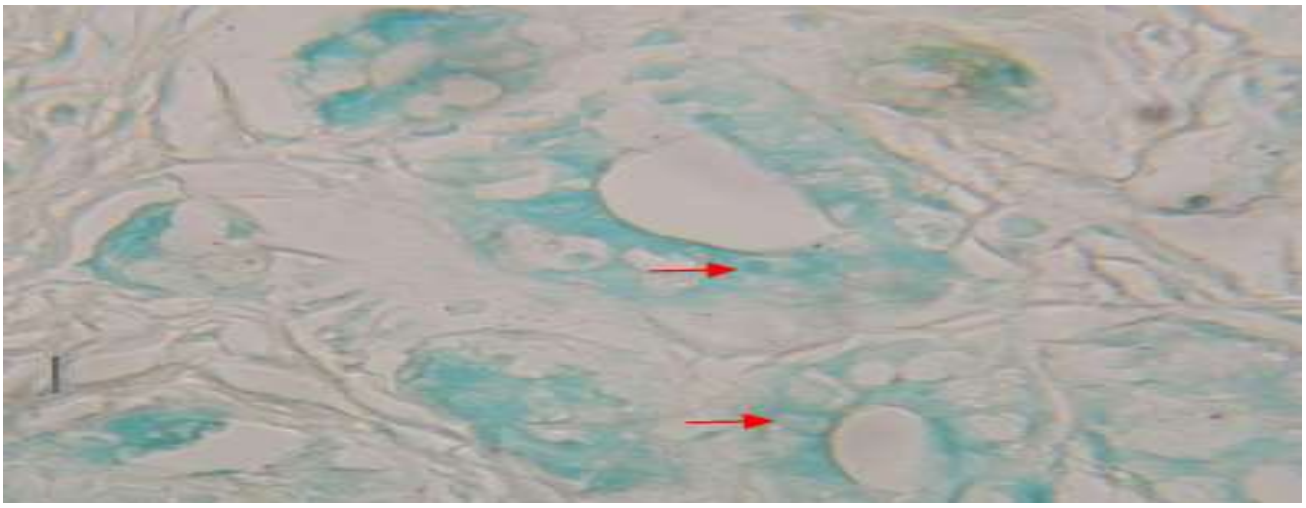

Figure 6: Normal breast tissue incision of Sprague Dawley rats with tunnel assay staining, magnification of 400x, light microscope. Arrows indicate breast gland epithelium that does not undergo apoptosis

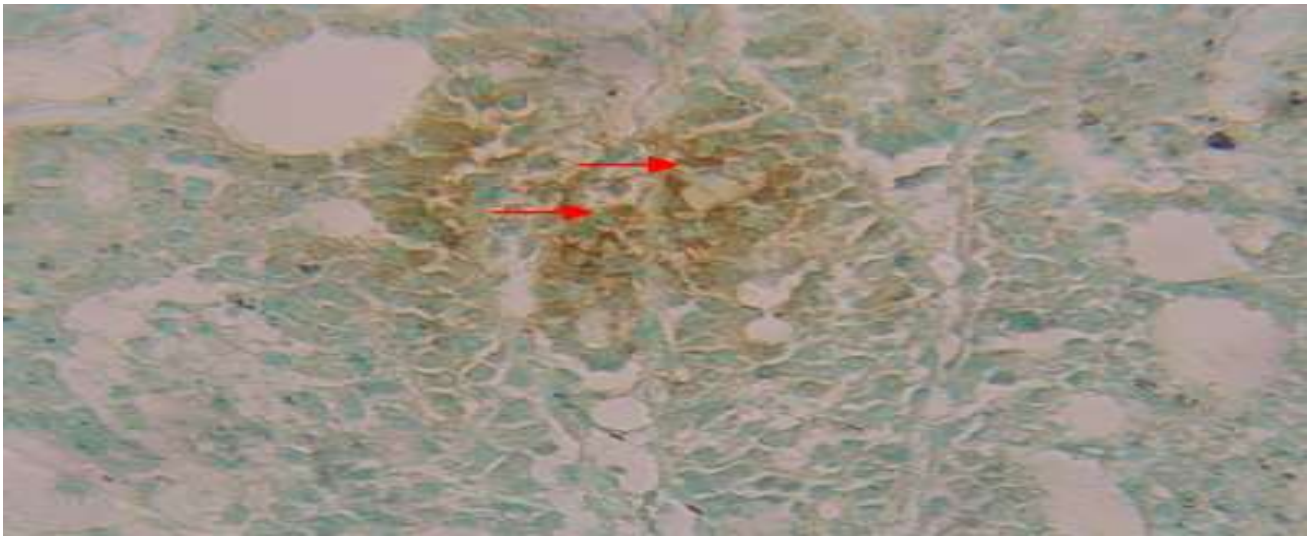

Figure 7: Incision of breast cancer tissue in Sprague Dawley rats receiving ethanol extract of *Cayratia trifolia*, by tunnel assay staining method, magnification 400x, light microscope. Cells undergoing cell apoptosis are brown, those not apoptotic are green (arrows indicate cells undergoing apoptosis)

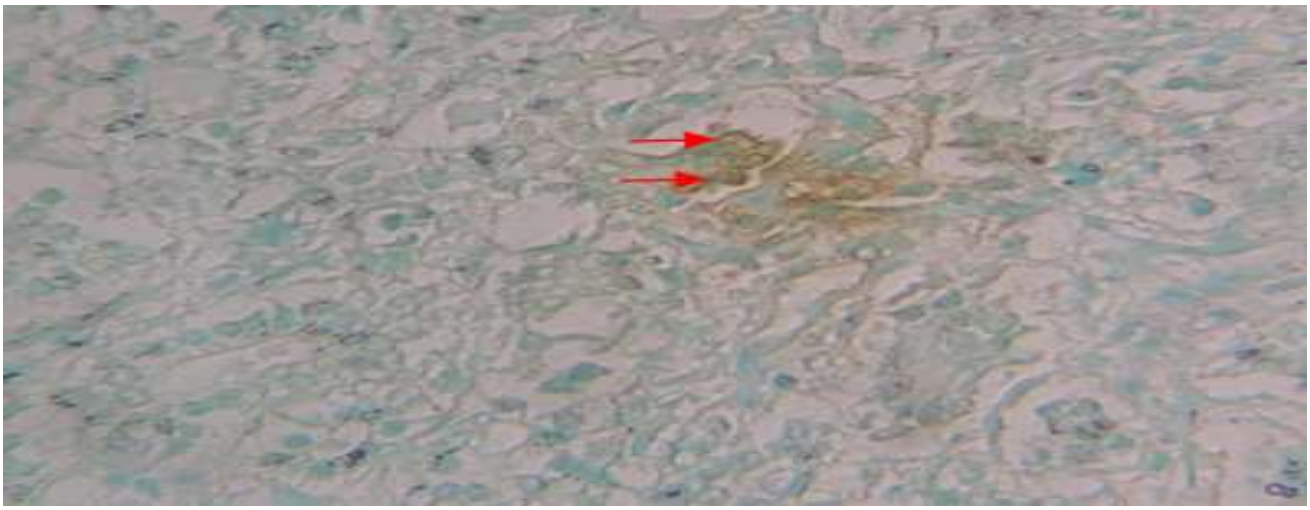

Figure 8: Breast cancer tissue incision of Sprague Dawley rats that did not receive *Cayratia trifolia* ethanol extract by tunnel assay staining method, magnification a 400x, light microscope. Cells that experience apoptosis in the nucleus are brown, those not apoptosis are blue (arrow indicates cells undergoing apoptosis)

Discussion

During the study, in K1 group in 3rd and 4th weeks two experimental animals died, so that group K1, which originally consisted of eight rats, had only six rats. It is possible, the K1 group, which consisted of rats with breast cancer but did not receive *Cayratia trifolia* ethanol extract, experienced a decrease in food intake, thus becoming malnourished. It is known that cancer cells can release cytokines.

Furthermore, the cytokines released by cancer cells induce the hypothalamus to release anorexic signal which results in a decrease in intake and/or appetite, resulting in malnutrition. Malnutrition is a major factor causing morbidity and mortality in cancer patients [10]. Besides as the cause of morbidity and mortality, malnutrition also results in a decrease in the immune system. Decreased immune system, besides causing susceptibility to infection, also leads to the inability to inhibit the progression of cancer.

Cellular immune system that acts to eliminate pathogens is phagocytes, ie. the monocytes and neutrophils [11], [12] while cellular immune system that plays a role in killing cancer cells is cytotoxic cells [13], [14] and NK cells [15, 16]. This decline in immune system triggered by malnutrition in the K1 group seemed to inflict fatality of the experimental animals. Whereas, in KP group no rats died during the treatment.

This was likely that by the administration of *Cayratia trifolia* ethanol extract the immune system in this group was better than that in K1 group, because the KP group, besides having increased resistance to infection, was also able to inhibit cancer progression. Previous study has shown that resveratrol (3,5,4 trihydroxystilbene) given to animals could increase IFN-gamma [17].IFN-gamma is a cytokine that acts to activate NK cells. These active NK cells destroy cancer cells, [15, 16] so that in KP group the cancer cells did not develop. However, unfortunately, this study did not measure experimental animals' body weight, serum albumin and serum immunoglobulin.

In this study, the number of cells expressing Bcl2 between normal/K0 group (X=0.0000; SD=0.0000) and DMBA/KI-induced group (X=22.4000; SD=16,90538) showed significant difference ($p<0.05$). It is reported that Bcl2 is one of the anti-apoptotic proteins [16, 18]. This study found that the number of cells expressing Bcl2 in experimental animals' breast tissue induced by DMBA was higher than that of normal experimental animals.

If we compare the results of this study with the Cox (1997) report, it appears that DMBA given to experimental animals results in an increase in the number of cells that have anti-apoptotic abilities. Thus, these cells can be regarded immortal. In addition, it is reported that DMBA is a substance that can induce breast cancer [21, 22]. DMBA, which is lipophilic in breast tissue, is converted to epoxide. The active metabolite has the capacity to damage DNA molecules. This is an event that initiated carcinogenesis [23].

It can be explained that the occurrence of tumors in a tissue shows that in these tissues excessive cell division occurs, whereas in this study many cells were found to be immortal. The nature of immortality and excessive cell division is characteristic of malignant cells. This study found that the number of cells expressing Bcl-2 between normal group/K0 (X=0.0000; SD=0.0000) and the group with breast cancer receiving ethanol extract of *Cayratia trifolia*/KP (X=6.1000; SD=3.39327) did not show significant difference (p>0.05).

Analysis of the data in Table 3 between groups with breast cancer who did not receive *Cayratia trifolia* ethanol extract, the KI group (X=22.4000; SD=16,90538), and group with breast cancer that received the ethanol extract of *Cayratia trifolia*, the KP group (X=6.1000; SD=3.39327), showed significant difference (p<0.05).

This shows that the administration of *Cayratia trifolia* ethanol extract in the breast cancer group caused decreasing number of cells expressing Bcl-2.According to some reports, chemicals such as piceids contained in the *Cayratia trifolia* ethanol extract are chemicals that can inhibit DNA synthesis [3]. There are supporting theories that resveratrol from *Cayratia trifolia* plants extracted with ethanol can inhibit tumor cell line growth [5].In addition, resveratrol is a very powerful ingredient for modulating NK cell activity [24].

NK cells are cells that play a role in destroying cancer cells [15, 16].Decreased number of cells expressing Bcl-2 in breast cancer tissue after the administration of the ethanol extract of *Cayratia trifolia*, in addition to inhibiting DNA synthesis or inhibiting tumor growth, indicated that *Cayratia trifolia* ethanol extract can induce NK cell activity, because NK cells are one of the responsible cells to destroy cancer cells or prevent growth and metastasis [25, 26].

Based on the results of the study, that the administration of *Cayratia trifolia* ethanol extract in experimental animals with breast cancer decreased the number of cells expressing Bcl-2, which showed a decrease in immortality, it can be inferred that the number of cells in breast cancer undergoing apoptosis as a result of the provision of *Cayratia trifolia* ethanol extract. This study showed that the number of apoptotic cells between K0 (X=0.1600; SD=0.11402) and KI groups (X=5.4333; SD=1.57184) did not show significant difference (p> 0.05), while between groups K0 (X=0.1600; SD=0.11402) and KP (X=17.5625; SD=13.70786) groups showed significant difference (p<0.05). Similarly, KI (X=5.4333; SD=1.57184) and KP (X=17.5625; SD=13.70786) groups showed a significant difference (p<0.05). This shows that in the group with breast cancer who did not receive the *Cayratia trifolia* ethanol extract, the number of apoptotic cells was very small, similar to the control group. This indicates that cell death in breast cancer tissue is physiological cell death. Whereas, cell death or apoptosis in the experimental breast cancer group receiving ethanol extract of *Cayratia trifolia* showed a significant increase.

Research also shows that kaempferol is a material that can induce apoptosis in gastric cancer and suppress proliferation and induce apoptosis [27]. According to Kumar (2011), one component contained in the *Cayratia trifolia* plant is kaempferol. A study conducted on ovarian cancer shows that the administration of kaempferol induces an increase in expression of p53 and Bax [28]. Increased expression of p53 and Bax in abnormal/malignant cells will suppress Bcl-2 function and subsequently trigger malignant apoptotic cells [29].

In addition to inducing increased expression of p53 and Bax, [28] kaempferol also inhibits proliferation factors and induces cell death in renal cell carcinoma [30].Kaempferol also inhibits cell proliferation in gastric cancer [27]. Besides kaempferol, the *Cayratia trifolia* plant also contains resveratrol [6]. Resveratrol is a very powerful ingredient for modulating NK cell activity [24] NK cells are one of the cells that play a role in destroying cancer cells [15, 16]. Given the effects of kaempferol and resveratrol chemicals that induce cancer cell death, while these two ingredients are contained by *Cayratia trifolia* plants, the administration of *Cayratia trifolia* ethanol extract in Sprague Dawley rats with breast cancer decreases Bcl2 expression and increases the number of apoptotic breast cancer cells.

Conclusion

Oral administration of the ethanol extract of *Cayratia trifolia* plant has an effect on the decrease in the number of cells expressing Bcl-2 and an increase in the number of breast

cancer cells undergoing apoptosis in the breast cancer tissue of Sprague Dawley rats. *Cayratia trifolia* ethanol extract is expected to be used as a base for the development of breast cancer therapy.

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