



The Effectivity of Supplementation *Artemisia vulgaris* for Adenocarcinoma Mammae Chemotherapy to Reduce CD 34 and Tumor Massa Diameter (Study in C3H Mice Given Adriamycin - Cyclophosphamide Chemotherapy)

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ABSTRACT

Introduction. Breast cancer is still a major health problem in the world. In the case of breast cancer, surgery is the main treatment option besides chemotherapy, radiation, and immunotherapy such as Artemisia vulgaris (AV). AV is cytotoxic selectively acts as a supplement to breast adenocarcinoma chemotherapy given the Adriamycin-Cyclophosphamide regimen, to improve chemotherapy response. The study was aimed to proving AV extract enhances the chemotherapy response in C3H mice with adenocarcinoma mammae given Adriamycin-Cyclophosphamide Chemotherapy. **Method:** This study used Post test only control group design on 24 females C3H mice that were randomly selected and divided into four groups: group K (control), P1 (chemotherapy), P2 (extract), and P3 (combination). Adenocarcinoma mammae comes from the inoculation of donor mice. Chemotherapy of Adriamycin 60 mg / m² and Cyclophosphamide 600 mg / m² were given in two cycles. AV 13 mg (0.2 ml) was given once daily orally. CD34 were evaluated by immunohistochemical staining and tumor mass diameter were counted by calipers. **Result:** The microvascular density CD34 and tumor mass diameter were obtained in groups of K, P1, P2, P3 respectively 60.76 ± 1.5; 39.70 ± 2.00; 57.10 ± 1.29; 35.26 ± 2.06 and 12.52 ± 1.49; 6.20 ± 1.04; 9.94 ± 1.21; 3.94 ± 0.76. Statistical analysis showed significant differences in CD34 between groups K vs P1, P2, P3 (p = 0.001, p = 0.014, p = 0.001), P1 vs P2 and P3 (p = 0.001, p = 0.033) and P2 (P = 0.001). Tumor mass diameter between groups K vs P1, P2, P3 (p=0.001; p=0.014; p=0,001), P1 with P2 (p= 0.001) P1 with P3 (p = 0.033) and P2 with P3 (p = 0.001). Correlation analysis between CD34 with tumor mass diameter was found to have significant correlation (p = 0.001 and r = 0.932). **Conclusion:** Artemisia vulgaris is a potential to reduce angiogenesis in terms of decreasing the microvascular density CD34 and tumor mass diameter of adenocarcinoma mammae of C3H mice treated with Adriamycin-Cyclophosphamide chemotherapy and can improve the effectivity.

1. Introduction

Cancer is one of the leading causes of death worldwide. Data from the International Agency for Research on Cancer (IARC) GLOBOCAN in 2012 noted that 1.7 million women were diagnosed with breast cancer or about 11.9% of all cancer incidence. WHO itself noted that the prevalence rate of breast cancer

reached 6.3 million worldwide at the end of 2012.⁽¹⁾ The highest incidence of breast cancer is in the age group of more than 50 years, and the estimated incidence rate is around 2 among 1000 women per year.^(2,3)

Cancer is basically not known with certainty, but it can be understood that this cancer is caused by

malfunctioning in the cells in controlling cell growth. Cancer in general can be caused by disruption of the transcription process at the cellular level which results in uncontrolled cell division. In this case Nuclear Factor-Kappa B (NF- κ B) has an important role in regulating regulation, including processes from inflammatory reactions, growth, formation of vascularity to oncogenesis.^(4,5)

Angiogenesis factor plays an important role in the growth of cancer cells, progression and metastasis. The microvascular tissue complex of cancer guarantees an adequate supply of tumor cells with nutrients, oxygen and good drainage of metabolites.^(10,11) The process of forming new blood vessels can be identified as a clinical parameter of microvascular density through the expression of glycosylated transmembrane proteins namely Capillary protein. Density (CD34) with a molecular weight of 116 kDa. The CD34 protein can differentiate hematopoietic cells from endothelial cells and lymphatic cells.⁽¹²⁾

The angiogenesis process involves NF- κ B which is an important angiogenic growth factor that stimulates cancer cells to grow and causes metastasis of the tumor.⁽¹³⁾ NF- κ B and VEGF are the main growth factors in the blood vessels around the tumor and can cause metastasis.⁽¹⁴⁾ related to providing the nutrients needed for tumor growth, invasion and metastasis.⁽¹⁵⁾ The process of angiogenesis in tumors begins with the formation of capillary endothelial cells and this process is not found in normal cells. NF- κ B can be an independent prognostic marker, because these vascular endothelial cells in tumor cells are more stable than normal cells, it could be a promising therapeutic target in new strategies for cancer therapy.⁽¹⁶⁾

In the process of breast cancer management, surgery is the main therapeutic modality. Other modalities include adjuvant therapy in the form of radiation and chemotherapy, especially if the resection is inadequate or there is metastasis. Some chemotherapy regimens commonly used for breast cancer are CAF / CEF (Cyclophosphamide, Adriamycin / Epirubicin, and 5-Fluorouracil), CMF (Cyclophosphamide, methotrexate and 5-Fluorouracil),

E-CMF (a combination of Epirubicin with CMF), MMM (Methotrexate mitozantrone, mitomycin). The response rate of each of the CAF regimens for all new therapies ranges from 20-40%, but until now there has been no therapy that has achieved a 100% response.⁽³⁾ Efforts should be made to increase the effectiveness of therapy so that the response rate is needed. be increased so that the survival rate can be increased.

Many studies have been conducted in order to find effective and efficient solutions in the treatment of breast cancer patients. The *Artemisia vulgaris* plant contains artemisinin compounds which are known to have anti-cancer properties. Artemisinin, was isolated and extracted from the dried leaves and flower buds of *Artemisia vulgaris*. Artemisinin is a sesquiterpene lactone compound that contains an endoperoxide radical without containing nitrogen atoms in its chemical structure.^(17,18) Previous research was carried out on C3H mice with liver carcinoma, at a dose of 100 mg / kg per day of artemisinin showing anticancer activity⁽¹⁹⁾. Artemisinin contains an endoperoxide moiety which can react with iron to form free radicals that are cytotoxic.^(20,21)

This study was conducted to determine the effectiveness of *Artemisia vulgaris* extract as supplementation against mammary adenocarcinoma in terms of CD34 microvascular density and tumor mass diameter in C3H mice receiving Adriamycin-Cyclophosphamide chemotherapy.

2. Method

Research design

This research is a laboratory experimental study with the design "Post test only control group design". The research subjects were divided into 4 groups, namely the group (1) control, tumor inoculated mice; (2) P1, the tumor inoculated mice, after the tumor arose, received chemotherapy Adriamycin - Cyclophosphamide; (3) P2, the tumor inoculated mice, after the tumor arose, they received *Artemisia vulgaris* extract 13 mg / times per day; P3, the mice were inoculated with tumors. After the tumor arose, they received AC chemotherapy and *Artemisia vulgaris* 13

mg / times per day.

Research samples

The experimental animal was the C3H strain mice (*Mus musculus*). Inclusion criteria: Female mice aged 8 weeks, inoculated with mammary adenocarcinoma, body weight 20-30 grams after acclimatization, no anatomical abnormalities were seen. Exclusion criteria: no tumor growth after inoculation, during inoculation and treatment the mice looked sick (movement was not active). The sample size according to WHO for each group is at least five animals with a reserve of 10%, in this study the number of samples used per group was six mice.⁽¹¹⁾

Time and location of research

Research and data collection were carried out for 5 months. The extract of *Artemisia vulgaris* was made at LPPT I, Faculty of Medicine, Gajah Mada University. The treatment of mice and the process of taking the tissue was carried out at LPPT IV, Faculty of Medicine, Gajah Mada University. The process of making paraffin blocks, HE staining and immunohistochemical staining were carried out at the Anatomical Pathology Laboratory, Faculty of Medicine, Sebelas Maret University, Surakarta.

Operational definition

Administration of *Artemisia vulgaris*: *Artemisia vulgaris* extract comes from the leaves extracted with ethanol solvent using the soxhletation method, with a dose of *Artemisia vulgaris* 100mg / kgBW / day orally (13mg / times). Administration of Adriamycin intravenously at a dose of 0.18 mg / time. Administration of Cyclophosphamide is intravenously 1.8 mg / time.

The microvascular density of CD34 tumors was a light brown color that appeared on the tumor capillaries after CD34 staining. The way to do the calculation is through the number of micro blood vessels per field of view which is calculated on five fields of view using 400x magnification.

The diameter of the tumor mass is used to show tumor growth. The diameter of the tumor mass was calculated by reducing the diameter of the tumor mass after and before treatment. The diameter of the tumor mass was measured using a tumor caliper tool, with an accuracy of 10^{-1} mm and measured in the largest one-dimensional tumor diameter, in millimeters (mm).

Materials and tools of research

During the experiment, the experimental animals were placed in cages and given food and drink ad libitum. Mice underwent an adaptation period of one week before treatment

Tumors in donor C3H mice will be inoculated in experimental animals for research and histopathological examination. *Simplisia Artemisia vulgaris* was obtained from the Biopharmaca Cultivation Conservation Unit, Center for Biopharmaca Studies, Bogor Agricultural University. The material used is *Artemisia vulgaris* extract, which is obtained by:⁽¹²⁾

- a. One kg of dried leaves of *Artemisia vulgaris* is finely ground, then the resulting powder is put into a soklet device (capacity of 50 mg) and the extraction is carried out by soaking using ethanol solvent with a cycle of 8-10 times.
- b. The extract was put into a rotary evaporator flask and vacuum distillation was carried out until it became concentrated (temperature 40°C).
- c. The extract was dried in an oven at 40°C for 1 hour to evaporate the ethanol.
- d. The results obtained were 5.5 mg of extract for every 1 kg of material (0.55%) and the results were diluted with aquabidest until a concentration of 0.2 mg / ml was reached.

Tumors containing adenocarcinoma cells from donor C3H mice were transplanted into recipient mice. The tumor from donor mice was then incised with a biopsy and a histopathological examination was performed to confirm the type of tumor.

Data analysis

After all data has been collected, data cleaning, coding and data tabulation are carried out. The research data is processed and presented in the form of tables and box plots to see the distribution of data. The data collected was tested for normality using the Shapiro-Wilk test. If the data is normally distributed, it is followed by the ANOVA test to see if there is a difference in CD34 and the diameter of the tumor mass in the four groups. The magnitude of the differences in each treatment group was further analyzed by using the Post Hoc Test. The correlation test between the CD34 difference variable and the tumor mass diameter obtained a normal distribution tested by the Pearson's correlation test. The degree of significance limit was $p \leq 0.05$ with a 95% confidence interval. Data analysis was performed with SPSS Ver software. 21.0. for Windows.

Ethical requirements

This research always applies animal ethics. Before the research was carried out, the study had received approval from the Ethics Committee for Health Research, Faculty of Medicine, Diponegoro University.

3. Results

Descriptive analysis

CD34 data description

Each group K, P1, P2, P3 was taken as a sample and preparations were made to determine the microvascular density of the tumor by staining CD34. The results of measuring the average microvascular density value of the tumor can be seen in Table 1.

The highest CD34 average was found in the control group, namely $60.76 \pm 1.53\%$, while the lowest average CD34 was found in the P3 group, namely $35.26 + 2.06\%$. Likewise, the highest and lowest medians were found in the control group and the P3 group, namely 61.50% and 35.60% , respectively. In the P1 and P3 groups with a population of C3H mice who received AC chemotherapy, the average was $39.70 + 2.00\%$ and $35.26 + 2.06\%$, respectively.

Tumor mass diameter data description

Measurement of tumor mass diameter growth in group K (untreated), P1 (chemotherapy), P2 (extract) and P3 (combination) obtained the following results:

The box plot as shown in Figure 2 below shows the median growth of tumor mass diameter in the P3 group is lower than that of the P1, P2 and K groups.

The test for normality and homogeneity of CD34 microvascular density and tumor mass diameter for each group used the Shapiro-Wilk test. The normality and homogeneity test of groups K, P1, P2, P3 showed a value of $p > 0.05$. These results indicate that each group is normally distributed and the data is homogeneous.

Microvascular density CD 34.

The Shapiro-Wilk test showed that the CD34 data were normal and homogeneous, so it was continued with the One Way ANOVA statistical test, with the following results:

From the results of the One Way ANOVA test, it was found that the value of $p = 0.000$, because $p < 0.05$, it can be concluded that there is a significant difference in CD34 in the four groups. Henceforth, the Post Hoc test was used to determine the differences between groups. From the results of the Post Hoc test, it was found that there were significant differences between each group with a value of $p < 0.05$.

The diameter of the tumor mass. The Shapiro-Wilk test found that the tumor mass diameter data were normal and homogeneous, so that the One Way ANOVA difference test was continued.

The results of statistical tests using One Way ANOVA showed a significant difference in tumor mass diameter between groups ($p = 0.001$) so that it was followed by a post hoc test using Bonferroni with a significance value of $p < 0.05$. The results of the post hoc test showed a significant difference between group K and group P1, P2, P3 ($p = 0.001$; $p = 0.044$; $p = 0.001$), group P1 with groups P2 and P3 ($p = 0.001$; $p = 0.033$) and group P2 with P3 ($p = 0.001$).

The relationship between CD34 and the diameter of the tumor mass

Assessment of the association between CD34 and tumor mass diameter was performed using the Pearsons parametric correlation test. The Pearsons test

revealed a very strong association between CD34 and tumor mass diameter ($p = 0.001$ and $r = 0.932$). Because the p value < 0.05 , it was concluded that there was a significant relationship between CD34 and tumor mass diameter, which was positive.

Table 1. Characteristics of CD34 data

Group	N	Min (%)	Max (%)	Mean \pm SD (%)	Median (%)
K	5	58.70	62.30	60.76 \pm 1.53	61.50
P1	5	36.70	41.60	39.70 \pm 2.00	39.70
P2	5	55.50	58.40	57.10 \pm 1.29	57.20
P3	5	32.40	37.70	35.26 \pm 2.06	35.60

Table 2. Characteristics of tumor mass diameter data

Group	N	Mean \pm SD (%)	Median (%)	Min (%)	Max (%)
K	5	12.52 \pm 1.49	12.00	11.10	14.80
P1	5	6.20 \pm 1.04	6.20	4.90	7.50
P2	5	9.94 \pm 1.21	10.10	8.10	11.20
P3	5	3.94 \pm 0.76	4.00	2.90	4.80

* The values in the table are the data on the growth of the tumor mass diameter in millimeters.

Table 3. Analysis of CD34 differences between treatment groups

Group	Microvascular density (mean \pm SD)	P
K	60.76 \pm 1.53	0.001*
P1	39.40 \pm 2.00	
P2	57.10 \pm 1.29	
P3	35.26 \pm 2.06	

* Tested with One Way ANOVA (significant $p < 0.05$)

Table 4. Post hoc analysis of CD34 microvascular density

Group	P1	P2	P3
K	0.001*	0.044	0.001*
P1	-	0.001	0.001
P2		-	0.001*

* Tested with Bonferroni (significant $p < 0.05$)

Table 5. Analysis of differences in tumor mass diameter between treatment groups

Group	Tumor mass diameter (%) (mean \pm SD)	P
K	12.52 \pm 1.49	0.001*

P1	6.20 ± 1.04
P2	9.94 ± 1.21
P3	3.94 ± 0.76

* Tested with One Way ANOVA (significant p <0.01)

Table 6. Post Hoc analysis of tumor mass diameter growth between groups

Group	P1	P2	P3
K	0.001	0.014	0.001
P1	-	0.001	0.033
P2		-	0.001

Table 7. Pearsons correlation test results

Variable	p	r
CD34		
The diameter of the tumor mass	0.001	0.932

* Tested with Bonferroni (significant p <0.05)

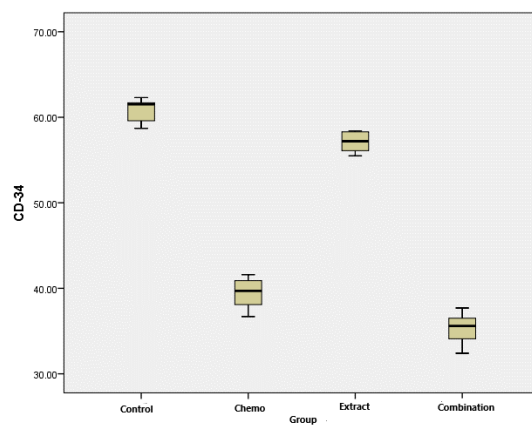


Figure 1. CD34 box plot graph

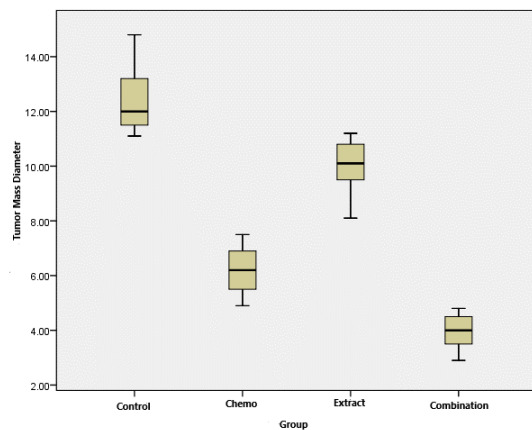


Figure 2. Box plot graph of tumor mass diameter

4. Discussion

This study found the expression of NF- κ B in C3H mice with mammary adenocarcinoma who received AC chemotherapy combined with *Artemisia vulgaris* extract was lower than that which was not combined. Thus, the first hypothesis is accepted. The Post Hoc test showed a significant difference in the comparison between the P1 and P3 groups ($p < 0.05$). Data analysis of the CD34 microvascular density variable data was found to be lower in the group (P3) than in the group (P1). Based on these data, it can be concluded that the effect of *Artemisia vulgaris* extract can reduce CD34 and can have a synergistic therapeutic effect on the administration of AC chemotherapy.

The benefits of *Artemisia vulgaris* extract as an anticancer are caused by the activity of artemisinin compounds. This potential activity is related to the endoperoxide bonding of the artemisinin compound. The peroxide bridge from artemisinin will react with ferrous ions from tumor cells which will produce free radicals or ROS which will induce oxidative DNA cell damage resulting in apoptosis. (23,24) This cell DNA damage is evidenced by decreased expression of NF- κ B which is a protein complex and acts as an important regulator of immune response, differentiation, cell proliferation, anti-apoptosis and angiogenesis. (25) The reduced expression of NF- κ B will cause a decrease in cellular activity. This process is caused by inhibited NF- κ B translocation activity to the cell nucleus, then the recruitment of other proteins (coactivators and RNA polymerases) will also be inhibited. (26)

Polyphenol compounds in medicinal plants have the ability to inhibit NF- κ B. (30) Polyphenol compounds work to inhibit kinases by preventing phosphorylation or ubiquitination which causes degradation of I κ B. This process further inhibits the translocation process of NF- κ B to the cell nucleus. (31) As a result, there is inhibition of NF- κ B in the NF- κ B-DNA complex. (32)

Post Hoc test on a population of C3H mice with mammary adenocarcinoma receiving AC chemotherapy, namely groups P1 and P3 obtained $p < 0.05$. These results indicate that there is a significant difference in the comparison between the P1 and P3

groups. By using the Pearson's correlation analysis between CD 34 and tumor mass diameter in the P1 and P3 groups, the correlation coefficient $r = 0.932$ with $p < 0.05$. There is a very strong association between CD34 and tumor mass diameter in C3H mice with mammary adenocarcinoma given combination chemotherapy Adriamycin-Cyclophosphamide and *Artemisia vulgaris* extract. Thus, the third hypothesis is accepted.

Other studies suggest that artemisinin compounds and their derivatives can inhibit angiogenesis, through inhibition of cell proliferation, migration, formation of tube formation in endothelial cells. (38) The inhibition process which reduces the microvascular density of tumors is triggered by inhibition of the NF- κ B pathway which causes downregulation of VEGFR2. VEGF and VEGFR2 are major regulators of the angiogenesis process. The blockade of the NF- κ B pathway by artemisinin against the VEGFR2 promoter will inactivate transcription activity, so that proteins that play a role in angiogenesis are not formed. (39) As a result, the growth in the diameter of the tumor mass decreases.

CD34 and tumor mass diameter growth has a very strong relationship. NF- κ B is a protein that functions to regulate the formation of blood vessels when they translocate into the cell nucleus. The transcription factor NF- κ B is activated when there are proinflammatory cytokines including TNF- α which will activate NF κ B, causing VEGF production and VEGFR expression to increase with clinical parameters in the form of increased CD34 microvascular density (25,40,41). This angiogenesis process can be assessed through clinical parameters in the form of microvascular density, an increase in angiogenesis activity will show an increase in microvascular density which has an impact on tumor size growth.

The results showed that the administration of *Artemisia vulgaris* extract had a synergistic effect on the administration of AC chemotherapy in a population of C3H adenocarcinoma mammae mice. This suggests that *Artemisia vulgaris* can be given as a supplement to AC chemotherapy.

5. Conclusion

Artemisia vulgaris extract has a synergistic effect by administering Adriamycin-Cyclophosphamide in increasing chemotherapy response by reducing CD34 microvascular density and decreasing the diameter of tumor mass in C3H mice with adenocarcinoma mammae.

6. Conflict of interest and funding.

The author does not receive funding or profit from industry or other places for conducting this research.

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