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Effect of *Artemisia vulgaris* Extract on VEGF Expression, CD34, Microvascular Density, and Diameter of Mammary Adenocarcinoma Tumors: In vivo Study

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ABSTRACT

Background. Breast cancer in Indonesia still has a high incidence. Surgery remains the main choice with other modalities such as chemotherapy, radiation, and immunotherapy such as *Artemisia vulgaris* (AV). This study aimed to prove that administration of AV extract reduced VEGF expression, CD34 and microvascular density (MVD), and tumor diameter in mammary adenocarcinoma. **Methods:** This study used a "Posttest only control group design" design for 24 female C3H mice which were selected randomly and divided into four groups, namely: group K (control), P1 (chemotherapy), P2 (extract), and P3. (combination). Mammary adenocarcinoma was derived from the inoculation of donor mice. AC chemotherapy (Adriamycin 0.18 mg and Cyclophosphamide 1.8 mg) was given in 2 cycles. AV is given at a dose of 13 mg (0.2 ml) once a day orally. VEGF and CD34 expression were assessed by immunohistochemical staining whereas MVD was assessed by the hematoxylin-eosin stain. Tumor diameter was measured using tumor calipers. **Results:** There was a significant relationship between CD34 expression and tumor diameter ($p<0.001$; $r=0.927$). There was a significant relationship between CD34 expression and MVD features ($p<0.001$; $r=0.906$). There was a significant relationship between CD34 and VEGF expression ($p<0.001$; $r=0.986$). There was a significant relationship between tumor diameter and VEGF expression ($p<0.001$; $r=0.903$). There was a significant relationship between tumor diameter and the appearance of MVD ($p<0.001$; $r=0.882$). There was a significant relationship between VEGF expression and MVD features ($p<0.001$; $r=0.893$). **Conclusion:** AV extract gave a higher response to chemotherapy in mammary adenocarcinoma of C3H mice given AC. a chemotherapy regimen ($p=<0.05$).

1. Introduction

Breast cancer is a malignancy that ranks highest in the number of new cancer cases worldwide based on GLOBOCAN (Global Cancer Incidence, Mortality, and Prevalence). Female breast cancer causes high morbidity and mortality rates, based on GLOBOCAN data in 2020 it is estimated that around 2.3 million new cases of female breast cancer each year globally and the 5th cause of death due to cancer with a mortality rate of 685,000 deaths annually. Based on GLOBOCAN data in 2018, approximately 291 out of 100,000 people in Indonesia suffer from cancer.[1] The estimated incidence of breast cancer in Indonesia based on IARC data in 2012 is 40.3 per 100,000 women with a mortality of 16.6 per 100,000 women.[2]

Vascular Endothelial Growth Factor (VEGF) induces endothelial cell mitosis and promotes fusion of surrounding blood vessels into a plexus and the formation of neovascularization. The VEGF/NRP-1 (neuropilin-1) axis can facilitate the progression of breast cancer formation through the facilitation of the epithelial-mesenchymal transition (EMT). The expression level of VEGF is influenced by internal and external factors. Cell internal factors that cause VEGF formation are activation of oncogenes, inactivation of tumor suppressor genes, as well as growth factors and hormones. Meanwhile, hypoxia and hypoglycemia are the main external factors in stimulating increased VEGF expression in tumor cells.[3,4] The process of new blood vessel formation can be identified as a clinical parameter of microvascular density through the expression of a glycosylated transmembrane protein, *Capillarity Density* (CD34) protein with a molecular weight of 116 kDa. [4]

The main treatment modality for breast cancer is surgery. In certain breast cases where adequate resection cannot be performed and there is metastasis to the surrounding lymph nodes, other modalities may be given in the form of adjuvant therapy, the form of radiation, and chemotherapy. Several chemotherapy regimens commonly used for breast malignancy 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, Mitozantron, Mitomycin*). The administration of each such as the CAF regimen. *The response rate* of all new therapies ranges from 20-40%, but so far no therapy can achieve a 100% response.[5,6] Efforts should be made to increase the effectiveness of therapy so that the *response rate* increases so that the survival rate can be increased.

Artemisinin is a promising medicinal molecule extracted from the flowers and leaves of *Artemisia vulgaris* with minimal side effects. The World Health Organization (WHO) has approved this drug as an antimalarial therapy. Based on recent research, it is known that artemisinin and its derivatives can function as a preventive therapy for ovarian cancer growth and metastasis, breast cancer, and other types of cancer. Artemisinin and its derivatives work in various ways, including; endocytosis and uptake of plasma Fe carrier proteins, stopping the multiplication of cancer cells, stopping the cell cycle, inducing apoptosis, inhibiting tumor angiogenesis, inducing autophagy and ferroptosis, as well as altering cell metabolism.[7,8] Previous studies conducted on mice with liver carcinoma, at a dose of 100 mg/kg per day of artemisinin showed anticancer activity.[9] it was found that the inhibitory effect of artesunate on the angiogenesis ability of cancer cells was dose-dependent in the range of 0.5-50 mol/L with signs of decreased VEGF expression, CD31 expression, and microvascular density.[10]

It is known that artemisinin contains an endoperoxide moety which can react with iron to form cytotoxic free radicals.[7,8] Artemisinin has the advantage that it can be used as an anticancer because it has selective toxic properties.[7,8] This is an important consideration in terms of security for its users. The selective cytotoxicity of *Artemisia vulgaris* research is preliminary at the preclinical stage. This study was conducted to determine the effect of *Artemisia vulgaris* at a dose of 13 mg/times per day, and chemotherapy doses of *Adriamycin* and *Cyclophosphamide* respectively 0.18 mg/times and 1.8 mg/times intravenously as adjuvant chemotherapy for mammary adenocarcinoma in terms of VEGF expression, tumor microvascular density, CD34 expression, tumor diameter performed on C3H mice.

2. Methods

The design of this is experimental research with a post-test-only approach with a control group design. Subjects research were C3H mice (Mus musculus), where the research was conducted at LPPT IV (Integrated Research and Testing Institute IV) Gadjah Mada University Yogyakarta and Anatomical Pathology Laboratory, Faculty of Medicine, Gadjah Mada University. A total of 24 mice (inclusion criteria of four weeks old female mice, strain C3H, bodyweight 20-30 grams after acclimatization, no visible anatomic abnormalities) were included in this study. Mice were

grouped into 4 groups (@6 mice), namely K (control group, mice induced by carcinogens), P1 (treatment group 1, mice induced by carcinogens, after the tumor developed, received chemotherapy *Adriamycin - Cyclophosphamide*), P2 (treatment group extract *Artemisia vulgaris*100 mg/kg/day), P3 (Treatment group 3, mice induced by carcinogens, after tumor emergence received AC chemotherapy and *Artemisia vulgaris*100 mg/kg/day). During the experiment, the experimental animals were placed in cages and given food and drink ad libitum. Before treatment, mice underwent an adaptation period of one week. Induction of mammary adenocarcinoma used *7,12-dimethylbenz(a)anthracene* (DMBA), which was dissolved in acetone reagent, and *12-O-tetradecanoylphorbol-13-acetate* (TPA) which was also dissolved in acetone.

Artemisia vulgaris leaves were obtained from the Biopharmaceutical Cultivation Conservation Unit, Center for Biopharmaceutical Studies, Bogor Agricultural University. One kilogram of dry leaves of *Artemisia vulgaris* was finely ground, then the powder was put into a soxhlet apparatus (capacity 50 mg) and extracted by soxhletation using ethanol solvent with a cycle of 8-10 times. The extract was put into a *rotary evaporator* and vacuum distillation was carried out until it became concentrated (temperature 40°C). The extract was dried in an oven at 40°C for 1 hour to evaporate the ethanol. The yield was 5.5 mg of extract for every 1 kg of material (0.55%) and the result was diluted with aquabidest until a concentration of 0.2 mg/ml was reached.

Adriamycin is administered intravenously at a dose of 60 mg/m^2 of body surface area (/BSA mice 0.03m2= 1.8 mg/time). Administration of Cyclophosphamide drug Cyclophosphamide given intravenously at a dose of 600 mg/m^2 body surface area (BSA mice 0.03 m2= 18 mg/time). VEGF expression was calculated on preparations that had been stained by immunohistochemistry, semiquantitatively counted cells expressing VEGF in one field of view observed by a pathologist and researcher who was accompanied by pathology with clinical agreement at least 95%. CD34 expression was calculated on preparations that had been stained with immunohistochemistry, semiquantitatively counted cells expressing CD34 in one field of view observed by a pathologist and researcher accompanied by a pathologist with a clinical agreement of at least 95%. Tumor microvascular density, assessed by the light brown color appearing in the tumor capillaries after hematoxylin-eosin staining. The method of calculating is through the number of capillaries per visual field which is calculated in five fields of view using a magnification of 400x. Mice were terminated and the tumor resected. The diameter of the tumor was then measured using a tumor caliper and the size was written down to the last two decimal places.

Data analysis was carried out using SPSS version 25 software where univariate analysis was carried out to determine the distribution and mean of the assessed variables and bivariate analysis (one-way ANOVA test) and multivariate analysis were carried out to compare the average levels of test variables (Post hoc bonferroni).

3. Results

Table 1. Analysis of differences in CD-34 expression between treatment groups

Groups	CD-34 levels (Mean ± SD)	P
K	60,76 ± 1,53	0.000*
P1	39,40 ± 2,01	
P2	57,10 ± 1,29	
P3	35,26 ± 2,07	

* *One Way ANOVA* (significant p < 0.05)

From the results of the One Way ANOVA test, p-value < 0.001 because p < 0.05, it can be concluded that there is a significant difference in CD-34 levels in the four groups. For further use the Post Hoc test to determine the differences between variables.

Table 2. Post hoc analysis of CD-34 levels between treatment groups

Groups	K	P1	P2	P3
K	-	0.000	0.027	0.000
P1	0.000	-	0.000	0.011
P2	0.027	0.000	-	0.000
P3	0.000	0.011	0.000	-

* Post Hoc *Bonferroni* (significant p < 0.05)

From the results, Post Hoc test found a significant difference between the control group with P1, P2, and P3. There was a significant difference between control and P1 (p=0.000), control with P2 (p=0.027), K and P3 (p=0.000), P1 and P2 (p=0.000), between P1 and P3 (p=0.011), and between P2 and P3 (p=0.000).

Table 3. Analysis of differences in diameter between treatment groups

Groups	VEGF Expression (%) (Mean ± SD)	P
K	12.52 ± 1.50	0.000*
P1	6.20 ± 1.04	
P2	9.94 ± 1.22	
P3	3, 94 ± 0.76	

* *One Way ANOVA* (significant p < 0.05)

From the results of the One Way ANOVA test, p-value < 0.001 because p < 0.05, it can be concluded that there is a significant difference in the diameter of the tumor mass in the four groups. For further use the Post Hoc test to determine the differences between variables.

Table 4. Post hoc analysis of tumor mass diameter between treatment groups

Groups	K	P1	P2	P3
K	-	0.000	0.017	0.000
P1	0.000	-	0.001	0.044
P2	0.017	0.001	-	0.000
P3	0.000	0.044	0.000	-

* Post Hoc *Bonferroni* (significant p < 0.05)

From the test results Post Hoc, there were significant differences between the control group and P1, P2, and P3. There were significant differences between control and P1 (p=0.000), control with P2 (p=0.017), K and P3 (p=0.000), P1 and P2 (p=0.001), between P1 and P3 (p=0.044), and between P2 and P3 (p=0.000).

Table 5. Analysis of differences in VEGF expression between treatment groups

Groups	VEGF expression (%) (Mean ± SD)	P
K	65,33 ± 1,77	0.000*
P1	38,24 ± 1,49	
P2	61,38 ± 2,12	
P3	33,41 ± 1,27	

* *One Way ANOVA* (significant p < 0.05)

From the results of the One Way ANOVA test, p-value < 0.001 because p < 0.05, it can be concluded that there are significant differences in VEGF expression in the four groups. For further use the Post Hoc test to determine the differences between variables.

Table 6. Post hoc analysis of VEGF expression between treatment groups

Groups	K	P1	P2	P3
K	-	0.000	0.012	0.000
P1	0.000	-	0.000	0.002
P2	0.012	0.000	-	0.000
P3	0.000	0.002	0.000	-

* Post Hoc Bonferroni (significant p < 0.05)

From the Post Hoc test results, there was a significant difference between the control group and P1, P2, and P3. There were significant differences between control and P1 (p=0.000), control with P2 (p=0.012), K and P3 (p=0.000), P1 and P2 (p=0.000), between P1 and P3 (p=0.002), and between P2 and P3 (p=0.000).

Table 7. Analysis of differences in microvascular density between treatment

Groups	Overview of microvascular density Mean ± SD	P
K	10.72 ± 1.76	0.000*
P1	5.33 ± 1.03	
P2	8.34 ± 1.16	
P3	2.22 ± 0.79	

* One Way ANOVA (significant p < 0.05)

From the results of the One Way ANOVA test, p-value < 0.001, because p < 0.05, it can be concluded that there is a significant difference in the value of the microvascular density in the four variables. For further use the Post Hoc test to determine the differences between variables.

Table 8. Post hoc analysis of microvascular density between treatment groups

Groups	K	P1	P2	P3
K	-	0.000	0.047	0.000
P1	0.000	-	0.009	0.007
P2	0.047	0.009	-	0.000
P3	0.000	0.007	0.000	-

* Post Hoc Bonferroni (significant p < 0.05)

From the test results Post Hoc there were significant differences between the control group and P1 (p = 0.000), the control group and P2 (p = 0.047), K and P3 (p = 0.000), P1 and P2 (p = 0.009), between P1 and P3 (p = 0.007), and between P2 and P3 (p = 0.000).

A correlation test between CD-34 level, a diameter of tumor mass, VEGF expression, and microvascular density was performed in the combination group. The data normality test for both variables was carried out using the Shapiro-Wilk test. From this test obtained normal data (p > 0.05) so the correlation analysis was continued using the Pearsons test.

Table 9. Pearsons correlation test results for CD-34 levels, the diameter of tumor mass VEGF expression, and overview of microvascular density

Variables	P	R
CD-34 levels – Tumor mass diameter	0.000	0.927
CD34 levels – Overview of microvascular density	0.000	0.906
CD34 Level – VEGF Expression	0.000	0.986
Tumor Mass Diameter - Overview of microvascular density	0.000	0.882
Tumor Mass Diameter - VEGF Expression	0.000	0.903
VEGF Expression - Overview of microvascular density	0.000	0.893

From table 9 *Pearsons* found that the correlation between CD34 levels and tumor mass diameter was p < 0.001 and r = 0.927. Because the p-value <0.05, it can be concluded that there is a significant relationship between CD-34 levels and the diameter of the tumor mass. The correlation between CD34 levels and microvascular density p < 0.001 and r = 0.906. Because the p-value < 0.05, it can be concluded that there is a significant relationship between CD34 levels and microvascular density. The correlation between CD34 levels and VEGF expression values p < 0.001 and r = 0.986. Because the p-value <0.05, it can be concluded that there is a significant relationship between CD34 levels and VEGF expression. The relationship between tumor mass diameter and VEGF expression values p < 0.001 and r = 0.903. Because the p-value <0.05, it can be concluded that there is a significant relationship between tumor mass diameter and VEGF expression. The relationship between tumor mass diameter and microvascular density p < 0.001 and r = 0.882. Because the p-value < 0.05, it can be concluded that there is a significant relationship between the diameter of the

tumor mass and the description of microvascular density. The relationship between VEGF levels and microvascular density *was* $p < 0.001$ and $r = 0.893$. Because $p < 0.05$, it can be concluded that there is a significant relationship between VEGF levels and microvascular density.

4. Discussion

Variable VEGF expression using *one-way ANOVA*, there is a significant difference between groups ($p = 0.000$).test post hoc, there were significant differences between the control group and treatment 1 ($p=0.000$), 2 ($p=0.012$) and 3 ($p=0.000$). This is following the theory of increased VEGF expression which plays a role in the angiogenesis process of tumor growth pathogens, wherein the control group there was no intervention, causing the angiogenesis process to continue and manifest in increased levels of VEGF.[51] In treatment group 1 who received *Adriamycin-Cyclophosphamide*, the decrease in VEGF levels could be associated with its function as a chemotherapeutic agent for anticancer drugs. results ($p=0.012$) were also found in treatment group 2 even though the mean and mean values showed a higher value (61.38 ± 2.12) than groups P1 (38.24 ± 1.49) and P3 ($33,41 \pm 1.27$). The decrease in VEGF levels in treatment group 2 receiving *Artemisia vulgaris* is supported by the theory of *Artemisia vulgaris* which contains artemisinin as an inhibitor of angiogenesis in tumors.[11-14] The decrease in VEGF levels in treatment group 3 who received chemotherapy and *Artemisia vulgaris* indicated a synergism of action between chemotherapy and the immunogenic effect of *Artemisia vulgaris*.

Variable CD34 using *one-way ANOVA*, there is a significant difference between groups ($p = 0.000$). test *post hoc*, there was a significant difference between the control group and treatment 1 ($p=0.000$), treatment 2 ($p=0.027$), and treatment 3 ($p=0.000$). This shows that the effects of chemotherapy and *Artemisia vulgaris* can reduce the CD34 marker which indicates a decrease in the formation of new micro blood vessels in the pathogenic process of tumor angiogenesis.[15-18]

In the microvascular density variable, significant results were obtained ($p=0.000$) between the control group and the P1 treatment group who received chemotherapy. The combination chemotherapy agent Adriamycin-Cyclophosphamide has been widely used for the treatment of cancer through its complex mechanisms such as lowering antioxidant levels, as antimitotic agents specifically targeting cells with high proliferation rates, and lowering the rate of angiogenesis, thereby reducing the microvascular density of tumors.[19-22]

The results were significant in treatment group 2 ($p = 0.047$) which was only given *Artemisia vulgaris*. This is in line with research conducted by abdolmaleki et al, where artemisinin compounds and their derivatives contained in *Artemisia vulgaris* have been shown to have anti-angiogenic effects so that they can inhibit the proliferation and growth of microvascular density.[23-25] In group P3 where chemotherapy and *Artemisia vulgaris* extract was given, there was a significant increase in microvascular density compared to control ($p=0.000$), a significant increase compared to P1 ($p=0.047$), a significant increase compared to P2 ($p=0.000$). This shows that the combination of Adriamycin-Cyclophosphamide extract *Artemisia vulgaris* has a synergistic effect on microvascular density in tumors although the effect of chemotherapy is more dominant.

The variable diameter of the tumor mass showed significant results between the control group with treatment 1 ($p=0.000$), treatment 2 ($p=0.017$), and treatment 3 ($p=0.000$). This shows that the combination of Adriamycin-Cyclophosphamide extract *Artemisia vulgaris* has a synergistic effect on reducing the diameter of the tumor mass although the effect of chemotherapy is more dominant. The use of chemotherapeutic agents, especially *Adriamycin-Cyclophosphamide,* has become an adjuvant therapy that has been shown to reduce the risk of recurrence and mortality in breast malignancy through its complex mechanism, although it can cause reversible and irreversible.[26-28]

In the correlation analysis between VEGF and CD34 expression variables on microvascular density and tumor mass diameter, significant results were obtained ($p<0.05$). This is consistent with the process of

angiogenesis in breast tumors where the growth of new blood vessels is induced so that there is a progressive increase in microvascular density and an increase in the diameter of the tumor mass, which is indicated by an increase in the expression of VEGF and CD34.[29-31] This angiogenesis has an important role in controlling tumor progression because tumor growth, invasion, and metastasis depend on angiogenesis.[32,33] Based on this theory, VEGF and CD34 are prognostic markers of clinical pathology on microvascular density and tumor size.[8] Administration of the chemotherapy agent Adriamycin-Cyclophosphamide works in a complex manner to decompose tumor cell DNA, suppressing angiogenesis and tumor development.[34-36] The addition of artemisinin compounds in *Artemisia vulgaris* as an anticancer that works through the effects of free radical toxicity, cell cycle arrest, inhibition of angiogenesis, and induction of apoptosis produces a synergistic mechanism with chemotherapy for breast tumor progression assessed based on the expression variables VEGF, CD34, microvascular density, and the diameter of the tumor mass.[7,8]

5. Conclusion

Artemisia vulgaris extract gave a higher response to chemotherapy in mammary adenocarcinoma of C3H mice given Adriamycin-Cyclophosphamide chemotherapy regimen (p=<0.05).

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