The Effectivity of Artemisia Vulgaris Extract on Cyclin-D1 and Ki-67 Decreased as a Supplementation of Adenocarcinoma Mammae Chemotheraphy (Study on C3H Mice Given AC Chemotheraphy Regimen)

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Abstract

Background: *The incidence of breast cancer worldwide is still high. Surgery remains the top choice with other modalities of chemotherapy, radiation, and suplementation such as Artemisia vulgaris (AV).*

Aim: *The study was aimed to demonstrate that administration of AV extract decreased the expression of Cyclin-D1 and the expression of Ki-67 in adenocarcinoma mammae.*

Method: *This study used "Posttest 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 / m^2 and Cyclophosphamide 600 mg / m^2 were given in 2 cycles. AV 13 mg (0.2 ml) was given once daily orally. Cyclin-D1expression and Ki-67 expression were evaluated by imunohistochemical staining.*

Result: *Mean of Cyclin-D1expression and Ki-67 expression were found in groups K, P1, P2, P3 were 35,30 + 0,72; 20,38 + 0,67; 33,50 + 0,71; 17,36 + 0,66; and 66,44 + 0,65; 35,40 + 0,65; 64,12 + 0,85; 32,32 + 0,61. The statistical analysis showed that there were significant differences in the expression of Cyclin-D1 between groups of K vs P1, P3 (p = 0,001), P1 vs P2 (p = 0,001), P1 vs P3 (p = 0,045), P2 vs P3 (p = 0,001), and in Ki-67 expression between groups of K vs P1, P3 (p = 0,001), P1 vs P2 (p = 0,001), P1 vs P3 (p = 0,041), P2 vs P3 (p = 0,001). Correlation analysis between Cyclin-D1 expression and Ki-67 expression showed significant correlation (p = 0,030 dan r = 0,914).*

Conclusion: *Artemisia vulgaris is potential as supplementation that can improve the effectivity of Adriamycin-Cyclophosphamide chemotherapy in terms of decreased expression of Cyclin-D1 and expression of Ki-67 adenocarcinoma mammae of C3H mice.*

Keywords: *Artemisia vulgaris*, adenocarcinoma mammae, expression *Cyclin-D1*, expression *Ki-67*.

Introduction

Cancer disease is known to be one of the major cause of death internationally. Based on the data from GLOBOCAN and International Agency for Research on Cancer (IARC), it is said that in 2012 there are 14.067.894 new newly diagnosed cancer cases and 8.201.575 death caused by cancer internationally. Breast cancer, prostatic cancer, and lung cancer are the most common types of cancer with the highest percentage of new cases. Meanwhile, lung cancer and breast cancer are the leading cause of death from cancer. In the female population, breast cancer still ranks first in new cases and deaths from cancer, which is 43.3% and 12.9% [1].

It is estimated that the incidence rate in Indonesia is 12 / 100,000 women with a high mortality rate of 27 / 100,000 or 18% of deaths found in women. This disease can also be suffered in men with a frequency of about 1%. In Indonesia, more than 80% of cases are found to be in an advanced stage, where treatment efforts are difficult to do. This disease can also be suffered in men with a frequency of about 1%. In Indonesia, more than 80% of cases are found to be in an advanced stage, where treatment success rates are low [2].

The modalities of breast cancer therapy include: surgery, radiotherapy, cytostatics, immunotherapy, and hormonal therapy. Surgery and

radiotherapy are definitive local therapy, while chemotherapy, immunotherapy, and hormonal therapy are systemic. Chemotherapy in breast cancer is done in the form of a regimen. The most common combinations of chemotherapy used are Fluorouracil, Doxorubicin and Cyclophosphamide (FAC); Fluorouracil, Epirubicin and Cyclophosphamide (FEC); Doxorubicin and Cyclophosphamide (AC) and Cyclophosphamide, Methotrexate and Fluorouracil (CMF). This chemotherapy is administered intermittently in intervals of three to four weeks. FAC, FEC and CMF are given in 6 cycles (within 18 to 24 weeks) while AC is given in 4 cycles (within 12 to 16 weeks) [3].

The success rate of chemotherapy is based on an objective evaluation of post-chemotherapy response rate(Complete Response and Partial Response - CR / PR). Chemotherapy for breast cancer has a CR / PR ranging from 20% - 40%, but this drug has toxic effects on the liver, kidneys, heart, and other organs of the body, and immunosuppression [4].

Research has been focused on safer chemotherapy development by exploring the anticancer properties of new compounds, one of which comes from medicinal plants. Many medicinal plant derivatives are known to be effective against various diseases with extensive antibiotic and antimalignant activity [5].

There have been many studies on medicinal plants used as chemotherapy supplementation, among others: Mahkota Dewa (*Phaleria macrocarpa*), Black Cumin (*Nigella sativa*), Green Tea (*Camellia sinensis*), and *Artemisia vulgaris*. One of the most commonly used herbs is *Artemisia vulgaris*. *Artemisia vulgaris* has a selective cytotoxic effect on tumor cells and has been used as a supplementation of colorectal cancer, kidney cancer, prostate cancer, liver cancer, pancreatic cancer, skin cancer and gastric cancer.

Utilization of *Artemisia vulgaris* as a medicinal chemotherapy supplementation plant for breast cancer still requires a proof. An early study of *Artemisia vulgaris* showed the presence of terpenoid group (artemisinin, artesunate), flavonoids, tannins and coumarin analogues (scopoletin). Artemisinin and artesunate are already used as antimalarial drugs, but also show cytotoxic activity against cancer cells through apoptotic induction and inhibit angiogenesis activity, cancer cell cycle disruption, and free radical toxicity. Although it is cytotoxic, artemisinin has the advantage of being used as an anticancer because it has selective toxic properties. This is an important consideration in terms of security for its users. The selective cytotoxic properties of *Artemisia vulgaris* are a contributing factor in preliminary research at the pre-clinical stage [6].

Previous research conducted on mice with hepatic carcinoma, with a dose of 100 mg / kg per day of artemisinin showed anticancer activity [7].

Cyclin D-1 is a protein weighing 45 kD, encoded by the CCND1 gene and located on chromosome 11q13. *Cyclin D-1* is part of a complex molecular regulatory system in the cell cycle, together with cyclin-dependent kinase (CDK) 4 and CDK6, forming an active complex that promotes the G1-S phase with phosphorylation and inactivation of retinoblastoma proteins [8]. These regulatory proteins commonly experience excessive expression in various types of human cancers. Recent studies have also revealed the important function of CDK-independent *Cyclin D-1* in the regulation of some transcription factors, as was first shown for ER [9-12].

Cyclin D-1 plays an important role in carcinogenesis because: (1) the excessive expression of *Cyclin D-1* accelerates the process of cell transformation and tumorigenesis and amplifies amplification in other cells; (2) antisense *Cyclin D-1* cDNA reverses the malignant phenotype of a mammary carcinoma.

Ki-67 shows the cell in a quiescent or cell condition in a resting condition, not proliferating. The *Ki-67* protein has a molecular weight of 359 kD -320 kD. *Ki-67* expression is low in not dividing cells or in G1 phase and early phase of phase S and will increase significantly in the mitotic phase. The *Ki-67* examination using this immunohistochemical technique is a marker for cell proliferation. The *Ki-67* expression describes a cell population undergoing cell proliferation and has a high accuracy compared with other markers used to determine cell proliferation activity [9].

This study wants to prove the effectiveness of *Artemisia vulgaris* extract as supplementation in chemotherapy for Adenocarcinoma mammae, in order to decrease *Cyclin D-1* expression and *Ki-67* expression in C3H mice given Adriamycin-Cyclophosphamide. The results of this study are expected to support the use of *Artemisia vulgaris* as one of chemotherapy supplementation of breast cancer.

Methods

Research Design

This research is a laboratory experimental research with design "Post test only control group design". The study group was divided into 4, control group (K), Treatment 1 (P1), Treatment 2 (P2), and Treatment 3 (P3). The distribution of treatment groups is as follows :

K: Control group, mice that were inoculated with cancer cells.
P1: Treatment group 1, mice that were inoculated cancer cells, after the lumps appear, received chemotherapy Adriamycin 0.18 mg and Cyclophosphamide 1.8 mg intravenously.
P2: Treatment group 2, mice that were inoculated with cancer cells, after the lumps appear, recived Artemisia vulgaris extract 13 mg once daily orally.
P3: Treatment group 3, mice that were inoculated with cancer cells, after the lumps appear, received chemotherapy Adriamycin 0.18 mg and Cyclophosphamide 1.8 mg intravenously and *Artemisia vulgaris* extract 13 mg once daily orally.

Research Sample

The experimental animals were C3H mice obtained from PT. IndoAniLab Bogor. Inclusion Criteria: Female mice aged 8 weeks, C3H strain that were inoculated with adenocarcinoma mammae, weight 20-30 gram after acclimatization, no visible anatomical abnormalities. Exclusion criteria: do not grow tumors after inoculation, during inoculation and treatment of mice looks sick (inactive motion). The sample size according to WHO for each group is at least 5 mices with 10% reserve, in this study the number of samples used for each group is 6 mices [13].

Time and Location of the Research

Research and data collection were done within 5 months. The making of *Artemisia vulgaris* extract was done in LPPT I of Faculty of Medicine, Gajah Mada University, Jogjakarta. Treatment in mice and tissue-taking process was done at LPPT IV Faculty of Medicine, Gajah Mada University, Jogjakarta. The process of making

paraffin blocks, HE staining and immunohistochemical staining was performed at the Anatomy Pathology Laboratory of the Faculty of Medicine, Sebelas Maret University, Surakarta.

Research Variable

The independent variable in this research are:

1. Administration of *Artemisia vulgaris* extract
2. Administration of Adriamycin-Cyclophosphamide
3. Administration a combination of Adriamycin-Cyclophosphamide and *Artemisia vulgaris* extract

The dependent variables in this research are:

1. *Cyclin D-1* Expression

Expression of *Cyclin D-1* was assessed by immunohistochemical examination of each preparation was investigated by five fields of view and the value of each field of view (on immunohistochemical examination) and calculated from 100 tumor cells as a value for obtaining *Cyclin D-1* excretion. Variable scale: ratio.

2. *Ki-67* Expression

The *Ki-67* expression was assessed by immunohistochemical examination of each preparation was investigated by five fields of view and the value of each field of view (on immunohistochemical examination) and calculated from 100 tumor cells as a value for obtaining *Ki-67* excretion. Variable scale: ratio.

Materials and Tools of the Research

The experimental animals were female mice of C3H strain with age of 8 weeks, and weight 20 - 30 gram. The mice are obtained from PT. IndoAniLab Bogor. During the experiment, the experimental animals were placed in a cage and fed and drank in ad libitum. Prior to the treatment, the mice underwent a one-week adaptation period.

Artemisia vulgaris simplicia was obtained from the Conservation Unit of Biopharmacy Cultivation, Biopharmacy Study Center, Institut Pertanian Bogor. The material used is *Artemisia vulgaris* extract, which is obtained by:

1. One kilogram of dried leaves *Artemisia vulgaris* was finely grounded, then the powder was inserted into the tool socket (50 mg capacity). Extraction was done by socletation using ethanol solvent with a cycle of 8-10 times.
2. The extract is into into a rotary evaporator flask and vacuum distillation was done until the extract became concentrated (40°C).
3. The extract was dried in an oven with a temperature of 40°C for 1 hour to evaporate ethanol.
4. It was obtained 5,5 mg extract for every 1 kg of material (0,55%) and the extract was then diluted with aquabidest until reach the concentration of 0,2 mg / ml.

Adenocarcinoma is obtained from the donor mice. Tumors containing adenocarcinoma cells from the donor mice will be transplanted into the recipient mice. In addition to being transplanted, the tumor from the donor mice will be incised by biopsy and histopathologic examination is done to confirm the type of tumor.

Data Analysis

After the data had been collected, data cleaning, coding and tabulation weredone. Data analysis includes descriptive analysis and hypothesis test. The descriptive analysis of Adenocarcinoma mammae *Cyclin D-1* expression and expression of *Ki-67* expression was presented in the form of mean table, SD, median and box plot graph. The data normality test was done by using Saphiro-Wilk test. Hypothesis test was done by using One Way Anova test, followed by Post-Hoc test in order to know the difference between groups. The correlation test between *Cyclin D-1* expression and *Ki-67* expression variable was done by using Pearson correlation test. The degree of significance is when $p \leq 0.05$ with 95% confidence interval. Data analysis was done with SPSS Ver software. 21.0 for Windows.

Research Ethic Requirement

The study applied animal ethics in managing experimental animals. Prior to this research, the submission of the approval from Medical Research Ethics Committee of Faculty of Medicine, Diponegoro University was done. All animals will be treated and managed according to animal maintenance standards.

Results

Descriptive Analysis

Cyclin D-1 Expression Data Description

After checking the control group (K) and the treatment group (P1, P2, P3) by calculating *Cyclin D-1* expression per 100 tumor cells using 400x magnification, in a paraffin block, the results of *Cyclin D-1* expression was as follows :

Table 1. *Cyclin D-1* Data Characteristics

Group	N	Min (%)	Max (%)	Rerata ± SD (%)	Median (%)
K	5	33,30	37,20	35,30 ± 0,72	35,60
P1	5	18,60	22,30	20,38 ± 0,67	20,10
P2	5	31,60	35,70	33,50 ± 0,71	33,30
P3	5	15,80	18,90	17,36 ± 0,66	17,10

Table 1 shows that the mean expression of *Cyclin D-1* adenocarcinoma mammae cell in P3 group was lower than in P1 group, and in P2 group was lower than in K group.

The box plot as shown in Figure 1 below shows that the median expresision of *Cyclin D-1* in P3 group was lower than in P1 group, and in P2 group was lower than in the K group.

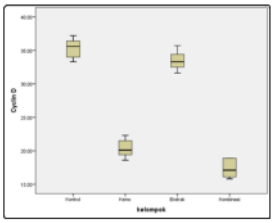

Figure 1: *Cyclin D-1* Box Plot Graph

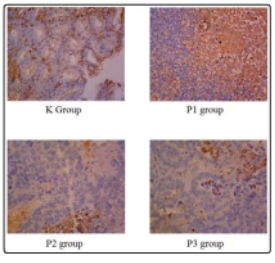

Figure 2: *Cyclin D-1* in IHC staining

Ki-67 Expression Data Description

The reading was performed on the control group (K) and the treatment group (P1, P2, P3) by calculating the *Ki-67* expression per 100 tumor cells using 400x magnification, from 5 field of view of each preparation, in a paraffin block, then the *Ki-67* expression was calculated using percentage.

Table 2: *Ki-67* Data Characteristics

Group	N	Min (%)	Max (%)	Rerata ± SD (%)	Median (%)
K	5	64,60	68,10	66,44 ± 0,65	66,50
P1	5	33,70	37,20	35,40 ± 0,65	35,50
P2	5	62,20	66,50	64,12 ± 0,85	63,70
P3	5	30,30	33,50	32,32 ± 0,61	32,90

Table 2 shows that the mean expression of *Ki-67* adenocarcinoma mammae cells in P3 group was lower than in P1 group, and in P2 group was lower than in K group.

The box plot as shown in figure 3 below shows that the median expression of *Ki-67* in P3 group is lower than in P1 group, and in P2 group is lower than in K group.

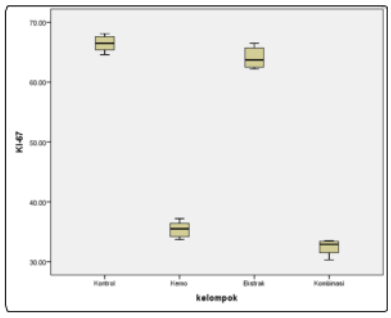

Figure 3: *Ki-67* Box Plot Graph

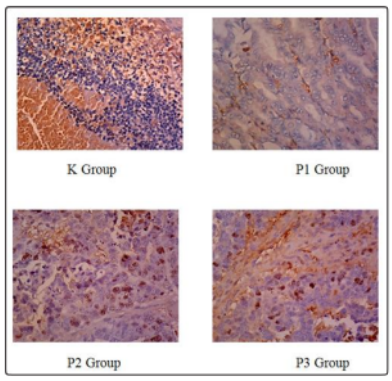

Figure 4: *Ki-67* in IHC Staining

Data Distribution

Data normality and homogeneity test from each group was done by using Shapiro-Wilk test because the sample size was less than 50. Data exploration of each variable from each group can be seen in the table below.

Table 3: *Cyclin-D1* Data Normality Test

Group	Shapiro-Wilk			Levene	
	Statistic	df	Sig. (p)	Statisic	Sig. (p)
K	0,949	5	0,729	0,023	0,995
P1	0,962	5	0,820		
P2	0,984	5	0,955		
P3	0,848	5	0,187		

Table 3 shows the p > 0,05 values in each group so it can be concluded that *Cyclin-D1* data distribution was normal and homogenous in all groups.

Table 4: *Ki-67* Data Normality Test

Group	Shapiro-Wilk			Levene	
	Statistic	df	Sig. (p)	Statisic	Sig. (p)
K	0,925	5	0,754	0,534	0,665
P1	0,951	5	0,748		
P2	0,900	5	0,408		
P3	0,874	5	0,283		

Table 4 shows the p> 0,05 values in each group so it can be concluded that *Ki-67* data distribution was normal and homogenous in all groups.

Hypothesis Test

Cyclin-D1

After the Shapiro-Wilk test was done, normal and homogenous data was obtained, so it was continued with One Way ANOVA difference test. The hypothesis test was performed to determine whether there was any difference of *Cyclin-D1* expression between study groups, the result was as follow :

Table 5: *Cyclin-D1* Expression Difference Analysis

Group	Cyclin-D1 Expression (%) (Mean ± SD)	p
K	35,30 ± 0,72	0,001*
P1	20,38 ± 0,67	
P2	33,50 ± 0,71	
P3	17,36 ± 0,66	

*being tested by One Way ANOVA test (significant if $p < 0,05$)

From One Way ANOVA test result, the p value was 0,001, because p <0,05 it can be concluded that there was significant difference of *Cyclin-D1* expression in all four groups. The *Bonferroni* test was then used to determine the differences between groups.

Table 6: *Bonferroni* analysis of *Cyclin-D1* expression between groups

Group	P1	P2	P3
K	0,001*	0,520*	0,001*
P1	-	0,001*	0,045*
P2		-	0,001*

*Being tested by *Bonferroni* test (significant if $p < 0,05$)

Bonferroni test results showed significant differences between K group with P1, P3 (p = 0.001); P1 with P2 (p = 0.001); P1 with P3 (p = 0,045); P2 with P3 (p = 0.001). The difference was not significant between K group and P2 group (p = 0,520).

Ki-67

After the Shapiro-Wilk test was done, normal and homogenous data was obtained, so it was continued with One Way ANOVA difference test. This statistical test was performed to determine whether there was any difference of *Ki-67* expression between study groups, the result was as follow :

Table 7: *Ki-67* Expression Difference Analysis

Group	*Ki-67* Expression (%) Mean ± SD	*P*
K	66,44 + 0,65	0,001*
P1	35,40 + 0,65	
P2	64,12 + 0,85	
P3	32,32 + 0,61	

*being tested by One Way ANOVA test (significant if $p < 0,05$)

From One Way ANOVA test result, the p value was 0,001, because p <0,05 it can be concluded that there was significant difference of Ki-67 expression in all four groups. The *Bonferroni* test was then used to determine the differences between groups.

Table 8: Bonferroni analysis of Ki-67 expression between groups

Group	P1	P2	P3
K	0,001*	0,197	0,001*
P1	-	0,001*	0,041*
P2		-	0,001*

*Being tested by *Bonferroni* test (significant if $p < 0,05$)

Bonferroni test results showed significant differences between K group with P1, P3 (p = 0.001); P1 with P2 (p = 0.001); P1 with P3 (p = 0,041); and P2 with P3 (p = 0.001). The difference was not significant between K group with P2 group (p = 0.197).

Cyclin D-1 and *Ki-67* Correlation

The correlation test between *Cyclin-D1* expression and *Ki-67* expression was performed in P3 group (combination). Data normality test of both variables was done by using Shapiro-Wilk test. Based on the test, it was obtained a normal data (p> 0.05) so that the correlation analysis was continued by using Pearson test.

Table 9: *Pearson* Correlation Test Result

Variable	p	R
Cyclin-D1 Expression	0,030*	0,914
Ki-67 Expression		

Based on *Pearson* Correlation Test Result in Table 9, the p value = 0.030 and r = 0.914. Because the p value was less than 0.05 it can be concluded that there was a strong positive relationship between the *Cyclin-D1* expression of the *Ki-67* expression.

Discussion

This study aims to prove the effect of *Artemisia vulgaris* extract on C3H mice with adenocarcinoma mammae, given either Adriamycin-Cyclophosphamide chemotherapy or not, in terms of *Cyclin-D1* expression and *Ki-67* expression. Discussion of the results is done sequentially from the expression of *Cyclin-D1*, the expression of *Ki-67*, then continued by looking at the relationship between those two variables.

In *Cyclin-D1* expression variable, the highest mean value was found in K group (control), meanwhile the lowest mean value was found in P3 group (combination). The difference analysis of *Cyclin-D1* expression between groups was done by using One Way ANOVA test, then followed by Bonferroni test showed significant differences.

The mean value of *Cyclin-D1* expression in P3 group was found to be lower than in P1 group (chemotherapy), indicating a significant value that the administration of Artemisia vulgaris extract has the potential to suppress *Cyclin-D1* activity thus preventing the cell from returning to G1 phase in mammae cell [14,15]. This is in accordance with previous study by Ferreira et al, 2010, one component of Artemisia vulgaris, which is artemisinin can decrease the expression of *Cyclin-D1* by activating E2 protein [16]. Another study by Firestone et al, 2009, explains that artemisinin plays a role in suppressing cell mitotic activity [17].

In *Ki-67* expression variable, the highest mean value was found in K group (control), meanwhile the lowest mean value was in P3 group (combination). The difference analysis of *Ki-67* expression between groups was done by using One Way ANOVA test, then followed by Bonferroni test.

There was a significant decrease of *Ki-67* expression mean value in P3 group (combination) when being compared to P1 group (chemotherapy). This suggests that Artemisia vulgaris extract has not been able yet to function as a primary therapy but can only be used as a supplementation on administration of Adriamycin-Cyclophosphamide chemotherapy. According to Jia et al, 2016, one of Artemisia vulgaris component, which is artemisinin, can inhibit cell proliferation to phase G1 from the cell cycle, this will lead to decreased expression of *Ki-67* [18]. Balzquez et al, 2013, states that artemisinin may be involved in the process of DNA transcription, which will inhibit the cell to begin the G1 phase of the cell cycle [19].

Pearson correlation analysis between *Cyclin-D1* expression and Ki-67 expression in P3 group (combination) showed a strong positive correlation relationship between the two variables. This suggests that the correlation of *Cyclin-D1* expression and *Ki-67* expression can be explained by the pathway in this study.

Based on this research, it can be concluded that Artemisa vulgaris extract has the potential to suppress cells. The use of Artemisa vulgaris extract is supplementative for primary therapy and as an alternative source of traditional medicine use. Researchers are also aware that there are still many limitations in the study that needed to be improved in order to complement and refine this research. If possible this study could be upgraded into clinical trials in humans.

Conclusion

Artemisia vulgaris is potential as supplementation that can improve the effectivity of Adriamycin-Cyclophosphamide chemotherapy in

terms of decreased expression of Cyclin-D1 and expression of Ki-67 adenocarcinoma mammae of C3H mice.

The Effectivity of Artemisia Vulgaris Extract on Cyclin-D1 and Ki-67 Decreased as a Supplementation of Adenocarcinoma Mammae Chemotheraphy (Study on C3H Mice Given AC Chemotheraphy Regimen)

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GRADEMARK REPORT

FINAL GRADE

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GENERAL COMMENTS

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