The Effectivity of Artemisia vulgaris Extract as Supplementation to Adenocarcinoma Mammae Chemotherapy for Increasing IL-12 and Apoptotic Index
(Study on C3H Mice Given Adriamycin-Cyclophosphamide Chemotherapy Regimen)

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ABSTRAK

Insiden kanker payudara di seluruh dunia masih tinggi. Pembedahan tetap merupakan pilihan utama dengan modalitas lain berupa kemoterapi, radiasi, dan imunoterapi antara lain Artemisia vulgaris (AV). Penelitian dilakukan untuk membuktikan efek pemberian ekstrak AV terhadap kadar IL-12 dan indeks apoptosis sel kanker pada adenokarsinoma mammae. Penelitian ini menggunakan desain post test only control group design menggunakan 24 ekor mencit C3H betina yang dibagi secara acak menjadi empat kelompok, yaitu: K (kontrol), P1 (kemoterapi), P2 (ekstrak AV), dan P3 (kombinasi kemoterapi dan ekstrak AV). Adriamycin 0,18mg dan Cyclophosphamide 1,8mg diberikan sebanyak 2 siklus. Ekstrak AV diberikan 13mg (0,2ml) perhari. Kadar IL-12 dinilai dengan pengecatan imunohistokimia sedangkan indeks apoptosis dengan hematoxilin eosis. Rerata kadar IL-12 dan indeks apoptosis didapatkan  K, P1, P2, P3 berturut-turut 60,8+1,54, 50,40+1,56, 75,40+1,35 dan 2,18+0,80, 18,00+1,58, 3,34+0,51, 20,32+1,39. Analisis statistik menunjukkan terdapat perbedaan bermakna pada kadar IL-12 antara kelompok K vs P1, P2, P3 (p=0,001), P1 vs P2 (p=0,001), P1 vs P3 (p=0,028), P2 vs P3 (p=0,001) dan indeks apoptosis antara kelompok K vs P1, P3 (p=0,001), P1 vs P2 (p=0,001), P2 vs P3 (p=0,035), P3 vs P3 (p=0,001). Terdapat hubungan positif kuat yang signifikan antara kadar IL-12 dengan indeks apoptosis (p=0,041 dan r=0,893). Pemberian ekstrak Artemisia vulgaris dapat meningkatkan kadar IL-12 dan indeks apoptosis sel kanker pada mencit C3H dengan adenokarsinoma mammae yang diberi regimen kemoterapi Adriamycin-Cyclophosphamide.

Kata Kunci: Adenokarsinoma mammae, Artemisia vulgaris, imunoterapi, indeks apoptosis, kadar IL-12

ABSTRACT

The incidence of breast cancer worldwide is still high. Surgery remains the top choice with other modalities of chemotherapy, radiation, and immunotherapy such as Artemisia vulgaris (AV). The study was aimed to demonstrate the effect of AV extract administration on IL-12 level and apoptotic index of cancer cells in adenocarcinoma mamma. This study used a posttest only control group design using 24 female C3H mice which were divided randomly into four groups, namely C (control), P1 (chemotherapy), P2 (AV extract), and P3 (combination chemotherapy and AV extract). Adriamycin 0,18mg and Cyclophosphamide 1,8mg were given in 2 cycles. AV 13mg (0.2ml) was given daily. IL-12 levels were evaluated using immunohistochemical staining while the apoptotic index was by Hematoxilin Eosin. Mean of IL-12 levels and apoptotic index in groups K, P1, P2, P3 were 60.8+1.54, 50.40+1.56, 75.40+1.35 and 2.18+0.80, 18.00+1.58, 3.34+0.51, 20.32+1.39 respectively. The statistical analysis showed that there were significant differences in the levels of IL-12 between groups of K vs P1, P2, P3 (p=0.001), P1 vs P2 (p=0.001), P1 vs P3 (p=0.028), P2 vs P3 (p=0.001), and in apoptotic index between groups of K vs P1 (p=0.001), P1 vs P2 (p=0.001), P1 vs P3 (p=0.035), P2 vs P3 (p=0.001). There was a significant positive correlation between IL-12 levels and apoptosis index (p=0.041 and r=0.893). The administration of Artemisia vulgaris extract can increase IL-12 levels and apoptosis index of cancer cells in C3H mice with mammary adenocarcinoma given Adriamycin-Cyclophosphamide chemotherapy regimen.

Keywords: Artemisia vulgaris, adenocarcinoma mammae, IL-12 levels, apoptosis index.

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INTRODUCTION

Cancer is still ranked as the second leading cause of death in the world after cardiovascular disease. GLOBOCAN data, the International Agency for Research on Cancer (IARC), revealed 14 million new cases of cancer were found in 2012. As many as 7.5 million people die from cancer, and 70% of cancer deaths take place in poor and developing countries. The most common type of cancer among women is breast cancer (38 per 100,000 women) (1). Riskesdas data in 2013 showed that the prevalence of cancer in Indonesia was 1.4 per 1,000 population and became the number 7 of death causes (5.7%). Globocon/IARC in 2012 reported that the estimated incidence of breast cancer in Indonesia was 40 per 100,000 women. This prevalence of breast cancer was higher compared to that in 2002, which was 26 per 100,000 women. Most cancer patients who were hospitalized throughout Indonesia in 2010 were breast cancer patients (28.7%) (1). Besides females, males can suffer from this disease with a frequency of approximately 1%. More than 80% of breast cancer cases are found at the advanced stage, where the therapeutic success rate is low (2).

Breast cancer therapy modalities include surgery, chemotherapy, radiotherapy, immune therapy, and hormonal therapy. Surgery and radiotherapy are local, while chemotherapy, immune therapy, and hormonal therapy are systemic. Chemotherapy in breast cancer is carried out in the form of a regimen (2). The combinations of chemotherapy for breast cancer used are Flurouracil, Adriaycin, and Cyclophosphamide (FAC); Flurouracil, Epirubicin, and Cyclophosphamide (FEC); Adriaycin and Cyclophosphamide (AC) and Cyclophosphamide, Methotrexate, and Flurouracil (CMF). This chemotherapy is given at a 3-4 week interval. FAC, FEC, and CMF regimens are given in 6 cycles (within 18-24 weeks) while AC is given in 4 cycles (within 12-16 weeks) (3). Complete Response and Partial Response - CR/PR shows the success rate of chemotherapy based on RECIST 1.1 (Response Evaluation Criteria in Solid Tumors). Chemotherapy for breast cancer has CR/PR ranging from 20% - 40% only, while this medication has a toxic effect on internal organs and immunosuppression (4).

Several research have focused on developing safer chemotherapy by exploring the anticancer properties of new compounds, one of which comes from medicinal plants. Many derivatives of medicinal plants are known to be effective against various diseases with extensive antibiotic and anti-malignant activity (5). There have been many research conducted on medicinal plants used as supplementation for chemotherapy, among others: God’s crown (Phaleria macrocarpa) (6), Black Cumin (Nigella sativa) (7), Green tea (Camellia sinensis) (8), Soursop (Anonna muricata) (9), and New China (Artemisia vulgaris). Artemisia vulgaris has a selective cytotoxic effect on tumor cells and has been used as a supplement in the treatment of gastric, colorectal, liver, gallbladder, pancreas, kidney, prostate, and skin cancers (10-13). The role of apoptosis in normal physiology is as important as mitosis. Apoptosis shows a complementary but opposite role to mitosis and cell proliferation in the regulation of various cell populations that function to maintain homeostasis in the adult human body. Cancer is an example of a condition when the normal mechanism of cell cycle regulation experiences dysfunctional, with over cell proliferation and decreased cell destruction. Suppressing apoptosis during carcinogenesis is thought to play a central role in the development of several types of cancer (21,22).

This study was conducted to examine the effectiveness of Artemisia vulgaris extract in increasing IL-12 level and apoptosis index of cancer cells in C3H mice with mammary adenocarcinoma given the Adriamycin-Cyclophosphamide chemotherapy regimen. The results of this study are expected to support the use of Artemisia vulgaris as a supplement to breast cancer chemotherapy.

METHOD

Research design

This experimental research used Post-test only control group design. There were four research groups, namely: (1) Control, C3H mice which were only inoculated with cancer cells, (2) P1, a group of C3H mice inoculated with cancer cells, received chemotherapy of Adriamycin 0.18 mg and Cyclophosphamide 1.8 mg intravenously, (3) P2, a group of C3H mice inoculated with cancer cells, received extracted Artemisia vulgaris 13 mg once daily orally, and (4) P3, a group of C3H mice inoculated with cancer cells, received Adriamycin 0.18 mg and 1.8 mg Cyclophosphamide.
chemotherapy intravenously and Artemisia vulgaris extract 13 mg once daily orally. Inoculation of cancer cells was subcutaneously given once in the armpit area towards mammae. The first dose of chemotherapy was given once per day for 21 days, and the second dose was given on day 22.

Research Sample
The research sample was obtained from PT. IndoAniLab Bogor with inclusion criteria: female mice aged eight weeks, C3H strains which were successfully inoculated adenocarcinoma mammary, weighted 20-30 grams after the adaptation period, and no visible anatomic abnormalities. Any sample was not included in the treatment if there was a regression of the tumor after inoculation and during inoculation, and if during treatment the mice appeared sick (inactive movement). According to WHO, the number of repetitions per group is at least five animals with a reserve of 10% (23). In this study, each group consisted of 6 mice. During the study, mice were placed in separate cages and fed according to the standard. Before treatment, mice underwent an adaptation period for one week.

Research Time and Location
This study was conducted for five months. The process of Artemisia vulgaris extracting was carried out at the LPPT I Faculty of Medicine, Gadjah Mada University. Treatment and tissue collection were carried out at LPPT IV Faculty of Medicine, Gadjah Mada University. Paraffin block making, HE staining, and immunohistochemical staining were carried out at the Pathology Anatomy Laboratory, Faculty of Medicine, Sebelas Maret University, Surakarta.

Research variable
The treatments in this study included the administrations of (1) extract of Artemisia vulgaris, (2) Adriamycin-Cyclophosphamid, and (3) a combination of Adriamycin-Cyclophosphamide and extract of Artemisia vulgaris. The measured outputs were the level of interleukin-12 (IL-12) and the apoptosis index. IL-12 levels were assessed using immunohistochemical examination; each preparation was examined in five fields of view. The calculated value of each field of view was the number of tumor cells with brown cores and was calculated from 100 tumor cells as a ratio measurement scale. The apoptotic index was calculated according to the method used by Aihara M et al., which was the apoptotic body calculated per 100 tumor cells in 5 fields of view, then the average results were taken with the ratio measurement scale.

Research Materials and Tools
Simplicia of Artemisia vulgaris was obtained from the Biopharmaca Aquaculture Conservation Unit of Bogor Agricultural Institute. Artemisia vulgaris extract was obtained through the following processes. The first stage, one kilogram of dried Artemisia vulgaris leaves was finely ground; then extraction was carried out by Soxhletation method using ethanol solvent with a cycle of 8-10 times. Extract result was put into the evaporator, and vacuum distillation was carried out. The extract was dried at 40°C for 1 hour to evaporate ethanol. From this process, 5.5 mg of extract was produced from every 1 kg of material (0.55%) and then diluted with distilled water until reaching a concentration of 0.2 mg/ml. Mammary adenocarcinoma was obtained from donor mice. In addition to being transplanted, a biopsy incision to confirm the type of tumor were conducted on the tumors from donor mice.

Data analysis
Cleaning, coding, and tabulation were carried out before analysis. Data analyses performed were descriptive analysis and hypothesis testing. Descriptive analysis of IL-12 level and adenocarcinoma mammary apoptosis index was presented in the form of tables and box plot graphs. Data normality test was conducted using Shapiro-Wilk test. The hypothesis test used was One Way ANOVA test, followed by post-hoc test to determine the differences among groups. Correlation test between IL-12 level and apoptosis index was done using Pearson correlation test. The limit of significance was p < 0.05 with a 95% confidence interval. Data analysis was done with SPSS Ver. 21.0 for Windows.

Research Ethics Requirements
This study applied animal ethics in the process of managing animal experiments and had obtained approval from the Health Research Ethics Commission of the Faculty of Medicine, Diponegoro University. All experimental animals were treated and managed according to animal care standards.

RESULTS
Artemisin effect on Interleukin-12 level

The results (Figure 1) show that the mean values of IL-12 levels of mammary adenocarcinoma cells which were lower than the control group were treatment 1 group that was given chemotherapy and treatment 3 group which received additional Artemisia vulgaris 13 mg extract once per day, given orally. On the other hand, the group that only received Artemisia vulgaris 13 mg/day per-oral showed a higher mean of IL-12 level of mammary adenocarcinoma cells.

One Way ANOVA test (Table 1) shows significant differences in IL-12 levels in all four groups. From the results of Bonferroni test, there were significant differences among the control group and all treatment groups P1, P2, and P3 (p = 0.001). The IL-12 level in the
group with chemotherapy administration was lower than the administration of artemisinin extract alone \( (p = 0.001) \) and combination therapy \( (p = 0.028) \). The addition of artemisinin to chemotherapy gives higher level of IL-12 compared to chemotherapy alone \( (p = 0.001) \). The highest value of IL-12 level was obtained in artemisinin therapy.

Table 1. Post Hoc analysis of IL-12 levels among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>0.001*</td>
<td>0.001*</td>
<td>0.001*</td>
</tr>
<tr>
<td>P1</td>
<td></td>
<td>0.001*</td>
<td>0.028*</td>
</tr>
<tr>
<td>P2</td>
<td></td>
<td></td>
<td>0.001*</td>
</tr>
</tbody>
</table>

Note: * Tested using Bonferroni (significant \( p < 0.05 \))

**Artemisin Effect on Apoptosis Index**

The results show that adenocarcinoma mammary cell apoptosis index was the highest in the group given combination therapy (P3) and the lowest in the control group (K). Administration of artemisinin alone provides a lower mean of apoptosis index than chemotherapy (Figure 2).

ANOVA test results show significant differences in apoptosis index values in the four groups. Bonferroni test results find a significant difference between the control group and chemotherapy or combination but is not different from the artemisinin therapy group alone. The combination of chemotherapy and artemisinin has been proven to provide statistically significant differences of apoptosis index if compared to chemotherapy or artemisinin alone. Therapy with artemisinin alone has a lower apoptosis index than chemotherapy, even equal to controls.

Table 2. Post Hoc analysis of apoptosis index among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>0.001*</td>
<td>0.792</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

**Correlation of IL-12 and Apoptosis Index**

Correlation test between IL-12 level and apoptosis index was carried out in the P3 group (combination). Pearson's correlation analysis was chosen because the data distribution of both variables was normal and homogeneous. The test shows there was a strong significant positive relationship \( (R = 0.893, p = 0.041) \) between IL-12 levels and apoptosis index.

**DISCUSSION**

This study was conducted to see the effectiveness of Artemisia vulgaris extract in increasing IL-12 level and apoptosis index of cancer cells in C3H mice with mammary adenocarcinoma given the Adriamycin-Cyclophosphamide chemotherapy regimen. The administration of chemotherapy was given in two cycles only of four cycles which are recommended according to human administration (26). The results show that the mean of the IL-12 level was found to be higher in the group of chemotherapy and Artemisia vulgaris extract combination than chemotherapy group. Higher IL-12 level in the combination therapy group compared to the chemotherapy group indicates that Artemisia vulgaris extract improves the immunosuppression effect of Adriamycin-Cyclophosphamide chemotherapy. One component of Artemisia vulgaris, artemisinin, increases the production of IL-12 by macrophages by inhibiting c-Jun N-terminal Kinase (JNK) pathway (24). Another study by Langroudi et al., 2010, also mentioned that Artemisinin plays a role in increasing immunity but in other way which is by suppressing the number of Treg cells (25).

The apoptosis index was found to be higher in the group of chemotherapy and Artemisia vulgaris extract combination compared to the chemotherapy group. This can be explained because artemisinin inhibits the G1 phase of cell cycle, increases the production of Reactive Oxygen Species (ROS) which causes the breakdown of the potential mitochondrial membrane, and triggers the release of cytochrome-c from the mitochondria into the cytoplasm, which eventually activates apoptosis mediated by Caspase-3 (17). Artemisinin and its derivatives have the role of pro-apoptosis not only by releasing cytochrome-3 and overexpression of Bax, but also by increasing Bax/Bcl-2 ratio, activating Caspase-3 and Caspase-9 (27). Also, Artemisinin may interact directly with DNA replication in the process of apoptosis induction (28). Artemisinin and its derivatives cause apoptosis in tumor cells by increasing intracellular calcium levels and p38 activation (29). Some of the mentioned studies show that there are many mechanisms of Artemisin as an active component of Artemisia vulgaris in inducing apoptosis.

This study also finds a strong significant positive correlation between IL-12 level and the apoptosis index in the group of a combination of Adriamycin Cyclophosphamide.
Chemotherapy and Artemisia vulgaris extract. This finding explains the correlation of IL-12 level and apoptosis index, but there are still other pathways that cause the apoptosis process (25-29).

It can be concluded that the addition of Artemisia vulgaris to chemotherapy increases the IL-12 level and apoptosis index of cancer cells in C3H mice with mammary adenocarcinoma given Adriamycin-Cyclophosphamide chemotherapy regimen. The correlation between IL-12 level and apoptosis index in C3H mice with adenocarcinoma mammary given chemotherapy combination of Adriamycin-Cyclophosphamide and Artemisia vulgaris extract shows one of the potential apoptotic pathways.

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