Concentration of Phalerin from *Phaleria macrocarpa* in Vena Porta: An Experimental Study on Adenocarcinoma Mammae in C3H Mice That Given Adriamycin and Cyclophosphamide

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Abstract

The therapy for cancer has used the combination regiment of adriamycin and cyclophoshamide that may cause deoxyribonucleic acid damage of cancer cells and organs. One of its side effects is the disturbance of absorption in the intestinal mucosa. Phaleria macrocarpa *has been reported to have anticancer activity. There has been no studies conducted on the phalerin level of* Phaleria macrocarpa *in portal vein. The aim of this study was to examine the concentration of phalerin in the* Phaleria macrocarpa *in C3H mice with adenocarsinoma mamma between treated group of* Phaleria macocarpa *(PH) and combination (PH, Adriamycin (ADR), and Cyclophosphamide (CYC)). The experiment was conducted using posttest only control group design, 12 female C3H with adenocarsinoma mamma were divided into 2 groups of 6 mice each to get one of the following treatment: PH (0.14 mg/day p.o) or combination (PH 0.14 mg/day p.o; followed by ADH 0.013 mg i.p. and CYC 0.0156 mg i.p.). High Performance Liquid Chromatography, detector Spectra System (Knauer, 2006),* spectroscopy detector systems UV 600 LP, Lichrosorb *coloumn RP.18 (250 x 4 x 5 µm) were used to assess the concentration of Phalerin. Phalerin as marker was obtained from medical herbal centre, Faculty of Medicine, Gadjah Mada University. Blood sample was taken from portal vein at 30, 60, 90 min. to asses the concentration of phalerin absorbance in time series. Data were presented in table and boxplot then analyzed with independent T-test to find out the difference between the groups with a significance level less than 0,05 ($p<0.05$) and Confidence Interval of 95%.* Phaleria macrocarpa *followed by adriamycin and cyclophosphamide in combination group reduced the absorption indicated by the significant difference in level of phalerin between treated group by time. It was concluded that the phalerin concentration in PH group have a higher value compare to combination group.*

***Key words**: concentration of phalerin,* Phaleria macrocarpa*, Adriamycin, Cyclophoshamide*

INTRODUCTION

The incidence rate of cancer has been relatively high and present a serious health problems in the world. According to the Association Cancer Society (2009) there have been about 1.2 million cases. The most common treatments for cancers include surgery, radiotherapy, immunotherapy and chemotherapy (Beretta G, 1991; Apantaku L.M, 2002). The therapy for cancer has used the combination regiment of adriamycin and cyclophosphamide which is aimed at reducing the tumor mass, distribution and the reoccurrence (Ashariati and Ami, 2007). The combination regiment has different

mechanism to kill cells (Siswando and Soekardjo, 2000). A problem arises that this therapy does not only kill cancer cells but also the normal tissue (Goodman and Gilman, 2006). The most affected tissue is found in organ with highly proliferative tissues, i.e. gastrointestinal tract (Komen SG, 2010)

The recommended dose for *Adriamycin* is 60-75 mg/m^2 of body surface area. *Cyclophosphamide* 100-2000 mg/m^2 (International Agency For Research on Cancer, 1976). One of the medicinal plants potential as natural anticancer agent is *Phaleria macrocarpa.* Studies have shown that *Phaleria macrocarpa* contains active substances inhibiting the tumor growth (Lisdawati, 2002; Nawawi, 2004; Susmastuti and Solimar, 2004; Mae dkk,2005, Faried dkk, 2007; Oshimi, 2008). Chemotherapy agent from medical plants has been known to contain antioxidants and anticancer capable to scavenge free radical (Meiyanto E dkk, 2008). According to Slamet et al. (2010) the administration of *Phaleria macrocarpa* combined with the chemotherapy *in vivo* in C3H mice has been shown to be harmless. The combination of chemotherapy with *Phaleria macrocarpa* has shown to be immunostimulator to solve the problem related to the immunosuppressant effect caused by chemotherapy. The aim of this study was to examine the concentration of phalerin from *Phaleria macrocarpa* in C3H mice with adenocarsinoma mamma between treated group of Phaleria macocarpa (PH) and combination (PH, Adriamycin (ADR), and Cyclophosphamide (CYC)).

The combination therapy must be developed for the attempt to search for the effective therapy to kill and inhibit cancer cells, as well as reducing the related side effects. Absorption disturbance in intestinal mucosa was determined by decrease of phalerin level at portal vein (has not undergone metabolism) by time. Portal vein is affected by the active substances absorbed because portal vein is the nutrition carrier for hepar cells. Therefore, the measurement of phalerin concentration obtained from portal vein is required.

MATERIAL AND METHODS

This was an experimental study using the design as described in *the post test only controlled group design* (Hanifah, 1993; Santoso G, 2007; Dahlan M.S, 2008),

Materials

Extraction Materials. ethanol extract (Merck), n-heksana p.a (Merck), etil acetate p.a (Merck), chloroform p.a (Merck), diclometane p.a (Merck), silica gel-60 (mesh 60-230 ASTM) and alcohol solution p.a. Merck,

Transplantation Tools for Tumor Tissue in Mice. Petri dish (6 cm), Petri dish (15 cm), Dish (10 cm, syringe 1cc, Trocar Needle, Straight scissors 10 cm, Curved scissors 10 cm, Anatomische Pinset (10 cm), Pinset (12 cm), fixation tool, micro pipet

Equipments for Measuring Concentration of Phalerin. 1 unit of High Performance Liquid Chromatography, 1 unit of extractor, 1 unit of rotary evaporator vacuum, centrifuge, vortex, Erlemeyer flask, pipette, analytical balance, tube.

Population. Population target in this study was female C3H mice kept intensively in the Pathology Anatomy Laboratory, Faculty of Medicine Indonesian University. The sample was taken randomly according to the criteria of inclusion and exclusion and the need of sample was based on the sample size calculation.

Sample. The laboratory animals used were female C3H mice because this spe-cies is freuently used for cancer research. In addition, this spesies is sensitive to carsinogenic agent compared to other spesies (Kusumawati, D, 2004, C Lanari dkk, 2009).

The sample size in this study was in accordance with the *World Health Organization* (WHO), 1993, that every group is of 5 mice with 10 % subtitute (1 mice). In this study, the samples were 6

female C3H mice which have been occulated and randomly divided into two groups of 6 mice each (X1 and X2). The criteria for the sample are as follows:

Inclusion criteria. a) female mice, b) *Strain* C3H, c) body wight of 20-30 gr after acclimation, d). Healthy, with active movement and no anatomical abnormalities

Exclusion criteria. a) Showing no tumor growth after inoculation, b) During inoculation and treatment appear unhealthy, c) the chance in weight during the adaptation < - 10% or > 10 %

Mice handling (Kusumawati, 2004)**.** Mice were kept in individual cage with the surface area of 200 cm^2/mice. The room temperature of 28-32^oC. Diet and drink were given by ad libitum. Before the treatment, mice were adapted for 1 week.

The laboratory animals both donors and recipients were female C3H mice obtained from the Laboratory of Anatomy Pathology of Indonesian University. The diet during the treatment was taken from LPPT Gadjah Mada University Yogyakarta.

Plant collection. *Phaleria macrocarpa* obtained from the plantation owned by the company of traditional medicine in Tasikmalaya, Jawa Barat, in February 2009 was used as samples.

Extraction procedure. One kg of *Phaleria macrocarpa* fruit was dried, powdered, and was Soxchleted (capacity of 50 g) using ethanolic solution (Merck) in 8-10 times of circulation. The extract was placed into the rotary evaporator and vacuum distillation equipment, followed by further drying in an oven at 40ºC for 1 hour to evaporate the ethanol. Five point five grams extract for every 1 kg plant material (0.55%), containing 3,4,5-trihydroxybenzoic acid 10% (Merck), and the extract was solved with aquabidest at the concentration of 0.2 mg/mL.

The dose is corresponding to the dose in human that is the powder of fruit 5 gram per day multiplied by a constant of therapeutic test in mice, 0.0026 was multiplied by a constant extract 0.0055, thus the dose given was 5000 x 0,0026 x 0.0055 = 0.0715 mg/day (0.36 mL). Then the dose was doubled into 0.14 mg/day. This dose serves as the single dose.

Blood Sampling Via Portal Vein. The treated group I was administrated with *Phaleria macrocarpa* extract at the dose of 0.14 mg/day, group II was given adriamycin at the dose of 0.013 mg iv, cyclophosphamide at the dose of 0.0156 mg iv. The level of Phalerin was assessed by a serial blood sampling. Blood collection was taken via the portal vein 3 times after intramuscular anesthesia with the combination of ketamine HCl 0.01 ml dan xylaxine 0.01 ml added with aquadest 0.0375 ml. On day 1, first week Peritoneal Excudate Cells (PEC) surgery was performed after the treatment, at minute 30, 60 and 90. Blood collection in portal vein was conducted after 90 minutes.

Procedure for phalerin preparation in plasma. Serum of 200 µl was added with 200 µl mixture (MeOH: CH3CN = 90:10) and Vortexed for 2 minutes, centrifuged at 10.000 rpm for 5 minutes at 17^oC. The clear solution was taken and placed in Eppendorf tube and dried in waterbath at 50^oC under continuous nitrogen purge. It was then redissolved in MeOH 200 µl, filtered with Millipore 0.45 µM filter and injected to HPLC at 20 µl of volume.

Preparation of the standard solution. Phalerin standard was obtained from Prof. Mae Sri H W, Apt., M.Kes from Faculty of Medicine Gadjah Mada University. Solution of 5300 ppm in MeOH was prepared, followed by dillution to obtain 0.33125; 0.6625; 1.325; 2.65; 5.3 ppm solution in MeOH. The solutions were then for injection to HPLC 20 µl.

Analysis. Data were presented in table and boxplot then analyzed with independent t-test to find out the difference between the groups with a significance level less than 0,05 ($p<0.05$) and confidence interval of 95%.

RESULTS AND DISCUSSION

Mean of level of Phalerin in portal vein in group treated with *Phaleria macrocarpa* (PH) at minute 30, 60 and 90 after the treatment were 0.283 ± 0,741 ppm, 0.190 ± 0.009 ppm and 0.174 ppm ± 0.007 ppm respectively. Mean of phalerin level for the group treated with combination of *Phaleria macrocarpa* (PH), adriamycin dan cyclophosphamide (AC) at minute 30, 60, 90 were 0.159 ± 0.005 ppm, 0.131 ± 0.023 ppm and 0.097 ± 0.026 ppm respectively. The level of phalerin in group treated with PH was higher compared to the group treated with PH and AC (figure 1).

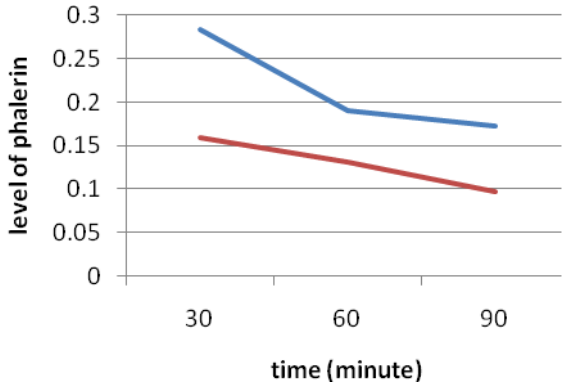

Figure 1. The level of phalerin at minute 30, 60, and 90 for the phalerin (PH) and combination group. — PH; — combination.

Shapiro-Wilk test applied to asses the normality of distribution of PH group after 30 and 90 minute resulted in p=0.145, p=0,072 and p=0,340 respectively. While the normality distribution test for the combination group after 30, 60, 90 minutes resulted in p=0.252, p=0.068 p=0.984 respectively. It means that all the treated groups both PH and PH, AC showed a normal distribution. Then it was followed by t-test to find out if there were significant difference in Phalerin level between PH group and combination group toward time of observation (figure 2-4).

The statistical analysis showed a significant difference in the level of phalerin at minute 30, 60 minutes and 90 between the group treated with *Phaleria macrocarpa* and combination of PH, AC with p value of < 0.05. This results showed that combination group has undergone an

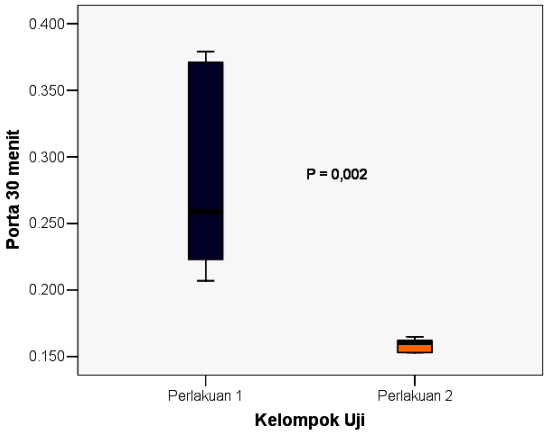

Figure 2. Boxplot of the phalerin level in PH group and PH, AC group at minute 30 in portal vein.

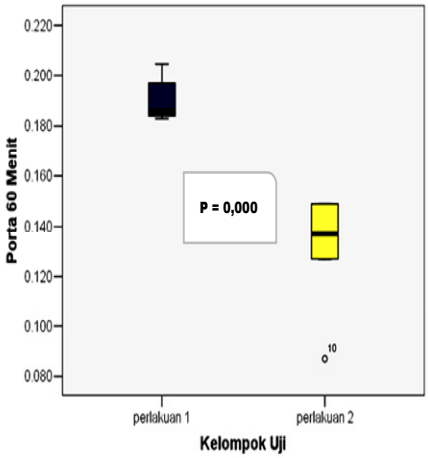

Figure 3. Boxplot of the phalerin level in PH group and PH, AC group at minute 60 in portal vein.

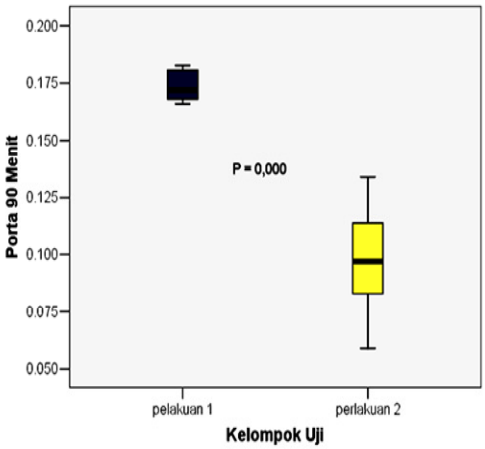

Figure 4. boxplot of the phalerin level in PH group and PH, AC group at minute 90 in portal vein.

absorption disturbance compared to the group treated with PH, indicated by the higher level of Phalerin. In other words, there was an absorption disturbance on the intestinal mucosa. The physical factors affecting the absorption include blood stream at the absorption site, the absorption area, the contact time with the surface area of absorbtion. According to Lipinski's Rule of Five, molecules with molecular weight above 500 is likely to have a poor absorption and permeability, lead to the disturbance of intestinal function, trigger the secretion of electrolyte and intestinal damage (Cameiro, 2004). One of the substance on the combination group is Doxorubicium leading to the gastrointestinal mucosal disturbance (Goodman and Gilman, 2006)

CONCLUSION

The level of Phalerin from *Phaleria macrocarpa* in the group treated with PH is higher than that of combination group (PH, ADH, CYC). There is a significant different among the group with $p<0.05$.

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