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The effect of co-chemotherapy of ethyl acetate fraction of Long jack roots (*Eurycoma longifolia* jack) on interleukin-6 expression of 7,12-dimethylbenz(α)anthrasen breast carcinoma model

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ABSTRACT

Background: Carcinogenic compound can cause cancer, one of which is 7,12- Dimethylbenz(α)anthrasen (DMBA). Therapy used is chemotherapy using doxorubicin. However, doxorubicin can cause side effects and not specific enough, so it can also impair normal cells and cause resistance, so Longjack roots are needed as co- chemotherapy agent.
Objective: To assess the effect of giving co-chemotherapy ethyl acetate fraction of Long jack roots and doxorubicin on the development of breast cancer through inhibition interleukin-6 expression.
Methods: A total of 35 female rats SD strain were divided into 5 intervention group, each group contained 7 rats, namely Group 1 (baseline given feed and water), Group 2 (DMBA 20 mg / kg given 2x a week for 5 weeks), Group 3 (DMBA 20mg / kg + Long jack roots Ethyl acetate fraction 100 mg / kg), Group 4 (DMBA 20 mg / kg + Doxorubicin 1,17mg / kg), Group 5 (DMBA 20 mg / kg + Long jack roots ethyl acetate fraction 100 mg / kg + Doxorubicin 1,17mg / kg), then on the 16th week, surgery was done to remove animal's mammae and organs and to conduct immunohistochemistry test with anti interleukin-6.
Results: Interleukin-6 expression test result% with the average value of group I, II, III, IV and V respectively, 6.56 ± 3.50%; 47.21 ± 9.48%; 20.83 ± 10.40%; 49.39 ± 9.87% and 58.37 ± 17.32%.
Conclusion: The administration of co-chemotherapy ethyl acetate fraction of longjack roots (Eurycoma longifolia Jack) and doxorubicin is effective in lowering aromatase expression, however not effective in reducing IL-6 expression compared to single regiment doxorubicin in DMBA-induced rat models.

Latar Belakang: *Senyawa karsinogen yang dapat menyebabkan kanker salah satunya adalah 7,12-Dimethylbenz(α)anthrasen (DMBA). Terapi yang biasa digunakan untuk mengatasi hal tersebut dengan kemoterapi menggunakan doxorubicin. Namun, doxorubicin dapat menimbulkan efek samping dan tidak spesifik, sehingga dapat merusak sel tubuh normal dan dapat menimbulkan resistensi sehingga perlu penambahan akar pasak bumi sebagai agen ko-kemoterapi.*
Tujuan Penelitian: *Penelitian ini bertujuan untuk mengetahui efek pemberian ko-kemoterapi fraksi etil asetat pada akar pasak bumi.*
Metode: *Sebanyak 35 ekor tikus betina galur SD dibagi dalam 5 kelompok perlakuan masing-masing kelompok dibagi 7 ekor tikus yaitu kelompok 1 (baseline diberikan pakan dan minum), kelompok 2 (DMBA 20 mg/kgBB diberikan 2x seminggu selama 5 minggu), kelompok 3 (DMBA 20mg/kgBB + Fraksi Etil Asetat Akar Pasak Bumi 100mg/kgBB), kelompok 4 (DMBA 20mg/kgBB+Doxorubicin 1,17mg/kgBB), kelompok 5*

(DMBA 20mg/kgBB + fraksi etil asetat akar pasak bumi 100mg/kgBB + Doxorubicin 1,17mg/kgBB), kemudian dilakukan pembedahan pada minggu ke-16 dan pengambilan organ payudaranya untuk dilakukan uji imunohistokimia dengan antibodi anti-IL-6.

Hasil: *Hasil pengujian % ekspresi IL-6 dengan nilai rata-rata pada kelompok I, II, III, IV dan V berturut-turut yaitu 6,56±3,50%; 47,21±9,48%; 20,83±10,40%; 49,39±9,87% dan 58,37±17,32%.*

Kesimpulan: *Ko-kemoterapi fraksi etil asetat akar pasak bumi dan Doxorubicin meningkatkan ekspresi IL-6 pada tikus galur Sprague Dawley yang diinduksi DMBA.*

INTRODUCTION

7,12-dimethylbenz(a)anthracene (DMBA) compound is carcinogenic and contributes in altering cell cycle thus causing mutation.[1] One chemotherapy agent used to decrease mortality is Doxorubicin. However, doxorubicin can cause side effects and are not specific. Thus it can destroy normal cells and cause resistance.[2] Doxorubicin is also reported to increase IL-6 expression in urine and kidney tissue.[3] Based on this, a co-chemotherapy agent is needed as a non-toxic or less-toxic chemoprevention to improve its efficacy by decreasing its toxicity towards normal cells.[4] Herbal ingredients can increase cell sensitivity to chemotherapy agent with minimal side effects.[5,6]

The combination of ethyl acetate fraction of Long jack roots and doxorubicin can decrease nodules and prevent adenocarcinoma mammae.[7,8] The combination of ethyl acetate fraction Long jack roots (*Eurycoma longifolia* Jack.,) with Doxorubicin has the synergistic effect on apoptosis, antiproliferation, and RAS expression on T47D cell.[9] A cytotoxic mechanism or chemopreventive of a compound can be assessed by measuring its effect on protein or gene expression that affect the cell cycle in both qualitative and quantitative.[10,11] UV radiation causes tissue inflammation marked by elevated IL-6 concentration, which is an apoptosis mediator.[12]

METHODS

Long jack roots determination (*Eurycoma longifolia* Jack)

The sample used in this study is Long jack roots from Banjarmasin, Kalimantan Selatan that was made into powder and dried in Laboratorium Teknologi Sediaan Farmasi UAD.

Extraction and fractionation of ethyl acetate longjack roots

Long jack root was extracted with ethanol solvent 70%. Thick ethanol solvent was diluted with 100 ml of hot water, continuously stirred until diluted and homogenous. Then inserted into a separating funnel, fractionated with liquid-liquid extraction using ethyl acetate solvent. 50 gr of the ethanol extract was fractionated using 20 mL ethyl acetate which was done by sparking and pouring five times. This fraction was then concentrated to obtain soluble and insoluble ethyl fraction in ethyl acetate. The results of soluble ethyl acetate fraction were qualitatively analysed to determine its chemical compound using *eurycomanone* standard.

Making of DMBA Solution

DMBA solution was prepared by diluting some DMBA according to dosage, with corn oil until the expected concentration so that when given to animal model will produce 0,5–1,5 ml volume.[13]

Intervention to Animal Model

35 healthy female *Sprague Dawley* rats, age two-monthold, was randomly assigned to 5 cages. Every cage consisted of 7 rat models.

Acclimatization was done for two weeks to homogenise rat's way of life, feeding, and conditioned with intervention. The Rat fed AD 1 and AD 2 feed and water ad libitum. Rat's bedding was sterilised coarse sawdust, changed twice a week.

Rat models in this study were 35 rats randomly assigned to 5 groups, seven rats each. Group I (normal) only fed standard feed (ADI/ ADII) and water ad libitum. Group II was given DMBA (20 mg/kgBW) from first to the fifth

week. Group III was induced with DMBA (20 mg/kgBW) and given Doxorubicin (1,17 mg/kgBW). DMBA compound was given twice a week every Tuesday and Friday from first to the fifth week, while Doxorubicin was given once a week from sixth to the tenth week. Group IV was induced with DMBA (20 mg/kgBW) then given intervention ethyl acetate fraction of Long jack roots (100 mg/kg BW) peroral. DMBA compound was given twice a week every Tuesday and Friday from first to the fifth week. Ethyl acetate fraction was given everyday from sixth to the eleventh week. Group V was given DMBA (20 mg/kgBW), Doxorubicin (1,17 mg/kgBW), and ethyl acetate fraction of Long jack roots (100 mg/kg BB). Palpation was done to every intervention groups twice a week.

Tissue Fixation

Mammillary organs obtained from dissection is cut accordingly. Mammillary organ with the size of 1x1x1 cm was fixed with formalin 10%. The strength of the fixation solution and length of fixation would affect results. Fixation was done for 24 hours. The mammillary organ was fixed with formalin (not more than 4% formaldehyde), dehydrated with alcohol 96; 80; 90; and 95% for 2 hours each. Results were cleared with benzene twice, for 1,5 hours each and infiltrated with liquid paraffin (58ºC/ not more than 60ºC), dried overnight in 37ºC temperature. Drying results were heated in an oven in 50-56ºC temperature for 1 hours and blocked and cut with a microtome to 4-5 μm thickness. Cutting results were mined with warm water to make the surface even and covered on to warm water mirror above hot plate (45ºC) for 0,5 hour.[14]

Parafin Section and preparation

The blocking was done to maintain morphology and resolution detail of the tissue. Fixed tissue was sectioned into certain size and mould into cubes 1x1x1 cm using paraffin. Liquefied parafin can infiltrate tissue spaces so that when it's solid, it could maintain tissue morphology. The preparation was made by cutting paraffin blocks in 4 μm density and attaching it to glass poly-l-lysine, for further staining.

Immunohistochemistry Staining Method

This study used mouse monoclonal antibody-IL-6. Procedure used for immunohistochemistry in this research is the procedure of Laboratorium Patologi Anatomi, RSUP Dr Sardjito Yogyakarta.

Data Analysis

Data in this study was obtained from tissues expressing IL-6, expressed genes were stained brown, and not-expressed genes were stained purple or blue, as well as the total quantity of cells. The observation was done using the optilab microscope with 400x magnification in 6 fields of views. The percentage of each group were statistically analysed using SPSS 19 for windows. Started with Kolmogorov Smirnov test to determine data distribution. If data distribution was normal with homogeneous variance, the analysis was done using one way ANOVA to determine the difference between groups. If the result is different, analysis continued using the LSD test, with a 95% confidence interval. If data were not distributed normally and not homogeneous, analysis was done using Kruskal-Wallis and Mann-Whitney with a 95% confidence interval.

RESULTS

Qualitative analysis for longjack roots compound (*Eurycoma longifolia* Jack)

The qualitative analysis used in this study was thin layer chromatography (TLC). This method was done to determine whether or not the ethyl acetate fraction of Long jack roots contained *eurycomanone*. The results of ethanol extract chromatogram showed more spots than ethyl acetate fraction; this is because ethanol solvent is a semipolar solvent that can filtrate a lot of compounds thus fractionation process was more spesific. Ethy acetate fraction and ethanol extract had the same Rf on the first spot, which was 0,55. This means that ethyl acetate fraction ethanol extract of Long jack roots contained eurikomanon, consistent with previous study by

Arifah dan Nurkhasanah (2014) that showed the standard eurikomanon had the same Rf which was 0,55.[15]

Immunohistochemistry test

The observation of IL-6 expression on the mammillary gland was done using immunohistochemistry. Observation was done in six fields of views using the microscope in 400 x magnification equipped with Optilab, and the percentage was counted. In Figure 1. It can be seen the expression of IL-6 in various intervention groups. In Figure 1. It can be seen the results of immunohistochemistry IL-6.

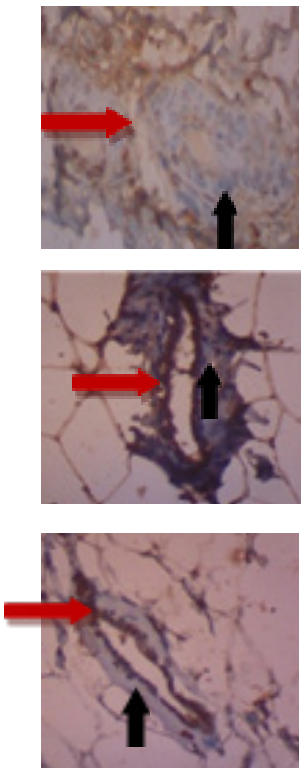

Figure 1. IL-6 expression on acynus epithelium from glandula (A) Group DMBA+*Doxorubicin* (B) Group DMBA+APB (C) DMBA+*Doxorubicin*+APB (staining antibody anti-IL-6, magnification 400X). Red arrow shows positive expression, black arrow shows negative expression.

Immunohistochemistry staining was done with the indirect method. Antigen was found to primary antibody directly, then secondary antibody was added which would bind to enzymes like peroxidase, alkali phosphatase, or glucose oxidase. Secondary antibody will bond with a primary antibody. Next, chromogen substrate was added which would be changed b enzyme into pigment.[14]

Measurement of IL-6 in this study used the amount of expressed gene cells divided by the total amount of cells times 100%.[12] The percentage of IL-6 expression in each group was then statistically anayse.

Data analysis started with normality and homogeneity test with the confidence interval of 95%. Normality test used *Kolmogorov Smirnov* and *homogeneity* test used Levene test. Table 1 showed the results of the statistical analysis of IL-6 expression.

Table 1. Mean of percentage IL-6 expression in female *Sprague Dawley* rats induced with DMBA and given different intervention

Groups	Percentage IL-6 expression (mean % + SD)
DMBA+Doxorubicin	20,38 ± 10,40
DMBA+APB	49,39 ± 9,87
DMBA+Doxorubicin+APB	58,37 ± 17,32

n=4, observed through 6 field of views with 400x magnification

DISCUSSION

IL-6 production has been found in various human tumour.[16] Interleukin-6 bind to the heterodimeric receptor, containing ligand that binds to IL-6α chain and cytokine receptor that transduces subunit gp130. IL-6 receptor involvement will induce JAK Tyrosin Kinase activation which then stimulates several pathways involving MAPKs, PI3Ks, statistic, and other signalling protein.[17]

Hakkak et al., (2005) reported that to be active, DMBA (as procarcinogen) is changed by cytochrome p-450 into the reactive metabolite, dihydrodiolepoxyde.[18] This metabolite can bind with DNA and causes mutation. CYP (*cyclophilin*) enzymes, like CYP1A1 and CYP1b1 in peripheral tissue (like mammillary tissue) and liver CYP1A2, is the enzyme that catalyse this reaction. In the DMBA-induced *mamillary tumour*, cyclin D1 and NF-kβ binding concentration increase, which are the transcription factors in cell

cycle regulation, related to cell proliferation.[19] Doxorubicin acts by intercalating with DNA, directly will affect transcription and replication. The use of doxorubicin efficacy is also decreasing due to drug resistance phenomenon. The mechanism that causes doxorubicin resistance is overexpression of p glicoprotein (PgP) which causes doxorubicin to be pumped out of cells thus reducing its concentration. Other biochemical changes in doxorubicin-resistant cells are increased gluthatione peroxidase activity, increased activity and mutation of topoisomerase II, as well as increased cell ability to repair DNA damage.[20]

Generally, doxorubicin is used in combination with other anticancer agents like cyclophosphamide, cisplatin, and 5-FU. Increased clinical response and reduced side effects are better in combination therapy compared to single regiment doxorubicin.[20]

IL-6 concentration increases along with tumor growth and *mammillary tumour* produce compared to normal mammillary tissue.[21] Interleukin-6 secreted by malignant cells has been proven to contribute to self-seeding process where tumour cells aggressively circulate within themselves.[22] Interleukin-6 promote the growth of cancer cells by activating STAT3 which will ultimately regulate oncogenic proliferation such as c-Myc and cyclin D1 as well as growth factor such as hepatocyte growth factor (HGF), VEGF and epidermal growth factor (EGF).[23]

Disturbance in DNA double-strand repair process will trigger cell damage, while overexpression of transcription for DNA repair may be involved in the drug-resistant phenomenon. Doxorubicin, with the presence of its quinone cluster, can also produce free radicals in both normal and cancerous cells.[24]

Chronic toxicity of doxorubicin might be facilitated by its metabolic conversion which involved various enzymes, such as carbonyl reductase. The primary mechanism of doxorubicinol toxicity occured due to its interaction with iron and the formation of reactive oxygen species (ROS) which damage cells macromolecules.[25]

CONCLUSION

Co-chemotherapy of Long jack roots ethyl acetate fraction and doxorubicin increases IL-6 expression in DMBA induced Sprague Dawley rats.

CONFLICT OF INTEREST

We declare that there is no conflict of interest.

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