

Effects of co-chemotherapy ethyl acetate fraction of *Eurycoma longifolia* jack roots and doxorubicin against apoptosis through expression p53 mutant and Bcl-2

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ABSTRACT

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Background: It was found mutations of p53 gene in Breast cancer. Mutant p53 protein caused a decrease in cell apoptosis mechanisms through increased expression of Bcl-2. Breast cancer therapy is commonly used chemotherapy using Doxorubicin. However, effectiveness of the use of this chemotherapeutic agent is limited due to the emergence of side effects and toxic to normal cells. Therefore, it is necessary to develop new drugs for combination of chemotherapy. *Eurycoma longifolia* Jack roots has the potential as co-chemotherapy of breast cancer and it is not toxic to normal cells.

Method: Rats were divided into 5 groups. Each group consisted of four female white Sprague Dawley rats. Group 1 (Normal), group 2 (DMBA 20 mg/kgB.W), group 3 (DMBA +Doxorubicin 1.12 mg/kgB.W), group 4 (DMBA +fractions 100 mg/kgB.W), group 5 (DMBA+Doxorubicin +fractions). All the rats were sacrificed at weeks 16 and to be taken their breast tissue. Immunohistochemistry was performed using a mouse monoclonal antibody mutant (BioGenex) and Bcl-2 (BIOSS).

Results: expression of mutant p53 percentage obtained for group I (9.35 ± 0.32)%, II (21.65 ± 1.60)%, III (10.72 ± 2.52)%, IV (11.63 ± 3.39)%, V (12.72 ± 3.44)%, While the percentage of Bcl-2 expression obtained for I (20.62 ± 10.09)%, II (52.83 ± 3.61)%, III (24.38 ± 3.54)%, IV (38.01 ± 6.25)%, V (27.99 ± 4.27)%. The data was statistically tested by Kruskal Wallis test (p< 0.005).

Conclusion: Co-chemotherapy of *E. longifolia* Jack roots and Doxorubicin can stimulate apoptosis through decreased in the expression of mutant p53 protein and Bcl-2 in breast tissue of rats induced by DMBA.

Latar Belakang: Mutasi pada gen p53 dapat dijumpai pada kanker payudara. Protein p53 mutan menyebabkan penurunan mekanisme apoptosis sel melalui peningkatan ekspresi Bcl-2. Terapi kanker payudara yang umum digunakan adalah kemoterapi dengan menggunakan Doxorubicin. Efektivitas penggunaan agen kemoterapi ini menjadi terbatas karena munculnya efek samping dan toksik pada sel normal. Oleh karena itu, perlu dikembangkan obat baru untuk kombinasi kemoterapi. *Eurycoma longifolia* Jack roots (akar pasak bumi) mempunyai potensi sebagai ko-kemoterapi kanker payudara dan tidak toksik pada sel normal.

Tujuan: Penelitian ini bertujuan untuk mengetahui efek ko-kemoterapi fraksi etil asetat *Eurycoma longifolia* Jack roots dan Doxorubicin terhadap apoptosis melalui ekspresi p53 mutan dan Bcl-2.

Metode: Tikus dibagi menjadi 5 kelompok. Masing-masing kelompok terdiri dari 4 ekor tikus putih betina Sprague dawley. Kelompok 1 (Normal), kelompok 2 diberikan DMBA (20 mg/kgBB), kelompok 3

(DMBA +Doxorubicin 1,12 mg/kgBB), kelompok 4 (DMBA+Fraksi 100 mg/kgBB), kelompok 5 (DMBA +Doxorubicin+Fraksi). Semua tikus dikorbankan pada minggu 16 dan diambil jaringan payudaranya. Imunohistokimia dilakukan dengan menggunakan mouse monoclonal antibody p53 mutan (Biogenex) dan Bcl-2 (Bioss).

Hasil: Persentase ekspresi p53 mutan yang diperoleh pada kelompok I ($9,35 \pm 0,32$ %), II ($21,65 \pm 1,60$ %), III ($10,72 \pm 2,52$ %), IV ($11,63 \pm 3,39$ %), dan V ($12,72 \pm 3,44$ %). Persentase ekspresi Bcl-2 I ($20,62 \pm 10,09$ %), II ($52,83 \pm 3,61$ %), III ($24,38 \pm 3,54$ %), IV ($38,01 \pm 6,25$ %), dan V ($27,99 \pm 4,27$ %). Data diuji statistika dengan menggunakan uji Kruskal Wallis ($p < 0,005$).

Kesimpulan: Ko-kemoterapi fraksi etil Eurycoma longifolia Jack roots dan Doxorubicin dapat memacu apoptosis melalui penurunan ekspresi protein p53 mutan dan Bcl-2 pada jaringan payudara tikus yang di induksi DMBA.

INTRODUCTION

Mutations of p53 gene (Tumor Suppressor Gene) is common in the variety of malignancies, one of it is in breast cancer.¹ Loss of p53 function due to the mutations may cause malignant transformation, tumor spread and tumor resistance to therapy that induces DNA damage.² The main function of protein 53 is for a cessation of cell cycle and initiation of apoptosis induced by DNA damage.³ The process of apoptosis is controlled by a balance between pro-apoptosis and anti-apoptosis genes. Among that genes control apoptosis which the most instrumental is BCL-2 gene that function to protect tumor cells from apoptosis process that allows the cells to keep growing.⁴ Breast cancer treatment using chemotherapeutic agents is still an option in the treatment of cancer. Chemotherapeutic agent commonly used in the treatment of breast cancer is Doxorubicin.⁵ The use of Doxorubicin chemotherapeutic agent becomes limited due to the problem of its toxic effects on normal tissues of the body and is also not selective.⁶

The potential approach in addressing the side effect of chemotherapy is by using co-chemotherapy agents. One of the agents potentially used in combination with chemotherapeutic agents is based on natural

materials.⁷ A natural material that has potential as a co-chemotherapy agent is the root of Eurycoma longifolia Jack. E. longifolia Jack root has a bioactive compound that is quassinoid. The main content of the quassinoid is eurycomanone which has anti-cancer effects.⁸

Some in vitro studies have proved that ethanol extract of E. longifolia Jack root combined with Doxorubicin by in vitro may enhance the effects of Doxorubicin work to be more synergistic with the increasing expression of p53 triggers apoptosis process.⁹ Ethyl acetate fraction of ethanol extract of E. longifolia Jack root and Doxorubicin as co-chemotherapy in an in vitro manner has the activity to inhibit cell T47D through the mechanism of increasing expression of Bax and decreasing expression of Bcl-2.¹⁰ Ethanol extract of E. longifolia root in breast cancer of female Sprague-Dawley (SD) rats at a dose of 400 mg/kgB.W were administered before and during induction of DMBA is able to decrease the expression of mutant p53 protein (pro-apoptosis) and inhibit the breast cancer growth in female Sprague-Dawley rats were induced by DMBA.¹¹ Mutations or abnormalities of p53 gene lead to absenteeism activation of the Bax apoptotic gene. The occurrence of inactivation in the Bax caused the cells do not undergo apoptosis. Besides, changes in the inhibition function of BCL-2 apoptotic gene would lead to an increase in gene expression or overexpression of the gene, resulting in the cells to do not undergo apoptosis more and more.³ It needs to do research to find out that the co-chemotherapy ethyl acetate fraction of E. longifolia root can lower the expression of mutant p53 and Bcl-2.

METHODS

Materials and tools

The test materials used were Eurycoma longifolia Jack plant from Martapura, South Kalimantan, CMC-Na 0.5%, corn oil (Superindo 365), 7.12 dimetilbenz(a)antrasen (DMBA) 20 mg/kgB.W (SIGMA-Aldrich), Doxorubicin (OGB Sanbe 2 mg/ml), 70% ethanol (Genera Labora), formaldehyde 10%, and NaCl physiology.

Experimental animals that used in this study were female Sprague Dawley (SD) rats with a body weight of 100-200 grams and 2 months old.¹² The tools that used were magnetic stirrer (LABINCO), Jar, analytical balance (HWH), rotary evaporator (Heidolph), a set of surgical tools, oven (Binder), chamber (Larrag), water bath (Memmert), glass tools (Pyrex), an optilab which is connected to a computer (OLYMPUS DP12).

Research Procedures

This study is divided into two phases, that are the making ethyl acetate fraction of *E. longifolia* Jack and the test of carcinogenesis.

Making ethyl acetate fraction from *E. longifolia* Jack roots

Identification of *E. longifolia* Jack plant was conducted in the Laboratory of Biology, Faculty of Pharmacy, University of Ahmad Dahlan, Yogyakarta. Simplicia powders of *E. longifolia* Jack roots were weighed each about 100 grams and then separately extracted with 70% ethanol using maceration method. The ethanol extracts viscous diluted with hot water of 100 ml, stirring constantly, until dilute and homogeneous, then put in a separating funnel, fractionated by liquid-liquid extraction with ethyl acetate solvent. As much 50 grams of ethanol extract was fractionated using 20 mL ethyl acetate and was done by precipitating-and-pouring by 5 times. The fraction is then concentrated to obtain the soluble fraction and insoluble fraction in ethyl acetate.

Co-chemotherapy test ethyl acetate fraction of *E. longifolia* Jack roots in an in vivo manner

Rats were maintained in enough ventilated room, provided with food in the form of pellets and drink in the form of water sufficiently. The animals were adapted to the cage for 1 week prior to treatment. Animal trials have obtained ethics approval letter (Ethical Approval) of the Research Ethics Committee of the University of Ahmad Dahlan (UAD KEP) under number

011 505 044. The carcinogen solution of DMBA (Sigma-Aldrich) diluted with corn oil. DMBA solution administered orally to the experimental animals with a dose of 20 mg/kg b.w. for 5 weeks with administration 2 times a week. DMBA solution always prepared immediately, before being given to the experimental animals.¹³ Ethyl acetate fraction of *E. longifolia* roots according to the dose suspended in a solution of 0.5% CMC-Na. Solution of ethyl acetate fraction of *E. longifolia* roots in 0,5% CMC-Na is always to be newly prepared prior to administration to the experimental animals. A single dose that used is 100 mg/kg b.w.¹⁴

Female Sprague-Dawley rats aged 2 months were divided into 5 groups. Each group consisting of 7 female Sprague-Dawley rats. Group 1 (Normal), group 2 DMBA 20 mg/kg b.w., group 3 DMBA 20 mg/kg B.W+Doxorubicin 1.12 mg/kg b.w, group 4 DMBA 20 mg/kg b.w+Ethyl acetate fraction of *E. longifolia* root 100 mg/kg b.w, and group 5 DMBA 20 mg/kg b.w +Doxorubicin of 1.12 mg/kg+Ethyl acetate fraction of *E. longifolia* roots 100 mg/kg b.w Observation of tumors incidence conducted by palpation at week 6 of treatment. DMBA administration time and treatment with Doxorubicin as well as Ethyl Acetate Fraction of *E. longifolia* roots can be seen in Table I.

Immunohistochemical Staining and analysis

This study used a mouse monoclonal antibody p53 mutant produced by BioGenex and Bcl-2 produced by Bioss. Procedures used for immunohistochemistry in this study was the procedure of Pathology of Anatomy Laboratory, Dr. Sardjito Generale Hospital.

The data obtained in this experiment is the number of cells expressed mutant p53 antibody and Bcl-2. Genes that expressed it were brown while those not expressed it were purple or blue and total cell number. Furthermore, based on the data it was computed in percent (%) immunohistochemical expression that was

Table 1. Timing of DMBA administration and treatment with Doxorubicin as well as Ethyl Acetate Fraction of *E. longifolia* root

Week	Fraction Treatment	Doxorubicin Control	DMBA Control	Normal
1-5			2 x 1 a week DMBA in corn oil orally	Feed + aquadest
6-10	Every day it was administered with ethyl acetate fraction of the <i>E. longifolia</i> root	Doxorubicin 1x1 a week intraperitoneally		Feed + aquadest
10-16	Every day it was administered with ethyl acetate fraction of the <i>E. longifolia</i> root			Feed + aquadest

calculated using the formula:

$$\% \text{ cell expression} = \frac{\text{number of cell expressed}}{\text{number of total cell}} \times 100\%$$

Percent expression of each group were statistically analyzed using SPSS 19 for windows. Statistical analysis of the results of mutant p53 and Bcl-2 immunohistochemistry value is done by using a nonparametric statistics Kruskal-Wallis test followed by using the Mann-Whitney level of 95 %.

RESULTS

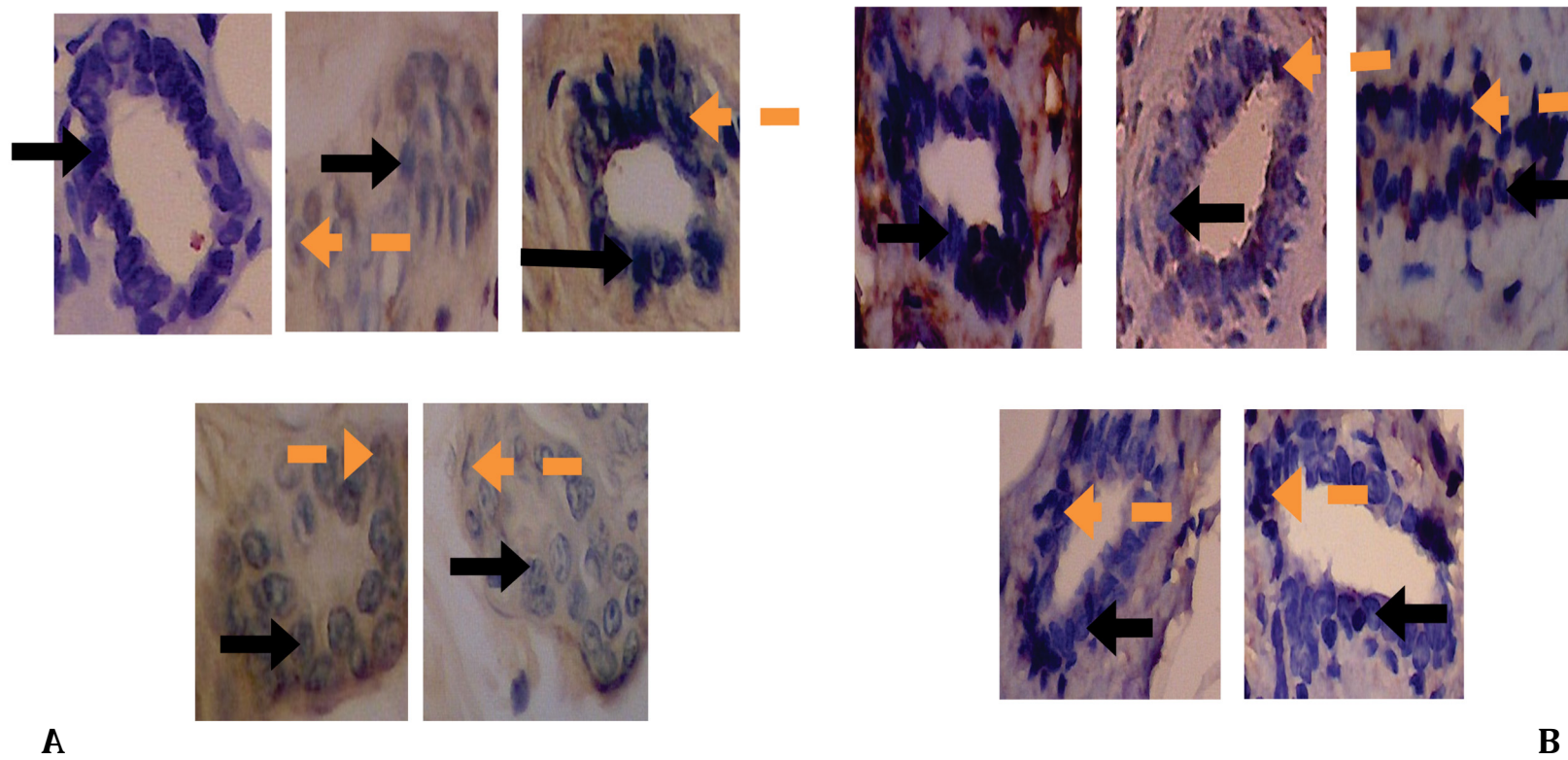
The extract obtained from 5 kg of dry powder of *E. longifolia* roots were extracted with 70% ethanol solution using maceration to produce ethanol extract of *E. longifolia* roots as much 121.36 grams with a yield of 2.92%. Subsequently, the extract was fractionated using ethyl acetate. Selection of the ethyl acetate was be based on the differences of polarity with ethanol to reduce the compounds component were extracted in the ethyl acetate solution.¹⁵ Ethyl acetate fraction of *Eurycoma longifolia* Jack roots has been proven to have high cytotoxic activity against human breast cancer cell in line-MCF-7 and selective against cells of normal breast MCF-10A.¹⁶ A total of 50 g of ethanol extract of *E. longifolia* roots was fractionated using 20 mL ethyl acetate and it was done through precipitating-and-pouring

by 5 times.

The results of DMBA induction at a dose of 20 mg/kg body weight by 10 times administration in observation for 16 weeks after the last administration of DMBA during palpation and nodules observations was not formed. It is likely due to the rats which used were old enough of 2 months. Appropriate age for the induction of mammary carcinogenesis is 1.5 months old due to it is an early puberty.¹² Microscopically, based on histopathological analysis the DMBA-induced rats showed the presence of epithelial hyperplasia and they underwent adenocarcinoma. The rats also suffered from bleeding on the nose and nails. This is due to the toxic effects of DMBA and Doxorubicin is already noticeable. The bleeding is presumably related to the side effects of DMBA that effects on the increasing production of VEGF and the occur of angiogenesis. Angiogenesis is the formation of new blood vessels functioning to supply nutrients and oxygen to cancer cells so that it can develop continuously.¹⁷

Expression of mutant p53 protein and Bcl-2 was observed after 16 weeks of administration of treatment using a microscope with a magnification of 400x which complemented with optilab.

In the DMBA group when it is compared to the healthy group showed differences in its color. Cells figure expresses the mutant p53 protein and Bcl-2 in breast tissue of rats shown in Figure 1.



Description: Expressed to be negative ———>
 Expressed to be positive - - - ->

Figure 1. (A) Microscopic photo of 400x magnification mutant p53; (B) Microscopic photo of 400x magnification BCL-2 (1) Normal Control (2) Group DMBA 20 mg/kg b.w (3) Group DMBA 20 mg/kg b.w+Doxorubicin 1,12 mg.kg b.w (4) DMBA 20 mg/kg b.w + Fractions 100 mg/kg b.w (5) DMBA 20 mg/kg b.w + Doxorubicin 1.12 mg/kg b.w + fractions 100 mg/kg b.w

The cells express the mutant p53 protein will appear brown-colored in the cytoplasm of that cells and the cells express Bcl-2 protein would appear colored brown.^{14,18} Percentage analysis of the mutant p53 and Bcl-2 expression was calculated by calculating the number of cells that

expressed divided by the number of total cells multiplied by 100%. The observation result of the percentage of mutant p53 protein and Bcl-2 expression after being treated can be seen in Table II.

Table II. Observation results on the percentage mean of mutant p53 protein and Bcl-2 expression after being treated with Doxorubicin and Ethyl Acetate Fraction of *E. longifolia* Roots in DMBA-Induced Rats

Group	Percentage of expression of mutant p53 (%) ± SD	Percentage of expression of Bcl-2 (%) ± SD
Normal	9.35 ± 0.32	20,62 ± 10,09
DMBA	21.65 ± 1.60	52,83 ± 3,61
DMBA + Doxo	10.72 ± 2.52	24,38 ± 3,54
DMBA + FAPB	11.63 ± 3.39	38,01 ± 6,25
DMBA + Doxo + FAPB	12.72 ± 3.44	27,99 ± 4,27

The significance value obtained in the Kruskal-Walis test was 0.000 (p < 0.05), followed

by Mann Whitney test, results can be seen on Table III.

Table III. Mann-Whitney test results of the expression levels of mutant p53 in normal group, DMBA 20 mg/kg b.w, DMBA 20 mg/kg b.w + Doxorubicin 1:12 mg/kg b.w, DMBA 20 mg/kg b.w + Fraction 100 mg/kg b.w, DMBA 20 mg/kg b.w + Doxorubicin 1:12 mg/kg b.w + Fractions 100 mg/kg b.w

Group	significance of mutant p53	interpretation of mutant p53	significance of Bcl-2	interpretation of Bcl-2	
Normal	DMBA	0,000	significant difference	0,000	significant difference
	DMBA + Doxo	0,160	not significant	0,887	not significant
	DMBA + Frac	0,378	not significant	0,000	significant difference
	DMBA + Doxo+Frac	0,007	significant difference	0,128	not significant
DMBA	DMBA + Doxo	0,000	significant difference	0,000	significant difference
	DMBA + Frac	0,000	significant difference	0,000	significant difference
	DMBA + Doxo+Frac	0,000	significant difference	0,000	significant difference
DMBA + Doxo	DMBA + Frac	1,000	not significant	0,000	significant difference
	DMBA + Doxo + Frac	0,242	not significant	0,520	not significant
DMBA + Frac	DMBA + Doxo + Frac	0,198	not significant	0,000	significant difference

DISCUSSION

Protein 53 has a crucial role in controlling the cell cycle, apoptosis and maintaining genomic stability. Loss of p53 function as a result of mutations may cause malignant transformation, tumor spread and tumor resistance to therapy that induces DNA damage. Mutations of p53 will produce an abnormal protein with very long half-life, caused the accumulation of these products, whose its expression can be detected by immunohistochemistry.² Most of the mutation of genes is missense mutation causing amino acid substitutions in the wild-type protein. A missense mutation causes a change in one amino acid and the type of this point mutation in the DNA-binding domain of p53 (exons 5 to 8) can coded proteins transcriptionally inactive or show aberrant transcriptional activity compared to normal or wild-type p53. Although the normal cells also generally have lower levels of protein 53 as a result of the half-life is shorter than wild-type proteins, missense mutation of p53 often encodes a protein product that resistant to degradation, and as a result, mutant p53 protein accumulates in the nucleus. This gene mutation causes the loss of function of the gene and it is the basis of changes in the occurrence of cancer. The main function of the protein p53 is to terminate cell cycle and initiate apoptosis triggered by DNA damage. The process of apoptosis is controlled by a balance between pro-apoptosis and anti-apoptosis genes. Among the genes that control apoptosis which the most instrumental is BCL-2 genes that protect tumor cells from apoptosis.

Figure 1. Results of research on groups of DMBA when compared to the normal group showed a difference of color. This indicates that the DMBA-induced test animals expressing mutant p53 for DMBA reactive metabolites to form DNA adducts and cause mutations mutation occurs in the gene p53 in mammary.^{19,20} p53 mutations can lead to increased expression of Bcl-2.²¹

The percentage of mutant p53 expression and Bcl-2 in the DMBA group than the healthy group indicate a percentage value that is greater than the normal group as shown in Table II and there

is a significant differences between the normal group and the group DMBA 20 mg/kg b.w as shown in Table III, in the expression of mutant p53 and Bcl-2 is shown with a significance value of 0.000 ($p < 0.05$). DMBA group of 20 mg/kg b.w of mutant p53 expression discount percent higher compared with the normal group in the amount of 21.65 ± 1.60 and for the normal group amounted to 9.35 ± 0.32 . This shows that the reactive metabolites of DMBA will cause damage to DNA.¹³ Damage to DNA can cause mutations in the p53 gene, p53 inactivation can lead to increased expression of Bcl-2. In the group of doxorubicin administration decreased the percentage of expression of mutant p53 and Bcl-2. It is proved that doxorubicin can decrease the expression of Bcl-2 with path induces the release of cytochrome c from mitochondria to the cytoplasm, activation of Caspase 9 and may induce apoptosis²² and can reduce the expression of mutant p53.²³ However Doxorubicin cause side effects such as changes in the use of cardiovascular,^{24;25} hepatotoxicity,²⁶ as well as their toxic effects on normal tissues of the body.²⁷ Furthermore, administration of co-chemotherapy ethyl acetate fraction of *E. longifolia* roots was observed for the purpose of reducing the side effects of Doxorubicin and improving the effectiveness of chemotherapy.

In the co-chemotherapy group ethyl acetate fraction 100 mg/kg + Doxorubicin 1.12 mg/kg shown to result in a significant difference with a significance value compared with DMBA 20 mg/kg + co-chemotherapy ethyl acetate fraction 100 mg/kg + Doxorubicin 1.12 mg/kg was 0.198 ($p < 0.05$). The percentage decrease in the expression of mutant p53 with co-administration of chemotherapy is not better with the provision of ethyl acetate fraction only. This is due to the low dose of ethyl acetate fraction used in co-chemotherapy, so the need to increase the dose of ethyl acetate fraction in order to maximize the effect of co-kemoetrapi in reducing the expression of mutant p53. Decreased expression of mutant p53 can induce the release of cytochrome c from mitochondria to the cytoplasm, activation of caspase 9 further activates another caspase such

as caspase 3 and 7, and caspase 3 will initiate apoptosis.²⁸

Effectiveness of chemotherapy can be seen by comparing groups of DMBA 20 mg/kg doxorubicin + 1.12 mg/kg to groups of DMBA 20 mg/kg + co-chemotherapy ethyl acetate fraction 100 mg/kg + Doxorubicin 1, 12 mg/kg. Statistical analysis showed a significant difference with a significance value of 0.128 ($p < 0.05$). However, the percentage decrease in the expression of Bcl-2 co-chemotherapy group was no better than the group with the administration of doxorubicin alone. It is caused by a combination of doxorubicin dose and ethyl acetate fraction is still not quite right, so that the decreased expression of Bcl-2 is better than the use of single doxorubicin. However, the use of co-chemotherapy still had an advantage compared to the use of single doxorubicin because doxorubicin has a lot of side effects on chronic usage that is irreversible, including the formation of congestive heart failure and can cause cardiotoxicity.²⁹ Doxorubicin is also capable of causing a fragmentation or normal cell DNA damage and subsequent effect on normal cell replication process. The ethyl acetate fraction does not cause damage to the heart's normal cells.¹¹ Thus the use of co-chemotherapy can reduce the side effects of doxorubicin and can trigger apoptosis with decreased expression of Bcl-2 and acts as an anti-cancer agent cytoselective for eurycomanone relatively non-toxic to normal cells.

The use of co-chemotherapy ethyl acetate fraction roots of the earth peg and Doxorubicin in this study were able to decrease the expression of mutant p53 and Bcl-2 that will happen activation Puma and Noxa. Both of these proteins activate the multidomain protein Bax or Bak. Activation of Bax and Bak by Puma and Noxa regulated by the anti-apoptotic protein Bcl-2, Bcl-xL, MCL1. On the condition of mutant p53 decreased, the expression of Bcl-2 also will decrease and an increase in the expression of Bax. Bax activation causes the release of cytochrome c to cytochrome c will form a complex with Apaf-1 and formed trimer Apaf-1/ cytochrome c/ procaspase 9

called apoptosom. Apoptosom formed to induce activation of procaspase 9 into caspase 9. Specific Aspartyl Cysteine Protease-9 (Caspase 9) further activates another caspase such as caspase 3 and 7, causing the cell to undergo apoptosis.¹³ This fraction can be further developed as a co-agent chemotherapy.

CONCLUSIONS

The conclusion of this study is that administrating co-chemotherapy of ethyl acetate fraction of the *E. longifolia* Jack roots and doxorubicin was able to induce apoptosis of breast cancer cells through the reduction of mutant p53 and Bcl-2.

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