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Research Article
Anticancer Activity of Pasak Bumi Root Extract (*Eurycoma longifolia* Jack) on Raji Cells

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Abstract

Background and Objective: The use of the roots of the pasak bumi (*E. longifolia* Jack) to treat cancer has been studied widely, however, the scientific basis of these plants used as an anticancer drug is widely unknown. The purpose of this study was to examine the anticancer activity of ethyl acetate and non-ethyl acetate fractions of pasak bumi roots in Raji cells. **Materials and Methods:** The cytotoxicity test is using the direct cell count method with trypan blue staining. The growth inhibition is using doubling time analysis of Raji cells. Observation of the apoptotic events of Raji cells used ethidium bromide staining, while observing the expression of p53 protein in Raji cells was done by immunohistochemical staining. **Results:** The results of the cytotoxicity and doubling time test showed that the activity of the non-ethyl acetate fraction was greater than that of the roots of pasak bumi. The lower concentration of non-ethyl acetate fraction of pasak bumi roots was able to delay the multiplication time of Raji cells which was greater than that of ethyl acetate. The results of the cytotoxicity and doubling time test showed that the activity of the non-ethyl acetate fraction was greater than that of the roots of pasak bumi. **Conclusion:** It can be concluded that the ethyl acetate and non-ethyl acetate fractions of the roots of pasak bumi have cytotoxic and antiproliferative activity on Raji cells, however they cannot induce apoptosis in Raji cells. The death of Raji cells is through the mechanism of inhibiting Raji cell proliferation as evidenced by an increase in p53 protein expression.

Key words: Pasak bumi, *Eurycoma longifolia* Jack, Raji cells, cytotoxic, antiproliferation, apoptosis, p53 protein

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Indonesia is a tropical country with more than 9000 species of plants that are efficacious for the treatment and most of them have been used as an alternative medicine to treat certain diseases such as malaria, diarrhea, fever, dysentery and cancer[1]. The use of plants as medicine has been done by nearly 80% of the world population, however scientific evidence has not been widely reported. It is necessary to carry out an inventory, screening, clinical tests, utilization and evaluation of various natural substances to their use.

The World Health Organization (WHO) states that 12% of the deaths that occurred out of 50 million deaths in 1997 were caused by cancer and two-thirds of that number occurred in developing countries including Indonesia. Cancer is a non-communicable disease that causes death at number two after cardiovascular disease[2]. On research at Dr. Sardjito Hospital between 1991-1995 showed that nasopharyngeal cancer ranks first of the top five malignant ENT tumors. In Indonesia, the exact number of cases has not been figured out but given the high frequency in several hospitals in Indonesia, the possibility high among indigenous Indonesians.

The existing cancer cures have not shown a remarkable results[3]. The use of anticancer chemotherapy has not provided optimal results because the drug works non-specifically so that it will cause other problems. Thus, it is necessary to find a more specific and sensitive anticancer.

There are quite several anticancer drugs derived from plants that have been researched and patented which have a positive impact on the prevention of various types of cancer. One of the medicinal plants that have been used empirically by the people traditionally to treat cancer is *E. longifolia* Jack. This plant is known by the local name of the pasak bumi (Kalimantan). Several studies have shown that the quassinoid of this plant can be used as an antiamoebic, cytotoxic, antitumor and antiplasmodial[4]. Research conducted by Sholikhah *et al.*[5] regarding the *in vitro* antiplasmodial and cytotoxic activity of the extract of the pasak bumi roots showed that methanol extract was the strongest extract with IC_{50} values varying from 0.6-1.9 mg mL^{-1} in the *P. falciparum* strain. Methanol extract also had the strongest in vitro toxicity with IC_{50} values ranging from 46.9-58.6 µg mL^{-1} in Hela cell culture. The methanol extract of the pasak bumi roots was then fractionated with ethyl acetate so a soluble fraction of ethyl acetate (ethyl acetate fraction) and an insoluble fraction of ethyl acetate (non-ethyl acetate fraction) was obtained. The anticancer activity of these two fractions of pasak bumi roots has not been widely reported. So, the purpose of this study was to examine the anticancer activity of ethyl acetate and non-ethyl acetate fractions of pasak bumi roots in Raji cells.

MATERIALS AND METHODS

Study area: This research project was conducted from April 20, 2019-March 20, 2020. Raji cell culture, cytotoxic, anti-proliferation and apoptosis tests were carried out at the Parasitology Laboratory of the Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Yogyakarta. Immunocytochemical test was conducted at the Laboratory of Anatomic Pathology, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University, Yogyakarta.

Chemical collection: Ethyl acetate and non-ethyl acetate fractions of pasak bumi (*E. longifolia* Jack) roots, provided by Prof. Dr. Mustofa, Apt, MKes. Media culture cell line: RPMI 1640 medium (Sigma-Aldrich, Saint Louis, Missouri, USA), Fetal Bovine Serum/FBS (Gibco, Grand Island, N.Y, USA), penicillin-streptomycin (Gibco, Grand Island, N.Y, USA), sodium bicarbonate (E. Merck, Darmstadt, Germany), hepes (Sigma-Aldrich, Saint Louis, Missouri, USA), distilled water (Laboratory of Biological Sciences, UGM), fungison (Gibco, Grand Island, N.Y, USA), NaOH (E. Merck, Darmstadt, Germany), HCl (E. Merck, Darmstadt, Germany). Viable dye: trypan blue (E. Merck, Darmstadt, Germany). The cell line used in this study was the Raji cell line, provided by Prof. D. Moss, QIMR, Brisbane, Australia. Chemicals for immunocytochemical testing are 0.5% hydrogen peroxide (E. Merck, Darmstadt, Germany), acetone (E. Merck, Darmstadt, Germany), anti p53 monoclonal antibody (Novocastra, Benton Lane, Newcastle, United Kingdom), Phosphate Buffer Saline (PBS) (E. Merck, Darmstadt, Germany), Novostain Super ABC kit (Novocastra, Benton Lane, Newcastle, United Kingdom), 3,3-diaminobenzidine tetrahydrochloride/DAB (Novocastra, Benton Lane, Newcastle, United Kingdom), hematoxylin (E. Merck, Darmstadt, Germany), ethanol (E. Merck, Darmstadt, Germany), whereas for the apoptosis test are ethidium bromide (Bio-Rad), methanol (E. Merck, Darmstadt, Germany), PBS (E. Merck, Darmstadt, Germany), RNA-ase (Promega, Madison, USA).

Methodology

Preparation of ethyl acetate fraction and non-ethyl acetate fraction of pasak bumi roots: The obtained test material is cleaned, then dried under indirect sunlight. The result of this

drying is followed by drying in the oven, then powdered in a blender and sieved to obtain the test powder. Before fractionation, the test material is extracted by maceration using MeOH solvent as follows: Approximately 50 g of the powder of the test material, put in an erlenmeyer vessel, then poured with 250 g of filter fluid (MeOH), closed and rested for 24 hrs protected from light while stirring repeatedly at room temperature. After 24 hrs, the pulp is squeezed out, the filtrate is collected, maceration is repeated three times by adding filter fluid to the obtained pulps, this is repeated until the final filtrate is clear. The filtrate that is stored in the vessel is then evaporated into a cup by aerating until a thick or almost dry extract is obtained. In the end, each extract was still evaporated in a freeze dryer vacuum until a dry extract was obtained. From the dry MeOHI extract of pasak bumi roots, then fractionated with ethyl acetate (EtOAc) solvent using a separating funnel, in order to obtain the soluble EtOAc fraction (relatively non-polar) and the insoluble EtOAc fraction (relatively polar). Then stored in the refrigerator at a temperature of -20°C.

Cytotoxicity test of the ethyl acetate and non-ethyl acetate fractions of pasak bumi roots: Into the 96-well microplate, containing 100 µL of test cells with a density of 2×10^4 cells/well, 100 µL of ethyl acetate and non-ethyl acetate fractions of pasak bumi roots was added at various concentration levels (1000, 500, 250, 100, 50 and 10 µg mL^{-1}) by triplicate. As a control, a culture medium that was considered to have 100% growth was used (Cell+media RPMI 1640 culture).

The culture containing the test material was then incubated in an incubator with a flow of 5% CO_2 at 37°C for 24 hrs. At the end of incubation, the number of living cells in each well was counted using trypan blue. Each well was resuspended first, then 10 µL of test cells were taken, put them in the eppendorf tube, add 50 µL of 0.5% trypan blue and mix sufficiently. Take 10 µL From the cell mixture, put in a hemocytometer and count the number of living cells under light microscope. Calculate the number of living cells from each well using the Eq.[4]:

$$\left(\frac{A}{4}\right)\times \text{Dilution}\times 10^4$$

where, A is the number of living cells obtained from the four hemocytometer count boxes.

Antiproliferative activity test (doubling time): Cells were starvated (fasted) for 24 hrs in culture medium containing 0.5% FBS. Furthermore, it was grown on a plate (multiple dishes) with a medium added with a sample at a non-lethal concentration, namely 3 series levels below the LC_{50} value (10, 1 and 0.1 µg mL^{-1}). Then incubated in 5% CO_2 incubator at 37°C for 24, 48 and 72 hrs. The number of living cells in each well was counted using a hemocytometer and a curve of the number of cells vs incubation time was made. The difference in the doubling time is calculated from the slope in the curve of log graph equation of the cell number vs observation time.

Apoptosis test with ethidium bromide staining: Into the 96-well microculture containing 100 µL of test cells with a density of 2×10^4 cells/well, 100 µL of the test compound was added at a concentration of 10 µg mL^{-1}. Then incubated in an incubator with 5% CO_2 flow at 37°C for 24 hrs. After being incubated overnight, 200 µL cells from each well were taken and put in eppendorf, then centrifuged at 1200 rpm for 5 min. The supernatant liquid is discarded, leaving the pellets, then resuspended. Take the cell suspension and place it on the slide. Then the cells were fixated with methanol with a volume of 1: 1 (10 µL cell: 10 µL methanol) and let dry for about 1 min. Add PBS containing RNA-ase with a level of 1 µg 10 mL^{-1}, then incubated at room temperature and add 10 µL of ethidium bromide, leave for 20-30 min until ethidium bromide permeated into cells, then covered with a deck glass. Observe the results under a fluorescent microscope. Apoptotic cells will appear orange, with the following morphological characteristics: (1) Shrinking cells, (2) Chromatin condensation, (3) Nuclear fragmentation and (4) Blebbing cell membranes[6].

Immunocytochemical detection of p53 protein: Into the 96-well microculture containing 100 µL of test cells with a density of 2×10^4 cells/well, 100 µL of the test compound was added at a concentration of 10 µg mL^{-1}. Then incubated in an incubator with 5% CO_2 flow at 37°C for 24 hrs. After being incubated overnight, 200 µL cells from each well were taken and put in eppendorf, then centrifuged at 1200 rpm for 5 min. The supernatant liquid is discarded, leaving the pellets, then resuspended. The cell suspension is taken and placed on a slide that has been coated with poly Lysine. Then the cells were fixated with acetone for 10 min. Washed with PBS for 5 min and dripped with 0.1% hydrogen peroxidase for 10 min. Then washed with running water, washed with PBS 1×5 min. Dripped with normal horse serum for 10 min 100 µL each. Then cleaned without washing with water. Then drip with the primary antibody, namely anti p53 protein and leave it for 24 hrs. Then washed with PBS 2×5 min. Dripped with a biotinylated secondary antibody for 10 min. Washed with PBS

2×5 min. Then dripped/incubated with Avidin Biotin enzyme reagent for 10 min. Washed with PBS 2×5 min. Later, incubated with peroxidase substrate (DAB) for 10 min or until staining appears. Washed under running water. Counterstain by using hematoxylin for 10-20 sec. Then washed with running water. Subsequently, dehydration was carried out using 95% ethanol then xylene for 10 min each. Then drop the mounting media, cover with a deck glass. The results were observed under light microscope at 400× magnification. Cells that are positive for p53 protein show the presence of a nucleus or cytoplasm that is brown.

Data analysis: The number of living cells was counted, compared to controls by considering the variations in sample levels on cell mortality. Cytotoxic analyzed using probit analysis and determined the LC_{50} value of each compound, that is the level needed to kill tumor cells by up to 50%. Probit analysis is obtained from the conversion of the percentage of cell mortality to its probit value, the percentage of cell mortality is calculated as follows[4]:

$$\text{Cell mortality (\%)} = [(\sum A-\sum B):\sum A] \times 100$$

where, ΣA is the number of living cells in control with no treatment and ΣB is the number of living cells due to treatment of compounds at various concentrations.

The LC_{50} average value of each test extract was then statistically analyzed by using the t-independent test with a confidence level of 95%.

The doubling time analysis is calculated by comparing the slope value of the log graph equation of the number of cells at various observation times. The doubling time is calculated by entering a log value of 2 times the original number of cells into the log graph equation for cell number vs. observation time. Furthermore, the average number of living cells at various times of observation was statistically analyzed using the t-independent test with a 95% confidence level to determine the differences between groups.

Observation of apoptosis is conducted by examination under a fluorescent microscope. The apoptotic cells will be orange in color with morphological characteristics such as shrinking cells, nuclear fragmentation, chromatin condensation and blebbing cell membranes. Cells that are positive for p53 protein, by immunocytochemistry will show the presence of a nucleus or cytoplasm that is brown. To determine the proportion of cells that tested positive for the p53 protein, it was determined by counting the entire nucleus or cytoplasm of the cells that tested positive per 100 cells.

RESULTS AND DISCUSSION

Cytotoxicity on Raji cells: In this cytotoxicity test, a direct cell counting method was used and using trypan blue staining, which is based on the principle that dead cells have lost their cell membrane integrity, so trypan blue can enter the cells. Thus, the dead cells will absorb the blue color, while the living cells will not absorb the blue color[5]. In this cytotoxicity test experiment, the LC_{50} value parameter was determined, that is the concentration that was able to produce up to 50% of cell death. The LC_{50} value indicates the toxicity potential of a compound to Raji cells. The smaller the LC_{50} value, the more toxic the compound.

The toxicity potential of ethyl acetate and non-ethyl acetate fractions of Pasak bumi (*E. longifolia* J.) roots to Raji cells was shown in Table 1. At the highest concentration of ethyl acetate fraction of pasak bumi roots which was 1000 μg mL^{-1}, 84.4% of cell mortality obtained, with the LC_{50} value of the ethyl acetate fraction against Raji cells was 25.7 μg mL^{-1}. The highest concentration of non-ethyl acetate fraction of pasak bumi roots was 1000 μg mL^{-1}, the mortality rate of 93.1% with the LC_{50} value of the non-ethyl acetate fraction of the pasak bumi roots to Raji cells was 17.8 μg mL^{-1}. The results of statistical calculations show that the LC_{50} value of the two test compounds was not significantly different ($p>0.05$). The graph of the relation between the concentration and the percentage of cell mortality was shown in Fig. 1.

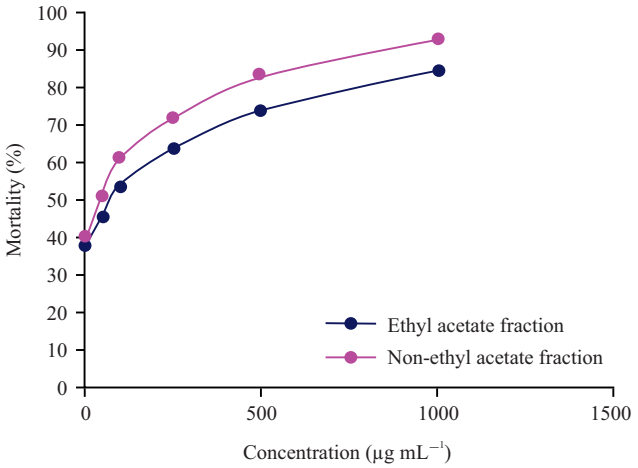

Fig. 1: Graph of the relationship between concentration (μg mL^{-1}) and percentage of Raji cell mortality after treatment with ethyl acetate and non-ethyl acetate fractions of pasak bumi (*E. longifolia* Jack) roots at various concentration levels

Table 1: Value of middle lethal concentration (LC_{50}) of ethyl acetate and non-ethyl acetate fraction of pasak bumi (*E. longifolia* Jack) roots to Raji cells

	Ethyl acetate fraction	Non-ethyl acetate fraction
Value of middle lethal concentration (LC_{50}) extract of pasak bumi roots (μg mL^{-1})		
	25.29	10.44
	25.59	22.50
	26.26	20.53
Average±SD	25.71±0.49	17.82±6.46

SD: Standard of deviation

Based on Fig. 1, it can be seen that the higher the concentration of ethyl acetate and non-ethyl acetate fractions of the pasak bumi roots, the greater the number of Raji cell mortality, although statistically it can be seen that the percentage of Raji cell mortality at the respective concentrations of ethyl acetate and non-ethyl acetate fractions of pasak bumi roots shows a no different number of deaths ($p>0.05$).

The results of the research of Nurani *et al.*[7] showed that the IC_{50} value of the ethyl acetate fraction of the ethanol extract of pasak bumi roots (44 μg mL^{-1}) was greater than that of doxorubicin (1.1 μg mL^{-1}) in lymphocyte cells. Based on the results of this study, the ethyl acetate fraction of the ethanol extract of the roots of pasak bumi had an IC_{50} value greater than doxorubicin, this indicated that doxorubicin had a more toxic effect than the ethyl acetate fraction of ethanol extract of pasak bumi roots on lymphocytes. It is possible that doxorubicin works not selectively because it is toxic to both cancer cells and normal cells, especially normal cells with high proliferation rates such as the spinal cord[8]. Meanwhile, the results of study conducted by Rehman[9] showed that the ethyl acetate fraction of the ethanol extract of pasak bumi roots (*Eurycoma longifolia* Jack.) had a cytotoxic effect on T47D cells with an IC_{50} value of 340 μg mL^{-1}. Meiyanto (2009) stated that the extract with a smaller IC_{50} value had a better anticancer effect. Compounds with smaller IC_{50} have more potential to be developed as anticancer[10], besides that the American National Cancer Institute (NCI) also states that the toxicity criteria for a compound against cancer cells are the IC_{50} value ≤20 μg mL^{-1} = very active, IC_{50} 21-200 μg mL^{-1} = moderately active, IC_{50} 201-500 μg mL^{-1} = weak[11]. Based on the research of Nurani[9], the IC_{50} value of the ethyl acetate fraction was categorized as quite active potentially for cancer cytotoxic compared to the insoluble fraction of ethyl acetate and ethanol extract from the roots of pasak bumi. The IC_{50} value category by NCI also illustrates that the smaller the IC_{50} value, the more toxic the compound is and conversely the greater the IC_{50} value, the smaller the toxicity potential. Compounds with a greater IC_{50} have a lower toxicity effect on normal cells[9,12,13].

Based on the research of Tee and Azimahtol[14], the methanol extract of pasak bumi roots was more effective than the water extract with IC_{50} values, respectively (7.80±0.45) and >99.99 μg mL^{-1}. Of the eleven fractions (F3-F13) obtained by silica gel permeation chromatography from methanol extract, the three main active fractions (F5, F6 and F7) showed IC_{50} values 6.17±0.38, 4.40±0.42 and 20.00±0.08 μg mL^{-1}. Other fractions showed IC_{50} values of more than 30 μg mL^{-1}. Results from purification of F7, F16, showed higher cytotoxic activity against MCF-7, (IC_{50} = 15.23±0.66 μg mL^{-1}) and a certain degree of selectivity against normal breast cell line, MCF-10A (IC_{50} = 66.31±0.47 μg mL^{-1}). This was because F5 and F6 contain eurycomanone, a potent cytotoxic agent found in the roots of *E. longifolia*. Eurycomanone is a bioactive compound in the roots of *E. longifolia* and is a strong cytotoxic agent against cell lines including human epidermoid carcinoma of the nasopharynx KB, Vincristine-resistant KB, fibrosarcoma, melanoma, colon cancer, human lung cancer A-549 and MCF-7, with IC_{50} values of 0.40 μg mL^{-1} each, 0.8, 0.2, 8.2, 1.2, 8.1 and 1.1 μg mL^{-1} [15-18].

Analysis of raji cells doubling time (antiproliferation) due to ethyl acetate and non-ethyl acetate fraction treatment: The doubling time test was carried out by counting the number of cells treated in time units (every 24 hrs). Each sample was counted with a hemocytometer, then draw a curve of the number of cells versus the incubation time. The differences in the doubling time can be seen from the slope on the curve or by calculation using extrapolation[6]. The concentrations of the test compound used in the doubling time test were three concentrations below the LC_{50} value, so it was expected that not too many cells died on the observation for 72 hrs, due to the cytotoxic properties of the compounds tested. Another purpose of this treatment is that the cell growth profile can be observed at 24, 48 and 72 hrs. Data analysis of doubling time of Raji control cells (without treatment) and with ethyl acetate and non-ethyl acetate fractions of pasak bumi roots can be seen in Table 2. In the treatment with ethyl acetate and

Table 2: Average number of Raji cells without treatment (control) and with ethyl acetate and non-ethyl acetate of pasak bumi roots at 24, 48 and 72 hrs observations

		Average number of Raji cell×10^4 (±SD)					
		Ethyl acetate fraction (μg mL^{-1})			Non-ethyl acetate fraction (μg mL^{-1})		
Time (hrs)	Control	10	1	0.1	10	1	0.1
0	18.0±15.5	18.0±15.5	18.0±15.5	18.0±15.5	18.0±15.5	18.0±15.5	18.0±15.5
24	47.0±15.5	24.5±0.8	28.5±3.0	33.5±0.8	19.0±0.9	22.5±1.5	22.5±1.5
48	61.0±3.1	29.0±1.5	34.5±1.5	37.5±1.5	22.5±0.8	26.0±0.8	30.0±1.3
72	73.0±3.8	33.0±3.0	39.0±2.6	43.5±3.0	27.0±1.8	31.0±3.4	37.0±2.4

SD: Standard of deviation

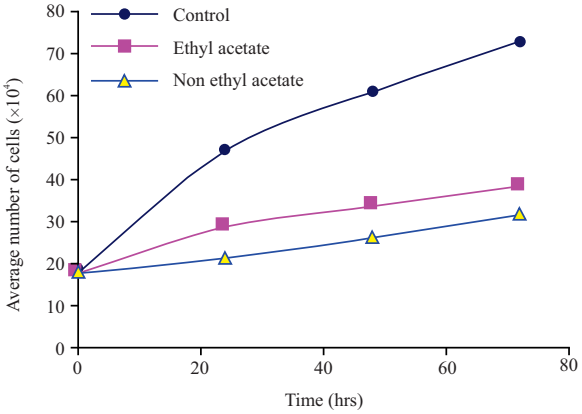

Fig. 2: Raji cell growth profile curve without treatment (control) and with the treatment of ethyl acetate and non-ethyl acetate fractions of the pasak bumi roots

non-ethyl acetate fractions of pasak bumi roots, concentrations of 10, 1 and 0.1 μg mL^{-1} at the 48th and 72nd hrs observations showed a significant difference in the mean number of living cells ($p<0.05$), while at the 24th hrs observations did not show a significant difference ($p>0.05$). This is because up to 24 hrs, the cells are still undergoing an adaptation phase after experiencing starvation, so the cells have not yet decided whether to divide or die[14].

Based on Fig. 2, it was observed that at 24th hrs observation, the percentage of the inhibition of Raji cell growth by treatment with ethyl acetate and non-ethyl acetate fraction of the pasak bumi roots was greater than the 48th and 72 hrs. It is likely that until the 24th hrs, the cells were still experiencing the adaptation phase after in a fasting condition for 24 hrs, so the cells lack growth signals from outside. In the treatment with ethyl acetate and non-ethyl acetate fractions of the pasak bumi roots, concentrations of 10.1 and 0.1 μg mL^{-1} at the 48th and 72nd hrs observations showed a significant difference in the number of living cells ($p<0.05$), while in the observation at 24th hrs showed no significant difference ($p>0.05$). From Fig. 2, it was shown that the ethyl acetate and nonethyl acetate fractions of pasak bumi roots concentrations of 10.1, 0.1 μg mL^{-1} resulted in a smaller slope value than the control. From the data, it was proven that the ethyl acetate and non-ethyl acetate fractions of the pasak bumi roots were able to inhibit the proliferation of Raji cells compared to control cells.

Tee and Azimahtol[14] found that methanol extract had higher antiproliferative activity against MCF-7 cells compared to water extract from *E. longifolia* roots. Likewise, the pasak bumi root fraction of F16 had an antiproliferative effect on MCF-7 cells by inducing apoptosis through modulation of Bcl-2 protein levels. Previous studies indicated that the methanol extract of *E. longifolia* root had antiproliferative activity against cancer cell lines (i.e., human HT-1080 fibrosarcoma, human cervix HeLa adenocarcinoma, human lung A549 adenocarcinoma, murine colon 26-L5 carcinoma, murine Lewis lung carcinoma LLC, murine B16-BL6 melanoma cells and P-388 murine lymphocytic leukemia)[16,19].

Apoptosis analysis and p53 protein expression of Raji cells

Apoptosis analysis of Raji cells by treatment with ethyl acetate and non-ethyl acetate fractions from the pasak bumi roots: Analysis of the antiproliferative character of ethyl acetate and non-ethyl acetate fractions of pasak bumi roots was carried out by observing changes in cell morphology and DNA, morphological changes such as shrinking cells, blebbing cell membranes, chromatin condensation and nuclear fragmentation are signs that cells are experiencing apoptosis[7].

In the staining of Raji cells without treatment (control), it showed that Raji cells did not experience apoptosis, this was seen in the absence of nuclear fragmentation and Raji cells did not shrink, while the results of Raji cell staining treated with 10 μg mL^{-1} concentration of ethyl acetate and non-ethyl acetate of pasak bumi roots also showed no apoptosis occurred, although Raji cells were reduced but there was no visible fragmentation of the nucleus (DNA) which is a characteristic of cells undergoing apoptosis. This was because the Raji cells are resistant to apoptosis due to mutations in

the downstream caspase 3 which are apoptotic mediators. A study by Thu *et al*.[20] showed that the results of treating Raji cells with C2-Ceramide and with diamide caused cell death after 48 hrs. This cell death was caused by a decrease in the potential of mitochondrial transmembrane followed by caspase 3 activation which is the cause of cancer cell death through apoptosis. In the study, cells don't undergo DNA fragmentation that is the characteristic of apoptosis.

In contrast to studies[14], F16 pasak bumi root fraction significantly increased apoptosis on MCF-7 cells, characterized by DNA fragmentation as evaluated by Tdt-mediated dUTP nick tip labeling tests and core morphology. Western blotting revealed down-regulation of the anti-apoptotic protein Bcl-2 expression. F16, however, did not affect the expression of the pro-apoptotic protein, Bax. Therefore, these results suggested that F16 has an antiproliferative effect on MCF-7 cells by inducing apoptosis through modulation of Bcl-2 protein levels. One of the key elements providing resistance to apoptosis is the anti-apoptotic protein of Bcl-2. Expression of the Bcl-2 protein was down-regulated in MCF-7 cells treated with F16. The initial event in cells that makes them sensitive to apoptosis is desuppression of the anti-apoptotic protein Bcl-2. In some models, Bcl-2 down-regulation alone can lead to cell commitment to apoptosis[21-23].

It was further suggested that administration of F16 decreased Bcl-2 expression in a time-dependent manner, which was evident at 24 hrs. A decrease in cell viability and an increase in the rate of apoptosis, along with a remarkable decrease in the level of the anti-apoptotic protein Bcl-2, suggest a Bcl-2-dependent apoptotic pathway by the active fraction F16. However, the mechanism by which F16 decreases Bcl-2 expression remains to be investigated. Decreased levels of Bcl-2 expression may play a positive role in increasing the susceptibility of these cells to apoptosis[14]. Treatment resulted in massive apoptotic cell death, which could be explained by low levels of the Bcl-2 protein in these cells, while Bax remained essentially unchanged. Bcl-2 is the dominant Bax negative inhibitor and decreased Bcl-2 expression sensitizes MCF-7 cells to apoptose. Thus, when the expression level of Bax is conserved and the expression level of Bcl-2 is low, the Bax homodimer is always formed and apoptosis is stimulated[24]. Previous studies have also found that decreased Bcl-2 expression sensitized MCF-7 and cells that are not derived from breast to apoptosis[23,24].

Eurycomanone, one of the most active medicinal compounds of *Eurycoma longifolia*, shows strong dose-dependent anticancer efficacy against lung carcinoma (A-549 cells) and breast cancer (MCF-7 cells), however, it showed moderate efficacy against stomach (MGC-803 cells) and intestinal carcinoma (HT-29 cells). The main mode of cytotoxicity of Eurycoma longifolia and its medicinal compounds is the induction of apoptosis (programmed cell death) through up-regulation of expression of p53 (tumor suppressor protein) and pro-apoptosis protein (Bax) and regulation of decreased expression. anti-apoptotic protein (Bcl-2)[20].

Cancer cells acquire resistance to apoptosis by overexpressing anti-apoptotic proteins (Bcl-2, IAPs and FLIP) and/or by down-regulation or mutation of pro-apoptotic proteins (Bax, Apaf-1, caspase 8 and receptor death)[25]. Apoptosis is the ability of cells to self-destruct by activation of the intrinsic cellular suicide program when cells are no longer needed or when they are severely damaged[21]. Apoptosis is an active form of physiological cell death, which is important for cellular and tissue development and homeostasis[26-29]. In cancer therapy, one approach that suppresses tumor growth is to activate the apoptosis engine inside cells[30-32]. In addition, the apoptotic process includes mechanisms that regulate both the packaging and removal of dead cells, thereby preventing inflammation of the surrounding tissue[33]. Evidence obtained over the last few years is beginning to establish that most cancer chemotherapy agents influence in vivo and in vitro tumor cell killing via the launch of the apoptotic cascade.[34] Apoptotic cells are characterized by loss of cell volume, release of plasma membrane, condensation of nuclei, aggregation of chromatin and endonucleolytic degradation of DNA into nucleosome fragments[35].

Analysis of protein p53 expression of Raji cells treated with ethyl acetate and non-ethyl acetate fractions of pasak bumi roots: From the detection results of p53 protein by immunocytochemistry, the amount of p53 protein expression per 100 cells was obtained. In Raji cells, whether in control or treated with ethyl acetate and non-ethyl acetate fractions of pasak bumi roots, most of the p53 protein expression was located in the cell nucleus, although some were located in the cytoplasm (Table 3).

The positive p53 protein expression in the cytoplasm indicated that inhibition of Raji cell growth occurs in the G1 phase of the cell cycle. The non-ethyl acetate fraction of pasak bumi roots could increase the expression of p53 protein in the cytoplasm compared to the ethyl acetate fraction of pasak bumi roots. It was possible that the quassinoid contained in the non-ethyl acetate fraction of the roots was more than that in the ethyl acetate fraction. According to Groeger[36], the p53 gene uses its function during the G1

Table 3: Location of positive p53 protein expression in Raji cells control and treated with ethyl acetate and non-ethyl acetate fractions of the roots of pasak bumi

	Treatments														
	Control					Ethyl acetate fraction					Non-ethyl acetate fraction				
Position of positive p53 expression	I	II	III	Total	%	I	II	III	Total	%	I	II	III	Total	%
Nucleus	26.0	17.0	15.0	58.0	95.1	35.0	42.0	36.0	113.0	91.9	63.0	68.0	72.0	203.0	90.2
Cytoplasm	2.0	1.0	0.0	3.0	4.9	3.0	3.0	4.0	10.0	8.1	7.0	7.0	8.0	22.0	9.8

phase of the cell cycle. Most of the p53 gene acts as: 'the guardian of the genome' , that is: (1) The level of p53 increased rapidly in response to DNA damage, (2) Causing inhibition of cell cycle during the G1 phase, (3) Allow time for the cell to repair the DNA damage, (4) If the damage is impossible to repair, p53 will induce programmed cell death (apoptosis).

Under normal circumstances, the wild type p53 protein was only expressed in very small amounts[37], but if there is DNA damage, the p53 protein expression will increase and this will stimulate p21 transcription. The p21 protein is an inhibitor of CDK and plays an important role in inhibiting pRb phosphorylation, thus inhibiting the release of the transcription factor of E2F and causing inhibition of DNA replication. In addition, the p21 protein also binds and deactivates Proliferating Cell Nuclear Antigen (PCNA). The bond between the p21 protein and PCNA will inhibit the interaction between PCNA and DNA polymerase, thereby inhibiting DNA replication. Thus, in cells that have DNA damage, the p53 protein will act through the p21 protein to stop DNA replication and cell division. However, under certain circumstances the p53 protein will stimulate Bax transcription which will inhibit Bcl-2 activity. Under normal circumstances, Bcl-2 inhibits activation of caspase 3, which is the central mediator of the apoptotic pathway. When bcl-2 activity is inhibited by Bax, caspase 3 activity is not inhibited, resulting in apoptosis. Thus, the p53 protein also plays a role in suppressing Bcl-2 transcription so that apoptosis occurs in cells that have DNA damage[20]. Bcl-2 and Bax are members of the Bcl-2 protein family that have been associated with apoptotic cell death both *in vitro* and *in vivo*[38]. Bcl-2 is mostly located on the outer mitochondrial membrane and is found on the membrane of the endoplasmic reticulum and nucleus[39]. The Bcl-2 molecule resembles a bacterial poison in its ability to control the entry and exit of ions and small molecules as gatekeepers[40]. Apoptosis is controlled by the ratio of various members of the Bcl-2 family[41]. Apoptotic 'on' and 'off' switching is determined by the ratio of anti-apoptotic and pro-apoptotic proteins, the Bcl-2/Bax ratio determines cell fate, i.e., the lower the ratio, the higher the probability of cell death[42,43].

It can be concluded that the ethyl acetate and non-ethyl acetate fractions of the pasak bumi roots can cause death in Raji cells. The death of Raji cells through the mechanism of inhibiting Raji cell proliferation but not through the mechanism of apoptosis as evidenced by an increase in p53 protein expression in Raji cells. In this study, there was an increase in p53 protein expression, so it is necessary to do further research to find a mechanism to inhibit proliferation, whether through increasing p21 expression and induction of DNA repair pathways by cytometry flow.

CONCLUSION

Ethyl acetate and non-ethyl acetate fractions of the roots of pasak bumi (*E. longifolia* Jack) have cytotoxic and anti-proliferation activity, yet unable to stimulate the process of apoptosis in Raji cells. Cytotoxic activity of fractions of pasak bumi roots against Raji cells is through inhibition mechanism of the cell cycle. Antiproliferation activity is by inducing the increased expression of p53 protein in each fraction by 41% (ethyl acetate) and 75% (nonethyl acetate).

SIGNIFICANCE STATEMENT

The findings of this study prove that the roots of pasak bumi can be developed as an anticancer agent. The mechanism of Raji cell death is through inhibition of Raji cell proliferation as evidenced by an increase in p53 protein expression. The presence of p53 protein expression in the cytoplasm indicates that the inhibition of Raji cell proliferation is through inhibition of cell cycle progression that occurs in the G1 phase. This provides an opportunity for genes that control DNA repair to repair existing DNA damage. The study of the biological effects of the pasak bumi root at the cellular level provides a molecular basis for its anti-proliferative function and helps build a platform for producing more potent chemopreventive and even chemotherapy agents.

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