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METABOLIC SYNDROME
Now and Future Trend

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IN VITRO CYTOTOXICITY ASSAY OF ETHYL ACETATE AND DIETHYL ETHER FRACTION OF BREADFRUIT LEAVES METHANOL EXTRACT ON HeLa CELL LINE

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Abstract: One of the traditional medicine that can be used to cure cancer is *Artocarpus altilis* (Parkinson) Fosberg. Based on that fact, this study conducted to determine the in vitro cytotoxic effects of ethyl acetate fraction and diethyl ether fraction of methanol extract of breadfruit leaves on HeLa cells using MTT assay method obtained LC_{50}. The results showed that the LC_{50} values of ethyl acetate fraction was 119.5363 µg/ml, while the fraction diethyl ether LC_{50} values was 53.8022 µg/mL. It can be concluded that both fractions potential as anticancer because the range of the LC_{50} under 1000 µg/ml, and diethyl ether fraction cytotoxic activity higher than ethyl acetate fractions.

Keywords: Cytotoxicity, Ethyl Acetate Fraction of Breadfruit Leaves, Diethyl ether Fraction of Breadfruit Leaves, HeLa cells, MTT assay

Introduction

Cancer is the abnormal tissue cells that constantly divide abnormally and uncontrollably, cancer cells can spread to other healthy tissues in the body and its normal function to cause death. The cause of cancer is very diverse, or so-called multifactor formed in a long time and progressing through different stages. Nutritional factors one of the most important aspects in cancer pathology. Lifestyle factors such as smoking, obesity, consumption of alcohol, nutrition, the exposure to chemicals, ultraviolet radiation, and physical inactivity is suspected as a major contributor to the growth of cancer (Bonita *et al.* 2001).

Treatment of cervical cancer depends on the specific cell type of cancer, the spread of disease, and the patient's performance status. Common treatments are surgery, chemotherapy, and radiation therapy. The use of anticancer therapy alone is not to provide optimal results. This is caused such treatments work not specific because in addition to attack cancer cells also damage normal cells. Therefore, necessary alternative therapy to minimize side effects, one of them using herbal medicine that was the leaves of breadfruit.
Traditionally, breadfruit leaves are known as anticancer agents. Previous research on the bioactivity of the breadfruit leaf has been done was methanol, ethyl acetate and chloroform extract of breadfruit leaves against the shrimp larvae of *Artemia salina* has LC_{50} values of each 392.826; 415.623; and 387.436 µg/ mL (Rosmawaty and Tehubijuluw 2013). The other study was conducted by Arung *et al.* (2009) which showed anticancer activity in diethyl ether fractions of methanol extract breadfruit wood containing artocarpine on T47D breast cancer cells with IC_{50} was 6.19 µg/ml.
Based on these studies, the researchers want to try to test the cytotoxicity diethyl ether and ethyl acetate fraction of methanol extract of breadfruit leaves against HeLa cells. The present study using breadfruit leaves, as expected in the same plant still has the same efficacy as well as anti-cancer, while cancer cells used are HeLa Cell Line in vitro. HeLa cells are human cells used for cell culture interests, and can grow rapidly and well in suspension (Anggrianti 2008). In cell culture cytotoxicity assay was performed using indirect calculation method MTT (Tetrazolium micro culture Technique) assay was then measured using an absorbance microplate reader.

Materials and Methods

This research was conducted on May until July 2016 in the Laboratory of Phytochemistry Faculty of Pharmacy and Science, Universitas Muhammadiyah Prof. DR. HAMKA, East Jakarta and Central Primate Studies Laboratory (PSSP) IPB University, Bogor.

Ingredients:

Breadfruits leaves (*Artocarpus altilis* (Parkinson) Fosberg) that have been determined at Herbarium Bogoriense, Bogor. Some of chemicals used to identify compounds (alkaloids, saponins, flavonoids, terpenoids and steroids). Other ingredients methanol, ethyl acetate, dimethyl sulfoxide (DMSO), culture medium Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), penicillin streptomycin,

sodium bicarbonate, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride (NaCl), trypan blue, aquabidest.

Preparation of methanol extract of leaves of breadfruit.

Making the methanol extract of leaves of breadfruit by maceration. First take as much as 1 kg of powdered leaves of breadfruit then extracted using methanol as much as 3 liters, maceration was done for 3 days, while stirring occasionally, then filtered, the waste in soak back with methanol as many as 3 liters, repeat the same thing up to 3 times. Evaporated it with a rotary vacuum evaporator at temperatures under 50°C to until the remaining methanol extracts were concentrated.

Fractionation of ethyl acetate and diethyl ether methanol extract of leaves of breadfruit

The condensed extract of breadfruit leaves 10 g dissolved in methanol: water (1: 2) 30mL put in a separating funnel, add n-hexane 20 ml, shake for 15 minutes, let stand to form two layers, namely the top layer fraction of n-hexane, the bottom layer phase of methanol: water (be repeated 3 times). Phase methanol: water fractionated again with diethyl ether20 ml, mixing for 15 minutes, let stand to form two layers, namely the top layer diethyl ether fraction, the bottom layer phase of methanol: water (repeat 3 times). Phase methanol: water fractionated again with ethyl acetate 20 mL mixing for 15 minutes, let stand to form two layers, namely the top layer of the fraction of ethyl acetate, the lower layer phase of methanol: water (repeat 3 times), the fraction of ethyl acetate obtained merged then evaporated with a water bath to obtain fraction viscous download diethyl ether and ethyl acetate fraction condensed for use in testing (Arung *et al.* 2009).

Phytochemical screening

This test was conducted to identify the content of alkaloids, tannins, saponins, flavonoids, steroids, and terpenoids.

Preparation concentration of diethyl ether and ethyl acetate fraction of breadfruit leaf extract for cytotoxicity assay

Diethyl ether and ethyl acetate fraction of methanol extract of breadfruit leaves weighed as much as 10 mg dissolved in 50 µl of DMSO and then added to 1 mL DMEM medium, to obtain the main solvent with a concentration of 10,000 µg/ mL (stock I). The concentration of the test solution will be made and retrieved from the stock first, then dilution with a concentration of 1000; 100; 50; 25; 12,5; 3,12 µg/ mL for Diethyl ether fraction of the methanol extract of breadfruit leaves, while the concentration 800; 400; 200; 100; 50; 25 µg/ mL for ethyl acetate fraction of the methanol extract of breadfruit leaves. All test solutions made with graded dilution. Preparation of the test conducted aseptically in the Laminar Air Flow (LAF)

Cell density

Hemocytometer used to calculate the density of the cells by mixing 20 µl of cell suspension with 180 µl of trypan blue. The rooms were clean count with glass lid was placed horizontally on the table as much as 10 µl pipette mix on the surface of the rooms count with a reference to the edge of the cover slip slowly. Cells were observed at 100 times magnification with a microscope. All cells contained in four large fields at the corners of the room the entire surface was calculated, the calculated amount. The average value was taken from the number of cells collected from the four major fields, and then multiplied by the dilution factor and a correction factor for each major field (Hughes and Mehmet 2003).

Amount cells/ mL = mean cell number x dilution factor x 10^4

Cytotoxicity assay methods of calculation of indirect (MTT assay)

Cytotoxicity assay was a quantitative test by way cell death. The parameters used to test the cytotoxic was LC_{50} value. LC_{50} value indicates the concentration resulting in cell death by 50% and demonstrate the potential toxicity of a compound to the cell. LC_{50} value can determine the potential of a cytotoxic compound. The test compound was said to be toxic if the LC_{50} value was smaller than 1000 µg/ml, LC_{50} values < 30 ppm potential as anti-cancer (cytotoxic), 30-200 ppm LC_{50} potential as antimicrobial, and 200-1000 ppm LC_{50} potential as a pesticide, so that the greater the value the $LC5_0$ less toxic compounds (Inayah 2012). MTT assay test was one method used in cytotoxic test. That method was a colorimetric method, which MTT reagent was a tetrazolium salt that can be broken down into formazan crystals by succinate tetrazolium reductase system which was in the path cell respiration in mitochondria was active in cells that are still alive. Gives purple formazan Crystal which can be read absorbance by using ELWAS A reader (Junedy, 2005).

Preparation of cytotoxicity assay against HeLa cells

The cell suspension in 100 mL media was inserted in the microplate 96 wells. Then incubating the cells for 24 hours. After incubation, remove the cell media, and then enter 100 mL of PBS into all wells are filled cell, then dispose of PBS. Next add the test solution and the positive control at various concentrations. Furthermore, the

culture medium DMEM were 100 µl and 100 µl of cell suspension was added to the other wells as control cells and then incubated at 37°C in a CO_2 incubator for 72 hours. In each of the wells was added 10 mL solution of MTT. Cells were incubated for 4 hours at 37 °C. MTT reaction was stopped by adding 50 µl SDS, incubated in the dark for 16 hours. Uptake read with microplate reader at a wavelength of 550-600 nm. In the MTT method, the percentage of cell death represents the difference between the absorbance of the control sample divided by absorbance control absorbance multiplied by 100%. Calculation of percent of cell death using MTT method using the following formula (CCRC 2010):

$$\text{Percent mortality} = \frac{abs\ negative\ control\ cells - abs\ test\ control}{abs\ negative\ control\ cells} \text{ X } 100\ \% \quad \ldots\ldots(1)$$

Results and Discussion

Breadfruit leaves extracted by maceration using methanol. Selection of extraction by maceration method because it was simple and easy execution. While the use of methanol as a solvent for extracts obtained be easily covered with mold and more volatile so that the time required for the concentration and drying becomes shorter.

Table 1: Phytochemical screening of diethyl ether and ethyl acetate fraction of methanol extract of breadfruits leaves.

No	Phytochemical constituents	Methanol extract	Diethyl ether Fraction	Ethyl acetate fraction
1	Alkaloid	+	+	+
2	Saponins	-	-	-
3	Flavonoid	+	+	+
4	Tannin	-	-	-
5	Terpenoids	+	-	+
6	Steroid	+	+	+

Qualitative screening of phytochemicals

A secondary metabolite was observed through qualitative phytochemical screening study. Ethyl acetate fraction and methanol extract of breadfruits leaves contains alkaloid, flavonoid, steroid, and terpenoids, while diethyl ether fraction showed three metabolites only are alkaloid, flavonoid, and steroid.

Assessment of cytotoxicity assay

Testing the cytotoxic activity using the MTT assay. The treatment was tested on the test consists of a cytotoxic treatment diethyl ether fraction and ethyl acetate fraction of methanol extract of breadfruit leaves was added to the cells as a control test using 6 concentrations and the cells without treatment control just using media as a negative control. Each treatment was incubated for 72 hours and the absorbance was read using a microplate reader to determine the percentage of deaths, then analyzed with Probit table, and put into the linear regression equation for determining the LC_{50} values. The cytotoxic potential of a compound known through LC_{50} values, the concentration that can kill HeLa cells by 50% after the incubation period.

Table 2 shows that the highest percentage of HeLa cell death diethyl ether fraction of methanol extract of breadfruit leaves occur at a concentration of 1000 µg/ml, at these concentrations diethyl ether fraction of methanol extract of leaves of breadfruit can kill cells by 91.87 %, whereas for ethyl acetate fraction can be seen in table 3. that the highest percentage of HeLa cell death ethyl acetate fraction of methanol extract of breadfruit leaves occur at a concentration of 800 µg/ml, at these concentrations can kill cells by 96,70 %. Based on these results show that the percentage of cell death continues to rise with increasing concentration, so it can be concluded that the higher the concentration the higher the ability to turn off cell growth.

Table 2: Inhibition and LC50 values of diethyl ether fraction of methanol extract of breadfruits leaves by MTT assay

Conc (µg/ml)	Average abs	Log conc (µg/ml) (X)	Percent mortality (%)	Probit (Y)	LC_{50} (µg/ml)
1000	0,032	3	91,87	6,3917	53,8022
100	0,100	2	74,68	5,6620	
50	0,247	1,6990	37,17	4,6708	
25	0,286	1,3979	27,43	4,3992	
12,5	0,308	1,0969	21,68	4,2142	
3,12	0,339	0,4941	13,97	3,9152	

Table 3. Inhibition and LC50 values of ethyl acetate fraction of methanol extract of breadfruits leaves by MTT assay.

Conc (µg/ml)	Average abs	Log conc (µg/ml) (X)	Percent mortality (%)	Probit (Y)	LC_{50} (µg/ml)
800	0,013	2,9031	96,70	6,8084	119,5363
400	0,027	2,6021	93,15	6,4758	
200	0,123	2,3010	68,78	5,4874	
100	0,251	2	36,29	4,6442	
50	0,343	1,6990	12,94	3,8689	
25	0,354	1,3979	10,15	3,7184	

Figure 1: A charts relationship between the concentration with percent mortality of diethyl ether fraction methanol extract of breadfruit leaves.

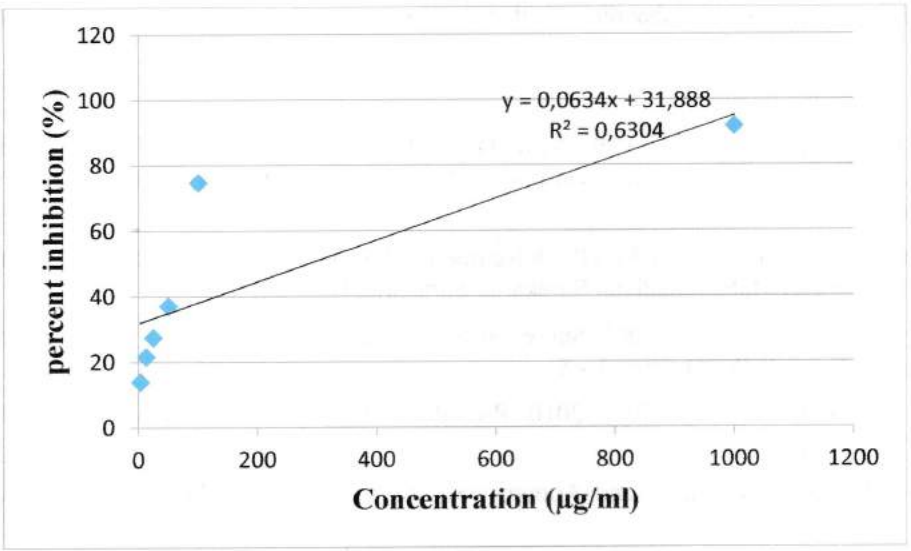
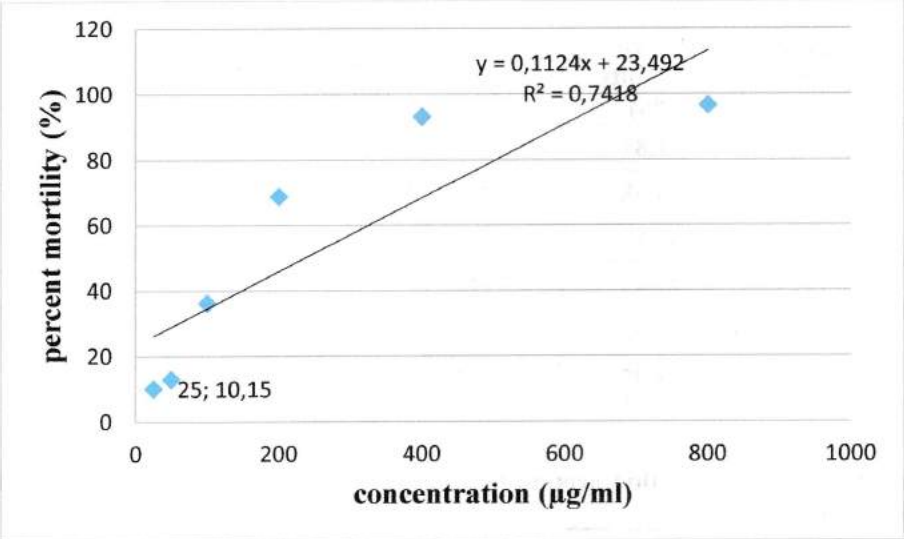


Figure 2: A charts relationship between the concentration with percent mortality of ethyl acetate fraction methanol extract of breadfruit leaves.



LC_{50} value generated from diethyl ether fraction of methanol extract of leaves of breadfruit amounted to 53.8022 µg/ml, whereas LC_{50} values of ethyl acetate fraction of 119.5363 µg/ml, a both values were included in the category of potential as anticancer because it was in the range of < 1000 µg/ml. Diethyl ether fraction of methanol extract of leaves of breadfruit more cytotoxic than the fraction of ethyl acetate, although very weak cytotoxic properties.

In this study were not given treatment positive control using anticancer drugs, because as we all know that the anticancer drugs have cytotoxic activity that works directly kill cancer cells, so it has been ascertained LC_{50} value of anticancer drugs was smaller than the LC_{50} value of the test sample, the smaller the LC_{50} value of a compound, the stronger the ability of these compounds in shutting down cell growth and the greater the potential of becoming an anticancer compound.

Conclusion

Diethyl ether and ethyl acetate fraction of methanol extract of breadfruit leaves have potential as an anticancer against HeLa cells based content flavonoid which can kill cancer cells with LC_{50} values of each of 53.8022 µg/mL and 119.53 µg/mL. It can be concluded that diethyl ether fraction more cytotoxic than the ethyl acetate fraction.

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