

The activity of *Rhaphidophora pinnta* Lf. Schott leaf on MCF-7 cell line

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ABSTRACT

Ekor naga's leaf (*Rhaphidophora pinnata* (Lf) Schott) is a type of vines and climbing plant. The leaves are elongated round and hollowed inside. This plant had been using for the treatment of breast cancer. Extraction with percolation method has been done in *ekor naga's* leaves with ethanol, and fractionated by n-hexane, chloroform and ethyl acetate using liquid-liquid extraction (LLE). Cytotoxicity assay of ethanol extract, n-hexane fraction, chloroform fraction, ethyl acetate fraction and water fraction against MCF-7 cells were done using MTT method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). Phytochemical screening results showed the presence of the compounds such as triterpenoida/steroid, alkaloid, flavonoid, tannin, and saponin. n-hexane fraction was positive for the presence of triterpenoida/steroid, chloroform fraction containing alkaloids, saponin and triterpenoid; ethyl acetate fraction contained, flavonoid, tannin, and the fraction of water indicated the presence of tannin and saponin. Secondary metabolite compounds in ethanol extract, chloroform fraction and ethyl acetate fraction gave positive results against MCF-7 cells. Cytotoxicity assay of MCF-7 cell line showed that crude ethanol extracts had 112.240 mcg/ml IC₅₀ chloroform fraction IC₅₀ was 59.082 mcg/ml, and ethyl acetate fraction IC₅₀ was 812.663 mcg/ml.

Keywords: *Ekor Naga's* Leaf; *Rhaphidophora Pinnata*; MCF-7 Cells; MTT Method; Cytotoxic Assay

1. INTRODUCTION

Breast cancer is one of the worldwide deathly cancer type after lung, liver and colon cancer. It is the main enemy of women and currently in the first rank of danger. Identification of breast cancer cases worldwide has in-

creased from about 640.000 in 1980 to 1.6 million by 2010 in developing country [1]. Patients with breast cancer are mainly caused by the diet, such as the selection of foods and drinks contain preservatives, coloring agents and beverages that contain alcohols [2].

Cause of cancer usually cannot be known with certainty because it is a combination of a set of factors including genetics, environment, foods containing chemicals, viruses, for example Papilloma Virus, which caused genital warts/genital, cytomegalo virus that caused Kaposi sarcoma/cancer of vascular system marked by blood red skin lesions, Hepatitis B virus, hormonal imbalance, free radicals, psychological and emotional factors [3].

The efforts for developing anticancer drugs had been continued intensively, both derived from chemicals and natural sources. Some research showed that many of the plants contain a variety of chemical compounds as potential anticancer. One of those things that has become scientists' observation is traditional medicine. This research is done because the potential of traditional medicine has long been believed by the people to cure the disease [4].

One of the plants that can be used for the cancer treatment is *ekor naga's* leaves (*Rhaphidophora pinnata* (Lf) Schott), from Araceae family. Some people have used the boiling water for the treatment of cancer. Polyphenolic compounds (flavonoids, tannins and saponins) are generally effective as antioxidant and could kill the growth of abnormal cells (tissue) and uncontrolled cell, which is one of the causes of cancer [5].

Pre-eliminary screening test for active compounds contained in the extract was the toxicity test on the shrimp larvae *Artemia salina* L. using the brine shrimp test method with LC₅₀ values of crude ethanol extract 19.686 mcg/ml, n-hexane fraction 505.82 mcg/ml, ethyl acetate fraction 28.84 mcg/ml and ethanol fraction 128.82 mcg/ml [6-9]. Acute toxicity test with oral administration in female mice up to doses of 5000 mg/kg BW did not reveal

any mortality up to 7 days and no physiological changes in the body of mice. Therefore, the LD₅₀ value of the ethanol extract of *ekor naga*'s leaves cannot be calculated and it was declared the value LD₅₀ was greater than 5000 mg/kg/BW in female mice.

One model of breast cancer cells used MCF-7 cells that had been patented by the institute of Michigan Cancer Foundation-7 (MCF-7). MCF-7 cells are cell type isolated in 1970 from a woman's breast tissue of Caucasian race. MCF-7 cells have been used for a variety of *in vitro* research on breast cancer because they have some of the same characteristics associated with breast epithelial estrogen in the form of the ability to process estradiol via estrogen receptors in the cytoplasm. It makes the MCF-7 cells give positive result to estrogen (ER) recipient in the control cells [10].

In order to find new drugs for the treatment of breast cancer, the *in vitro* cytotoxicity test of the ethanol extract (*crude*) and fractions (n-hexane, chloroform, ethyl acetate and water) of *ekor naga* leaves against MCF-7 cells had been done.

2. MATERIALS AND METHODS

2.1. Materials and Reagents

The material used in this study was *ekor naga*'s leaves. The chemicals used were, n-hexane (Merck), 96% ethanol (Merck), chloroform (Merck), ethyl acetate (Merck), a solution of dimethylsulfoxide (DMSO) pro 99.5% GC (Sigma), Roswell Park Memorial Institute Medium (RPMI 1640), hepes (Sigma), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Fetal Bovine Serum (FBS), Penicillin-Streptomycin, silica gel GF₂₅₄, H₂SO₄p (Merck), methanol (Merck), acetone (Merck), Amphoterisin B, Sodium dodecyl sulphate in 0.01N HCl, Doxorubicin, MCF-7 cell line.

2.2. Phytochemical Screening

Phytochemical screening of *ekor naga* leaves powder, ethanol extract and fractions of class compounds included examining alkaloids, glycosides, anthraquinone glycosides, saponins, flavonoids, tannins, and triterpenoida/steroid.

2.2.1. Examination of Alkaloids

Crude leaves powder weighed 0.5 g and then diluted with 2 N hydrochloric acid and distilled water, heated over a water bath for 2 minutes, cooled and filtered. Filtrate was tested with the addition of reagent of alkaloid such as Mayer, Bauchardat, and Dragendorff.

2.2.2. Examination of Glycosides

Crude leaves powder was refluxed for 2 hours with a mixture of ethanol-water (7:3) and 2 N hydrochloric acid,

then filtered. Filtrate was added with a solution of lead (II) acetate 0.4 M, then shaken, and filtered. Filtrate was extracted with isopropanol-chloroform mixture (2:3). Water extract was collected and evaporated at a temperature of not more than 50°C. The remaining part was dissolved in methanol and added with 2 ml of water and reagent Molish. Then slowly 2 ml of concentrated sulfuric acid was added through the tube wall. The formation of a purple ring was formed.

Examination of anthraquinone glycosides Crude leaves powder was heated with 5 ml of 2 N sulfuric acid, then cooled it room temperature, the mixture then was added with 10 ml of benzene, shaken and allowed to stand. Benzene layer was separated and shaken with 2 ml of 2 N NaOH, allowed stand. Water layer is red and colorless benzene layer showed the presence of anthraquinone.

2.2.3. Examination of Saponin

Crude leaves powder was inserted into a test tube, then hot water was added. The mixture was cooled then shaken vigorously for 10 seconds. If the 1 - 10 cm tall foam was formed and stable for not less than 10 minutes and did not disappear with the addition of 1 drop of 2 N hydrochloric acid, then it was positive for saponin.

2.2.4. Examination of Flavonoid

Crude leaves powder was shaken with hot water for 5 min and filtered under hot conditions, the filtrate was added magnesium powder, and concentrated with hydrochloric acid and amyl alcohol, then shaken. It showed flavonoid with positive if a red or yellow or orange colour in the lining of amyl alcohol was formed [11].

2.2.5. Examination of Tannin

Crude leaves powder was boiled in water for 3 minutes, cooled and filtered. The filtrate was added with 1 - 2 drops of 1% iron (III) chloride. If there is blue-black or blackish green indicates the presence of tannins [11].

2.2.6. Examination of Steroid/Triterpenoida

Crude leaves powder was macerated with 20 ml n-hexane for 2 hours, then filtered. Filtrate should be evaporated in the evaporating dish, then added with a few drops of Liebermann-Burchard reagent. Onset of blue or blue-green color showed the presence of steroid, while the color is red, pink or purple that indicated triterpenoid [12].

3. PLANT MATERIAL AND EXTRACTION OF EKOR NAGA'S LEAVES

Extracts were performed using 96% ethanol by percolation method [13].

Dried *ekor naga*'s leaves were weighed approximately

900 g and soaked with n-hexane for 24 hours to eliminate the fat content. The residue was filtered and aerated until the n-hexane solvent evaporated, then soaked in ethanol for 3 hours for percolation the mass was moved into the percolator while carefully pressed and slowly poured the solvent until the liquid began to drip and the sample was still embed in the solvent. After that the percolator was closed and left for 24 hours. Then the tap was opened and the liquid was allowed to drip at rate of 1 ml per minute. Solvent was added continuously until there was a layer of liquid on top of sample. Percolation was continued until the last out of liquid evaporated leaving no residue. Resulted liquid was evaporated using rotary evaporator with temperature $\pm 40^{\circ}\text{C}$, and then freeze dried until highly viscous extract was obtained as much as 138 g. Proceed the fractionation by liquid-liquid extraction.

3.1. Liquid-Liquid Extraction of Ethanol Extract

Fractionation was performed using solvents with different polarity from non-polar to polar such as: n-hexane, chloroform, ethyl acetate, and water.

A total of 100 g of ethanol extract was dissolved in ethanol and then added with distilled water, the mixture was put in a separating funnel and added with 200 ml of n-hexane, shaken, allowed to stand until there were 2 separated layers. n-hexane layer (upper layer) was taken with decantation method. Fractionation was done if the n-hexane layer was colorless (clear) and did not give color with Liebermann-Burchard reagent. The fractionation was continued with addition of 200 ml of chloroform in the shaker (separating funnel). The sample was shaken and then allowed to stand until there were 2 separated layers. The chloroform layer was taken (bottom layer). Fractionation was done until the chloroform was layer colorless gave negative result with Dragendorff reagent, then add 200 ml of ethyl acetate in water layer, shaken, and then allowed to stand until there are 2 separate layers. Fractionation is done until the color of the layer and the ethyl acetate is clear then fraction of water is taken, all the fractions obtained were evaporated by vacuum rotary

evaporator. Each of the obtained fractions was assay for cytotoxic activity.

3.2. In Vitro Assay for Cytotoxic Activity

Five milligrams of ethanol extract and the fractions (n-hexane, chloroform, ethyl acetate and water) were dissolved with the aid of 100 μL of DMSO, then diluted with culture media in order to get a series of concentration (500, 250, 125, 62.50, 31.25 mcg/ml). The culture media was used as negative control and doxorubicin was used as positive control with series concentration of (200, 100, 50, 25 mcg/ml). Each concentration was made replication for 3 times.

3.3. Cytotoxic Assay with MTT Method

MCF-7 cells were grown in 96 wells microplate in order to obtain the density of 1×10^4 cells/wells and incubated for 24 hours to get good growth. After the medium was replaced with a new one it was added with 100 μL of test solution in a variety of concentrations and incubated again for 24 hours. At the end of incubation, the media was removed and washed with PBS solution, and then each of the wells was added with 10 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) 0.5% in PBS. Incubation was continued for 4 hours at 37°C until formazan was formed. The living cells were converted MTT into dark-blue formazan. MTT reaction was stopped by the stopper reagent (100 μL of Sodium dodecyl sulfate), and then incubated overnight at room temperature. The absorbance was read by ELISA reader at a wavelength of 595 nm. The absorbance results were converted into percentage of life reads. MTT method was performed according to Mossman [14] with modification of stopper reagent.

3.4. Data Analysis

Data obtained in the form of absorbance values of each which converted into percentage of living cell and calculated with the following formula:

$$\% \text{ Living Cells} = \frac{\text{Absorbance Cell With Treatment} - \text{Absorbance Control Media}}{\text{Absorbance of control cell} - \text{Absorbance Control Media}} \times 100\%$$

Then IC_{50} concentrations calculated were by probit analysis using SPSS 17, in order to obtain linearity between log concentration and percentage of live cells. IC_{50} is the concentration that causes cell death for 50% of the population.

4. RESULTS AND DISCUSSION

Extraction of plant material was done to get secondary

metabolites from plant material which is used as medicine. Percolation extraction had been done by ethanol with obtained 117.4 g from 900 g of dried "*ekor naga*" leaves.

4.1. Phytochemical Screening

The identification of leaves powder and ethanol extract of *ekor naga*'s leaves chemical compounds were done

with some color reagents to obtain the class of secondary metabolites information. The results could be seen in **Table 1**.

Flavonoids as antioxidants can prevent and cope with of cancer. Mechanism of action: flavonoids in cancer treatment can inhibit the cancer cells growth through the inhibition of cell cycle mechanism, induce apoptosis, inhibition of angiogenesis, antiproliferative or a combination of these mechanisms [15].

4.2. Liquid-Liquid Extraction

Solvent extraction with n-hexane, chloroform and ethyl acetate obtained 3.681 g of n-hexane fraction, 1.680 g of chloroform fraction, 0.572 g of ethyl acetate fraction, and 24.809 g of water fraction. The screening results of n-hexane fraction with Lieberman-Burchard (LB) reagent gave a blue purple color, which was likely to contain triterpenoid/steroid compound. The chloroform fraction gave pink color which may contained triterpenoid. Triterpenoid is an antioxidant as a catcher of free radicals that can kill brain cells and also could revitalize blood vessels

Ethyl acetate fraction with FeCl_3 reagent gave a positive result, which was likely to contain polyphenolic compounds that can inhibit the growth of cancer cells through the inhibition of cancer cell growth, induce apoptosis, inhibition of angiogenesis, antiproliferative or a combination of these mechanisms [15].

4.3. Assay Anticancer

The results of the extract and some fractions treatment on MCF-7 cells can be seen in **Figure 1**. The morphology of MCF-7 cell due to the treatment of n-hexane and water fractions did not provide significant changes to the untreated cells (control cells). While the ethanol extract (*crude*), chloroform and ethyl acetate fractions with high concentration, *i.e.* 500 mcg/ml could be seen dead cells which was round (**Figure 1**).

Cytotoxicity assay were performed to confirm the cy-

totoxic ability of the sample solution against MCF-7 cells with a quantitative colorimetric method based on the measurement of the color intensity that occurred as a result of metabolism of a substrate by living cells into colored product. This study used yellow MTT {(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)} to form a dark blue formazan which insoluble in water and attached to the cells. The ability of the succinate dehydrogenase enzyme to reductase tetrazolium system were included in the mitochondria of living cells [14,16]. The method was fast, sensitive, accurate and could be used to test large samples which automatically using a spectrophotometer [16], that also proved more reliable than cell counting using a hemocytometer [17].

The results of t-test analysis of the sample treatment with several concentrations, positive control, control cells, and media control can be seen in **Figure 2**.

From **Figure 2**, it was known that the ethanol extract (concentration of 31.25 - 250 mcg/ml). The chloroform fraction (concentration of 31.25 - 500 mcg/ml), ethyl acetate fraction (concentration of 31.25 - 500 mcg/ml), n-hexane fraction (concentration of 500 mcg/ml) and water fraction (concentration of 62.5 - 500 mcg/ml), showed a significant difference ($p < 0.05$) to the positive control, media control and control cells.

Cytotoxic test gives an overview of the potential test-

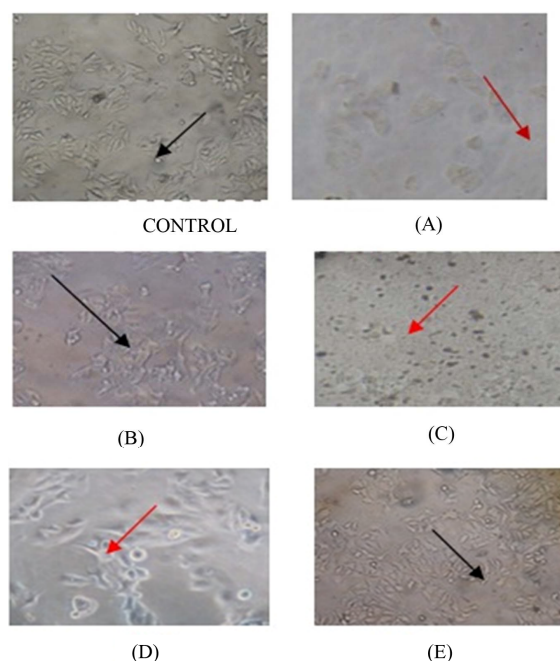


Figure 1. The effect of ethanol extract (A), hexane fraction (B), chloroform fraction (C), ethyl acetate fraction (D), and water fraction (E) of *ekor naga*'s leaves on MCF-7 cells after incubation for 24 hours. The observation of cell morphology at 24 hours was performed using an inverted microscope with 400 \times magnification. Living cells were indicated by black arrows, whereas dead cells were indicated by red arrows.

Table 1. Phytochemical screening simplex and ethanol extract.

No	Secondary metabolites	Leaves powder	Ethanol extract
1	Tannin	+	+
2	Alkaloids	+	+
3	Flavonoid	+	+
4	Saponin	+	+
5	Glycosides	+	+
6	Anthraquinone glycosides	Yellowish-brown	-
7	Steroid/Triterpenoid	+	+

ing material in inhibiting cell growth. The parameters used are the 50% of inhibition concentration (IC_{50}). The smaller value of IC_{50} the more potential in inhibiting cell growth, as **Figure 3** shows chloroform fraction IC_{50} was smaller than ethanol extract and ethyl acetate fraction.

Doxorubicin (Adriamycin) is used as a positive comparator and a chemotherapeutic agent against MCF-7 cells because it is currently one of anti-tumor chemotherapy drug that had been using successfully and extensively, although the side effects will increase with the increase the dose. Based on the results of the research, it was known that it has cardiotoxic effects associated with cumulative dose [18,19]. In addition beside it is toxic against normal tissues, it was also known as the cause of tumor cell that resistance to drugs, such as resistance of MCF-7 cells [20].

IC_{50} analysis results indicate that a parameter for cytotoxic potential of a test substance using probit analysis SPSS 17, was calculated from the relation between different concentrate with the percentage of live cells resulted ethanol extract IC_{50} 112.240 mcg/ml, chloroform fraction IC_{50} 59.082 mcg/ml, ethyl acetate fraction IC_{50} 812.663 mcg/ml, while the fraction of n-hexane and

water didn't inhibit the growth of MCF-7 cells. Doxorubicin has IC_{50} of 1.989 mcg/ml, when compared with doxorubicin, the samples still had lower IC_{50} concentration sample, which can be used to combine the sample fraction that had smaller IC_{50} with doxorubicin which can reduce the effect of cardiotoxic and resistancy of MCF-7 cells.

5. CONCLUSION

The IC_{50} values of ethanol extract obtained by cytotoxic assay results was 112.240 mcg/ml, chloroform fraction was 59.082 mcg/ml, and ethyl acetate fraction was 812.663 mcg/ml, while the fraction of n-hexane and water did not inhibit the growth of MCF-7 cells.

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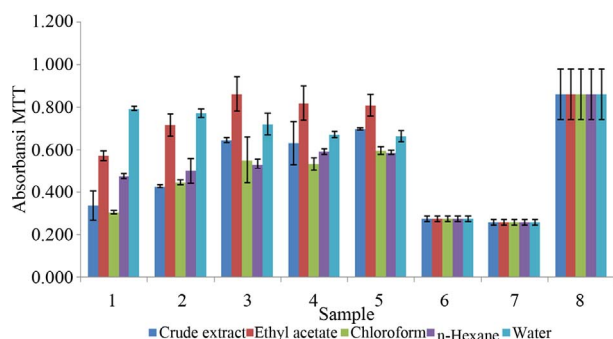


Figure 2. Effects of samples treatment to of the MTT absorbance with different concentrations. Description: 1. Extract concentration 500 mcg/ml; 2. 250 mcg/ml; 3. 125 mcg/ml; 4. 62.5 mcg/ml; 5. 31.25 mcg/ml; 6. positif control; 7. media controls; 8. control cells.

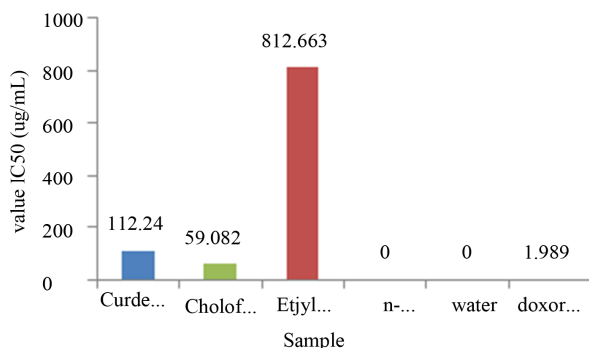


Figure 3. IC_{50} results with some samples Description: IC_{50} of n-hexane and water fractions is not detected.

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