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Original Article

Cytotoxicity of Soursop Leaves (*Annona muricata*) against Cervical HeLa Cancer Cells

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ABSTRACT

Background: Cervical cancer is the cancer with highest prevalence and the leading cause of women death in Indonesia. Current treatments available for cervical cancer are chemotherapy, radiation, surgery, and nuclear therapy. Unfortunately, these treatments still have several limitations due to serious side effects, development of resistance, and very expensive price. Therefore, it is necessary to develop effective and low-cost therapy to treat cervical cancer. One of which is by utilizing natural sources available in Indonesia such as soursop (*Annona muricata*) leaves which has been used in folk medicine as a treatment for various diseases, including cancer. However, studies about its cytotoxicity against cervical cancer in Indonesia are still limited. **Objective:** The aim of this research is to analyze the potency of *A.muricata*leaves extracts originated from Indonesia as a novel alternative treatment for cervical cancer. **Materials and method:** *A.muricata* leaves obtained from Serpong, West Java, Indonesia were grounded and macerated in three different solvents with various polarity, namely ethanol (polar solvent), ethyl acetate (semipolar solvent) and hexane (non-polar solvent). Subsequently, the extracts were diluted into 8 various concentrations. Cytotoxicity of *A.muricata*leaves extracts against HeLa cervical cancer cells were determined by MTT assay and expressed by IC_{50} value. **Results:** The results showed that three extracts of *A.muricata* have strong cytotoxicity against cervical HeLa cells. The highest cytotoxic activity was shown by etanol extract with an IC_{50} of 35.51 µg/mL, followed by ethyl acetate (IC_{50}: 5.91 µg/mL), and hexane (IC_{50}: 8.39 µg/mL). **Conclusion:** *A.muricata* leaves extracts are potential to be developed as a novel alternative therapy for cervical cancer.

Key words: *Annona muricata,* Soursop, Cytotoxicity, HeLa cells.

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INTRODUCTION

Cancer is the leading cause of 8.8 million death globally in 2015 and ranked as the 2nd disease with highest mortality.[1] According to National Basic Health Report (*Riset Kesehatan Dasar*), in 2013 the prevalence of cancer in Indonesia reached 1.4 per 1,000 population. Cancer with the highest prevalance is cervical cancer with 15,000 cases annually which make it as the leading cause of women-death in Indonesia. Cervical cancer is a type of cancer that arises from the cervical surface epithelium, located on the opening of the uterus.[2,3]

Currently, modalities of treatment available for cervical cancer consist of chemotherapy, radiation, nuclear therapy, and surgery based on stadium of the cancer. Unfortunately, these treatments have several limitations due to serious side effects such as disrupting cell metabolism, nausea, vomitting, nephrotoxicity, anemia, ototoxicity. In addition, development of resistance to the drugs also considered as limitation of the current therapy. Another obstacle encountered is the expensive cost of treatments.[4] Therefore, it is necessary to develop a novel alternative therapy which is more effective and low-cost to help the patients.

Indonesia is a tropical country with various natural resources, including herbal plants, that can be utilized as an alternative treatment. One of herbal plants that known has been widely used in Indonesian folk medicine is soursop (*Annona muricata*) (Figure 1). People commonly used its leaves to treat several conditions such as dermatitis, diarrhea, dysentry, fever, hypertension, diabetes, and also cancer.[5-7]

Based on previous research conducted by Minami *et al*, soursop leaves extract is reported had an anticancer activity against breast cancer cells through induction of apoptotic process.[8] Another study reported that soursop leaves extracts were known to increase expression of p53 gene and lower hsp70 expression, resulted in activation of apoptosis process and stable homeostasis in Raji cells.[9] However, research about cytotoxicity of *Annona muricata* leaves originating from Indonesia against cervical cancer are still limited. Therefore, this research aimed to analyze cytotoxicity of *A.muricata* leaves against cervical cancer because it is the leading cause of cancer-related death.

On the other hand, polarity of the solvents used in extraction process of herbal plant will contribute to the biological activity of the plant itself, including its cytotoxicity. Secondary metabolites contained in the plant tend to be more dissolve in the solvent with a same polarity.[10] Thus, this study also explore cytotoxicity of *Annona muricata* extract in three different polarity solvents, which are ethanol (polar),

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Figure 1: Morphology of *Annona muricata.*
Kingdom: Plantae
Divisio: Tracheophyta (Magnoliophyta)
Classes: Manoliopsida
Ordo: Magnoliales
Familia: Annonaceae
Genus: Annona L.
Species: *Annonamuricata L.*

ethyl acetate (semipolar), and hexane (nonpolar) to determine at which polarity *A.muricata* extracts show the greatest cytotoxicity against cervical cancer.

METHODS

Collection and extraction of soursop leaves (Annona muricata)

Soursop leaves originated from Serpong, Banten Province, Indonesia were washed and grinded to approximately 1000g of dry powder sample. Then, the powder were macerated in 500 mL of three different polarity solvents which are ethanol (polar), ethyl acetate (semipolar), and hexane (nonpolar). Maceration were done three times in 24 hours. The result of maceration then filtered and concentrated with vacuum dry technique to obtain ethanol, ethyl acetate, and hexane extracts of *Annona muricata* leaves. Afterwards, the three extracts of *A.muricata*were diluted using serial dilution method into 8 different concentrations (1.5, 3.125, 6.25, 12,5. 25, 50, 100, and 200 µg/mL).

In vitro cytotoxicity evaluation

Cytotoxicity of *Annona muricata* extracts against HeLa cervical cancer cells in this research were evaluated by MTT Assay. Malignant HeLa cervical cancer cells were obtained from Department of PathologyAnatomy Faculty of Medicine University of Indonesia. Then, HeLa cervical cancer cells were cultured and diluted. Afterwards, the cells were seeded into 96-microwell with each well consist of 100 µL. The well was incubated for 24 hours in CO_2 incubator. Complete media used in this study was mixture of DMEM, Penistrep and Fetal Bovine Serum. After 24 hours, the cells were ready to be treated with extracts of *Annona muricata*. Ethanol, ethyl acetate, and hexane extracts of *Annona muricata* were prepared by diluting them into 8 various concentration using serial dilution. Then, 100 µL of each sample concentrations were added into the well with three times of duplication (triplo). Positive control used in this present study was Cisplatin and negative control was cells and culture media without addition of extract. Afterwards, the cells were incubated in CO_2 incubator for another 24 hours to evaluate cytotoxicity of the sample.

On the next day, 100 µL of MTT reagent with a concentration of 5mg/mL in phosphat-buffer saline was added into the wells to determine the number of living cells. Then, the microplate was left incubated for 4 hours. After that, 100 µL of DMSO was added into each well to dissolve purple formazan crystal in the medium. Furthermore, absorbance of each well was read with ELISA reader at 590 nm. Absorbance data from the ELISA reader were transformed into inhibitory percentage using the following equation:

$$\%\text{Inhibiton} = 1 - \left(\frac{\text{absorbance of group with extract}}{\text{absorbance of control group}}\right) \times 100\%$$

IC_{50} value is determined by plotting of log concentration of *Annona muricata* extract in x-axis versus the percentage of inhibition in y-axis to produce linear regression equation, which is y = (log x) a + b. The IC_{50} value (x) were obtained by substituting y= 50% to the equation.

RESULTS

Percentage of inhibition of soursop leaves (Annona muricata) against HeLa cervical cancer cells

The percentage inhibition of soursop leaves extracts against HeLa Cervical Cancer was shown in Table 1. Based on the result, percentage inhibition of extracts *A.muricata* ranges from 12.8% to 88.3% against cervical HeLa cells. The lowest percentage of inhibition (12.6%) was shown by ethyl acetate extract at the concentration of 1.5 µg/mL. Whereas, the highest percentage (88.3%) was shown by hexane extract at the concentration of 200 µg/mL.

Figure 2 illustrated correlation between % inhibition and concentration of each extract. As shown, it is clear that the extracts showed cytotoxicity in a dosage dependent manner. The higher concentration of the extracts resulted in higher percentage of inhibition against HeLa cervical cancer cells.

Table 1: Percentage inhibiton of *A.muricata* leaves extracts against HeLa cells.

Concentration (µg/mL)	Log Concentration	Average of % Inhhibition		
		Ethanol	Ethyl Acetate	Hexane
1.5	0.176091	28.7	12.6	22.4
3.125	0.49485	40.5	33.5	40.4
6.25	0.79588	43.7	49.3	43.1
12.5	1.09691	64.0	71,3	63.8
25	1.39794	81.7	79.4	80.8
50	1.69897	83.6	84.6	85.3
100	2.00000	86.2	86.3	86.5
200	2.30103	88.5	87.5	88.3

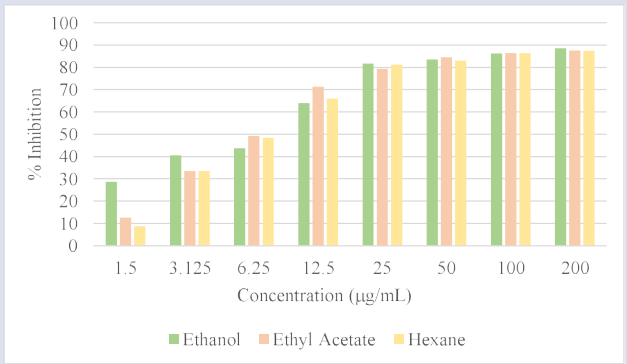

Figure 2: Correlation between concentration of extract and % inhibition.

Table 2: IC_{50} value of *A.muricata* extracts.

Extract of A.muricata	IC_{50} (µg/mL)
Ethanol	5.91
Ethyl acetate	7.56
Hexane	8.39

Cytotoxic activity of soursop leaves (Annona muricata)

Cytotoxicity of soursop leaves extracts is depicted by IC_{50} value. Based on the calculation using formula $10^{(50-b)/a}$ derived from the linear regression equation, the IC_{50} value of *A.muricata* extract were summarized in Table 2. The IC_{50} value of the ethanol, ethyl acetate, and hexane extracts was found to be 5.91; 7.56; 8.39 µg/mL, respectively. Ethanol extract of *A.muricata* showed the greatest cytotoxicity against HeLa cervical cancer cells compared to others, followed by ethyl acetate and hexane. The IC_{50} value of these three extracts were still higher when were compared to Cisplatin as a positive control (IC_{50} value = 1.78 µg/mL).

DISCUSSION

This present study used MTT Assay as a method to analyze cytotoxicity of *A.muricata* extracts against cervical cancer HeLa cells. The basic principle of MTT assay is that it measures the reduction of yellow tetrazolium salt into purple formazan crystal. By reductase enzyme found in mitochondria of viable cells. Thus, the higher absorbance of the sample indicates the higher number of living cells. Percentage of Inhibition describes the number of cancer cells growth that can be inhibited by the extract.[11] Table 1 showed the inhibitory percentage of *A.muricata* extracts against HeLa cervical cancer cells. The result indicates that there is a positive correlation between concentration of the extract and the inhibition percentage.

Cytotoxicity is depicted by IC_{50} value which means the concentration needed for the extract to inhibit 50% activity of the cancer cells. The lower IC_{50} value of an extract indicates the greatest cytotoxicity against cancer cells. Table 2 summarized IC_{50} value of three *Anonna muricata* leaves extract. The lowest IC_{50} value (5.91 µg/mL) was shown by ethanol extract, suggesting that ethanol extract has the greatest cytotoxicity compared to other extracts.

According to American National Cancer Institute, crude extract of a herbal plant is considered to have *in vitro* cytotoxicity if it shows IC_{50} less than 20 µg/mL.[12] As displayed in Table 2, the IC_{50} value of all*A. muricata* extracts are less than 20µg/mL. Thus, it can be concluded that all extracts have active cytotoxicity against HeLa cervical cancer cells.

Previous studies have reported various mechanism of *A.muricata* as an anticancer agent. According to Torres *et al,* soursop leaves showed inhibit tumorgenicity and metastasis in pancreatic cancer by inhibiting cell metabolism.[13] Another study conducted by Liu *et al* reported that soursop leaves could activate apoptosis process of hepatic cancer through reticulum endoplasm stress.[14] In addition, Moghadamtousi also proved that soursop leaves extract could induce apoptotis process of lung cancer through mitochondria pathway.[15]

Anticancer activity of *Annona muricata* could be affected by its biological compounds. Research suggest that it comes from special compound owned by genus *Annonaceae* called *Annonaceous acetogenin*. This molecule is derivate of long chain fatty acids (C32 or C34).[16] The cytotoxicity mechanism of this molecule is by inhibiting complex I in mitochondria and reduce the ATP production in cytoplasm, resulting in disruption of oxidative phosphorylation and leads to activation of apoptosis process. Cancer cells require more ATP than normal cells because they are constantly growing. Thus, it makes cancer cells more susceptible to *Annonaceous acetogenin* rather than normal cells.[17] On the other hand, *Annonaceous acetogenin* is a polar compound which is more easier to dissolve in polar solvent. This could explain the result from this study which showed that ethanol extract with the highest polarity has the greatest cytotoxicity compared to other extracts.

Another biological compound contributes to anticancer effect of *Annona muricata* is flavonoid, as reported by Yang *et al.* The administration of soursop leaves extract has been shown to inhibit the growth of prostatic cancer. Flavonoid is also a polar compound and it is more likely to dissolve in polar solvents. Mechanism of flavonoid as an anticancer including induction of apoptosis, inhibition of cells proliferation, and inhibition of lipid peroxidation.[18]

CONCLUSION

This research clearly demonstrate that three extracts of *A.muricata* have a strong cytotoxicity against HeLa cervical cancer cells with the greatest cytotoxicity was shown by ethanol extract (IC_{50} = 5.91 µg/mL). Thus, extracts of *A.muricata* are potential to be developed as a novel alternative therapy for cervical cancer.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

ABBREVIATIONS

MTT: (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide); IC_{50}: Inhibition Concentration 50%; %: Percentage; µg: Microgram; nm: Nanometer; g: Gram; mL: Millimeter; nm: Nanometer; PBS: Phosphate- Buffered Saline; HeLa: Henrietta µl: Microliter; CO_2: Carbon dioxide; µg/mL: Microgram/milliliter.

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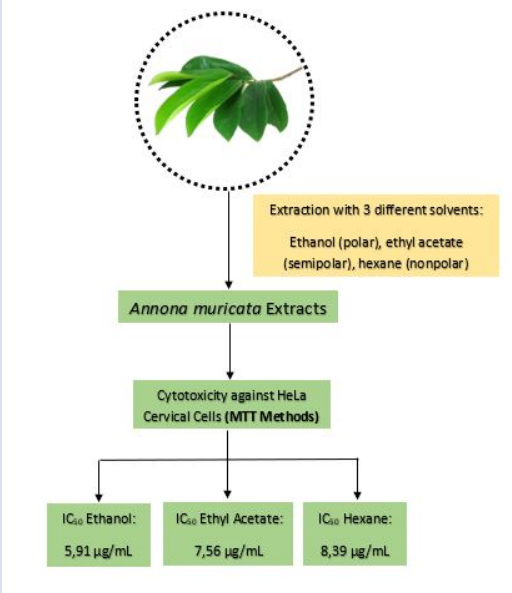
GRAPHICAL ABSTRACT



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