

## Cytotoxic Activity of Ethanol Extract in Namnam Leaves (*cynometra cauliflora* L.) to Hela Cell

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### Abstract

Cervical cancer is one of the most prominent death cases after breast cancer for a woman in Indonesia. Several treatments can be conducted to cure cancer such as chemotherapy, radiotherapy, tumor surgery, and others. In addition, people can consume some medicines or natural substances that can suppress cancer growth. The natural substances contain active compounds that have the potential as anticancer. One of the natural substances used in this research is the Namnam leaves which grow in Southeast Asia. The purpose of this research was to determine the potential compounds in Namnam leaves extract by cytotoxic activity testing by using HeLa cells. The active compound in the Namnam leaves extract was obtained by using the maceration method with ethanol for 3x24 hours. The extract was then tested by BSLT method and proliferation by using HeLa cancer cells (ATCC CCL-2). The toxicity results showed that LC50 value was 125,89 µg/mL. This result indicated that the extract belongs to the moderate toxic category and has potential as an anticancer agent. Proliferation test in inhibiting of HeLa cancer cells used Microculture Tetrazolium Technique (MTT) method. The result showed that the extract with a concentration of 25 µg/mL could inhibit the proliferation of HeLa cancer cells by 57,51%.

**Keywords:** Cytotoxic activity; ethanol extract of Namnam leaves; HeLa cell

### Introduction

Cancer is a noninfectious disease caused by free radicals that are concerned in the world including Indonesia. Cancer or tumor prevalence had an increasing number around 0.4 per-mille according to doctor diagnoses in Indonesia. This data showed in Basic Health Research (*Risikesdas*) that conducted by the Ministry of Health in 2018 (*Badan Penelitian dan Pengembangan Kesehatan*, 2018). The rate of cancer disease in Indonesia had 8<sup>th</sup> level in Southeast Asia and 23<sup>rd</sup> level in Asia by 136.2/100,000 population. The highest occurrence for breast cancer was 42.1 per 100,000

population women following by cervical cancer 23.4 per 100,000 with a mortality rate of 13.9 per 100,000 population (Kemenkes, 2019). Several treatments were conducted to cure cancer such as chemotherapy, surgery, and radiotherapy. Chemotherapy is one of the treatments that used anticancer drugs to inhibit cancer cells growth. This treatment is not as effective as others because it has to design anticancer compounds that have high anticancer properties but low side effects to normal cells and hit the target of the cell cancer accurately (Yudistira, 2017)

Many patients have side effects after doing chemotherapy such as hair loss,

tiredness, anemia, loss of appetite, and others (Society, 2020). Therefore, they try another treatment by consuming some natural medicines. These medicines are obtained from natural substances like a plant. Medicine made from natural substances is the best solution to prevent and cure cancer because it is safer than chemotherapy (Mutiah, 2017). A natural substance that has potential as a medicine is a plant with the *Cynometra* genus from *Fabaceae/Leguminosae familie*.

Aziz and Iqbal identified this plant about phytochemical compounds from the extract of the plant. It contains tannin, saponin, flavonoid, terpenoid, and glycoside that had potential as antioxidants, antidiabetics, and anticancer (Sumarlin *et al.*, 2018; Maharani, Sukandar and Hermanto, 2016). The antioxidant of Namnam leaves extract was 66,36 µg/mL. The leaves had higher antioxidant activity than other parts of the plant. The antioxidant properties were proven to prevent cancer cells and had a lot of advantages (Mulia, Endang Zainal Hasan, and Suryani, 2016). This extract has several phytochemical compounds that can be used potentially as antidiabetics and anticancer. The purpose of this research was to determine anticancer properties by cytotoxic activity from extract Namnam leaves to HeLa cancer cells. The research was begun with identifying the toxicity of Namnam leaves extract by using ethanol. The value of toxicity was used to determine the cytotoxic activity to test the use of HeLa cancer cells by MTT assay.

## Research Methods

### Tools and Materials

The tools used in this experiment were rotary vacuum evaporator (Buchii B480, incubator (Memmert), and ELISA. The ingredients used in this research were ethanol (p.a Merck), *shrimp*, DMSO, Dulbecco's modified eagle's medium (D-MEM), RPMI 1640, Fetal Bovine Serum (FBS) 5%, Penicillin, Streptomycin, HeLa cell (ATCC CCL-2), and Namnam plant. Namnam plant can be found in Rangkasbitung, Banten Province. The plant was identified by the

Indonesian Academy of Sciences (LIPI) with the number B-994/IPH.3./KS/IV/2019. It stated that the Namnam plant is included *Cynometra cauliflora* L species and a family of *Leguminosae/Caesalpinaceae*.

## Procedure

### Extraction Process

The leaves were dried, grounded, and weighed 100 grams to have a maceration process by using ethanol by ratio 1:5 solvent. The maceration process took 3x24 hours. This method was a modification from (Maharani, Sukandar, and Hermanto, 2016) method and procedure from BIOFARMAKA, Bogor laboratory. The extract was condensed by removing solvent and using a rotary evaporator at 45-50°C. This extract was called crude extract (Maharani, Sukandar, and Hermanto, 2016).

### Toxicity Test

According to Lestari, Kartika, and Marlina (2019), the toxicity of the extract then was tested by using BSLT method and shrimp larvae of *Artemia salina*. The shrimp larvae egg (10 mg) was incubated in 250 mL seawater using a lamp and aerator for two days. Ten shrimp larvae that had been hatching were added to 1000 µL seawater. Each vial contains ten shrimp larvae, seawater, and additional extract of the concentration 10 ppm; 50 ppm; 100 ppm; 500 ppm; and 1000 ppm consecutively. Each concentration replicated the test three times. For control, it used ten shrimp larvae and seawater without adding extract. The solution was observed for 24 hours to see shrimp larvae mortality. To determine shrimp larvae mortality, it can be calculated by the following formula:

$$\%mortality = \frac{\sum \text{larvae mortality}}{\sum \text{total larvae}} \times 100\% \quad (1)$$

The value of LC<sub>50</sub> was obtained from Probit analysis by using a regression linear curve which log<sub>10</sub> concentration as x-axis and Probit as y-axis (Mshelia E.H., Maigari and, Yohanna Christopher., and Ismail, 2016).

### Microculture Tetrazolium (MTT) Assay

The Primate Animal Study Center (PSSP) IPB, Bogor, has ever held proliferation test by using the procedure. HeLa cancer cells can be grown by a concentration of 5000 cells in 100 $\mu$ L on a growing medium. The extract was added after the cell reached confluent 50% in 24 hours. Microculture Tetrazolium Technique (MTT) assay was done after day 3 by adding MTT (5mg/mL) 10  $\mu$ L per well then incubated for 4 hours at 37°C. Formazan crystals were dissolved with ethanol. Absorbance was measured at wavelength 595 nm by ELISA reader. The absorbance was converted into inhibition percentage to determine the cytotoxic activity of the extract.

### Results and Discussion

The extract of Namnam leaves used ethanol solvent by ratio 1:5 with maceration method for 3x24 hours. This solvent was categorized as a polar solvent that easily attracts polar compounds such as phenolic compounds (Manongko, Sangi, and Momuat, 2020). It's also an organic solvent that was used to dissolve an organic compound or being used as a solvent extract (Ismiyati, 2015). To determine the toxicity of the extract, the use of BSLT method had several advantages such as simplicity, rapidness, inexpensiveness, and having high degrees of repeatability (Nofita, Maria Ulfa, and Delima, 2021). This method was used to test if such compounds had potential as anticancer properties by calculating the mortality rate of shrimp larvae *Artemia salina*. The number of shrimp larvae mortality on each concentration was shown in table 1. The value of LC<sub>50</sub> was obtained from the graph by plotting probit analysis using a regression linear curve.

Table 1 shows that the extract had a toxicity value of LC<sub>50</sub> at 125.89  $\mu$ g/mL. Meyer et al stated that extract from natural substances was classified as toxic if it had a value of LC<sub>50</sub> < 1000  $\mu$ g/mL, while its pure compound had a value of LC<sub>50</sub> < 200  $\mu$ g/mL. It means that it had potential as an

anticancer. Lestari *et al* stated that if the value of LC<sub>50</sub> > 1000 ppm, its category was non-toxic substances (Nofita, Maria Ulfa, and Delima, 2021).

Table 1. Lethal Concentration of *A. Salina* against Different Extract Concentrations

Concentration ( $\mu$ g/mL)	Average Mortality Shrimp Larve <i>Artemia salina</i> Leach	LC <sub>50</sub> ( $\mu$ g/mL)
10	2	
50	2	
100	3	125,89
500	8	
1000	9	

There are several classifications of toxicity by LC<sub>50</sub> values. If the value of LC<sub>50</sub> is 500-1000 ppm, it is classified as low toxic, 100-500 ppm as moderately toxic, and 0-100 ppm as highly toxic. The value of LC<sub>50</sub> of this research showed that ethanol extract of Namnam leaves was in the moderate toxic category and had the potential for anticancer substances (Mshelia E.H., Maigari and, Yohanna Christopher., and Ismail, 2016).

Cancer causes progeny from normal cells that have lost their cellular activities in controlling proliferation. It could have happened because of growth factors in the specific location that received instruction to grow and division of cells (Berridge, 2012). HeLa cell culture was adopted from a woman who suffered cervical cancer named Henrietta Lack who died in 1951 (Suraduhita, 2017). This cell is a continuous cell line that can be used as a cancer cell model and learn about the cellular transduction signal. It's also a safe cell to be used in the research and human cell in general which can be used in cell culture (Kurniawan, Chandra; Siagian, Jonathan Willy; Hutomo, 2016). The proliferation test was conducted by MTT method. This method had a principal reduction of yellow tetrazolium MTT salt by using the reductase enzyme. The succinate tetrazolium reacted into respiration cycles in mitochondria cells and formed a purple formazan crystal. This crystal was not

dissolved into the water by adding a stopper reagent. The crystal was dissolved and measured in ELISA reader at wavelength 595 nm (Tatiana and Ria, 2020).

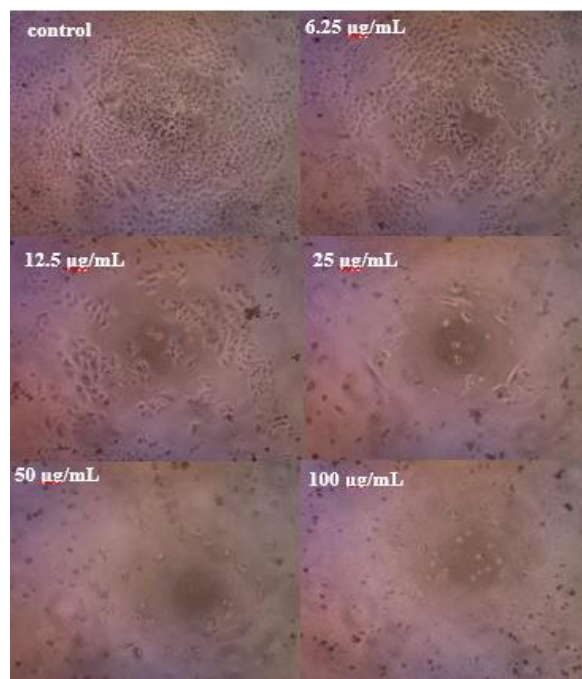
Based on the concentration result in the toxicity test by using BSLT method, the concentration extract that used in this MTT assay was 100 ppm; 50 ppm; 25 ppm; 12.5 ppm; 6.25 ppm. The extract and HeLa cancer cells were combined then measured in ELISA reader. The potential anticancer agent of the extract Namnam leaves was shown in percentage inhibition data which inhibit HeLa cancer cells growth. The data was shown in table 2 and the picture below.

**Table 2.** Proliferation Result of Extract Namnam Leaves in HeLa Cell

Concentration (ppm)	% Inhibition	IC <sub>50</sub> (µg/mL)
100	94.49	
50	93.08	
25	57.51	~25
12,5	nd	
6,25	nd	

Table 2, it showed the value of IC<sub>50</sub> at concentration 25 µg/mL. This value was obtained from percent inhibition of HeLa cancer cells that could inhibit or equal to 50% from initial concentration (Fassy *et al.*, 2017). The picture above has shown the morphology of HeLa cancer cells after treatment with ethanol extract of Namnam leaves by various concentrations. Increasing extract concentration of Namnam leaves indicates that the HeLa cancer cell could be inhibited higher than 50%. It's shown in morphology by using the colony of the cell was apart from center growth (control). It is also shown that some of HeLa cancer cells were damaged and others were still the same with control. This extract contained flavonoid and tannin compounds. Flavonoids in the extract of Namnam leaves could presume to inactivate the carcinogen, inhibit of angiogenesis process, antiproliferation, inhibit the cycle of cells, restore resistance to medicine, and combine from all the mechanism (Diba, Salni and Subandrate, 2019). Tannin compounds contained in an extract could inhibit the growth

of cancer cells (Fitriana, 2019). This extract of Namnam leaves could be developed as medicine and curing of cervical cancer by doing this research results to fractionate the extract. Therefore, the pure compound obtained from the extract could assess the mechanistic target of cervical cancer or proliferation cycles to the cancer cells.



**Figure 1.** Morphology of HeLa cell in Ethanol Extract of Namnam Leaves

## Conclusions and Suggestion

### Conclusions

The toxicity of ethanol extract in Namnam leaves was tested by using BSLT method. It showed that the value of LC<sub>50</sub> was 125.89 µg/mL with the category as toxic and had potential as an anticancer agent. Proliferation inhibition of HeLa cancer cells by MTT method showed a value of 57.51% at the concentration of 25µg/mL of extract. The extract had big potential to be developed as an anticancer agent for cervical cancer.

### Suggestion

To improve the potential of Namnam leaves extract, the research is possibly conducted on other cells or by in vivo process.

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