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Research Article

Cytotoxic Activity of Ethyl Acetate Extract from *Voacanga foetida* (Bl.) Rolfe Leaves Against T47D Breast Cancer Cells

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Abstract

The cytotoxicity of ethyl acetate extract from Tampa badak (*Voacanga foetida* (Bl.) Rolfe) leaves against *Artemia salina* leach larvae was determined using the brine shrimp lethality test (BSLT) method and was evaluated against T47D breast cancer cells using MTT assay method. The result of BSLT showed a consistent result with MTT assay, which is the result obtained that ethyl acetate extract is very toxic against A. salina Leach larvae with LC_{50} value of 8.61 µg/mL and very cytotoxic against T47D breast cancer cells with IC_{50} values of 0.87; 0.66; and 0.95 µg/mL at the 24, 48 and 72 hours of incubation times, respectively. The MTT assay data were analyzed using a two-way ANOVA statistical method to see the effect of the dependent variable (concentration and time) on the independent variable (% viability). Based on the statistical test result, there is a difference in % viability between concentrations of 0.1; 1; and 10 µg/mL ($p < 0.05$), but the length of incubation does not affect % viability ($p > 0.05$).

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INTRODUCTION

Breast cancer attacks the epithelial tissue of the breast (carcinoma) and generally originates from the glands, gland ducts, and supporting tissues of the breast[1]. The risk factors for breast cancer include a family history of breast cancer, obesity, consumption of fast food that contains a lot of saturated fat, alcohol consumption, menopause at an older age (> 50 years), early menarche, namely the first menstruation at a relatively young age (≤12 years), long-term use of hormonal contraceptives, radiation exposure, having had a benign breast tumor or breast cancer, never giving birth or giving birth for the first time at the age of more than 35 years, and not breastfeeding are also risk factors for breast cancer[2,3].

Treatment of cancer patients can be done with surgery, radiotherapy, and chemotherapy. Chemotherapy is a cancer treatment using cytotoxic chemicals[4]. The working principle of chemotherapy is to kill cancer cells, control their growth, and stop their growth from spreading or reduce the symptoms caused by cancer[5]. Treatment with chemotherapy has not given satisfactory results because it does not work precisely. It can also cause normal cell damage and cause some side effects, such as hair loss, nausea, vomiting, diarrhea, susceptibility to infection, thrombocytopenia, neuropathy, and myalgia. Treatment with radiation causes side effects such as nausea and vomiting[6].

Meanwhile, surgical treatment cannot entirely remove body tissue damaged by cancer[7]. Because of these conditions, it is necessary to look for alternative drugs, one of which is developing anticancer agents derived from natural ingredients or

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chemopreventives[8]. Anticancer drugs from natural ingredients can treat the source of the disease by repairing damaged cells, tissues, and organs by increasing the immune system[9].

Tampa badak (*Voacanga foetida* (Bl.) Rolfe) is an Indonesian medicinal plant from the Apocynaceae family. Based on our previous work, the leaves methanolic extracts, their fractions (*n*-hexane, ethyl acetate, and butanol fractions), and some isolated compounds from this plant were reported to have highly cytotoxic activity against L1210 blood cancer cells[10]. One isolated compound from this plant's butanol fraction of leaves ethanolic extract was also tested against various cancer cell lines (K562, A549, HeLa), and the isolate exhibited a highly cytotoxic activity[11]. In addition, the ethanolic extract also demonstrated in vivo anticarcinogenic activity on the dose of 200 mg/kgBW, and based on the acute toxicity test, this extract was also categorized as not toxic (LD_{50} >15000 mg/kgBW)[12]. Therefore, it becomes fascinating to explore more widely the potential of *V. foetida* as a natural source of bioactive agents to treat various types of cancers.

Our previous work also reported that the leaves ethyl acetate extract of *V. foetida* exhibited highly cytotoxic activity against HTB-38 colon cancer cells[13]. However, the potency of the cytotoxic activity of this extract against other cancer cell lines has never been explored. Therefore, this work aimed to investigate the cytotoxic potency of ethyl acetate extract of *V. foetida* leaves by BSLT method and then continued with an *in vitro* evaluation against T47D breast cancer cells.

MATERIALS AND METHODS

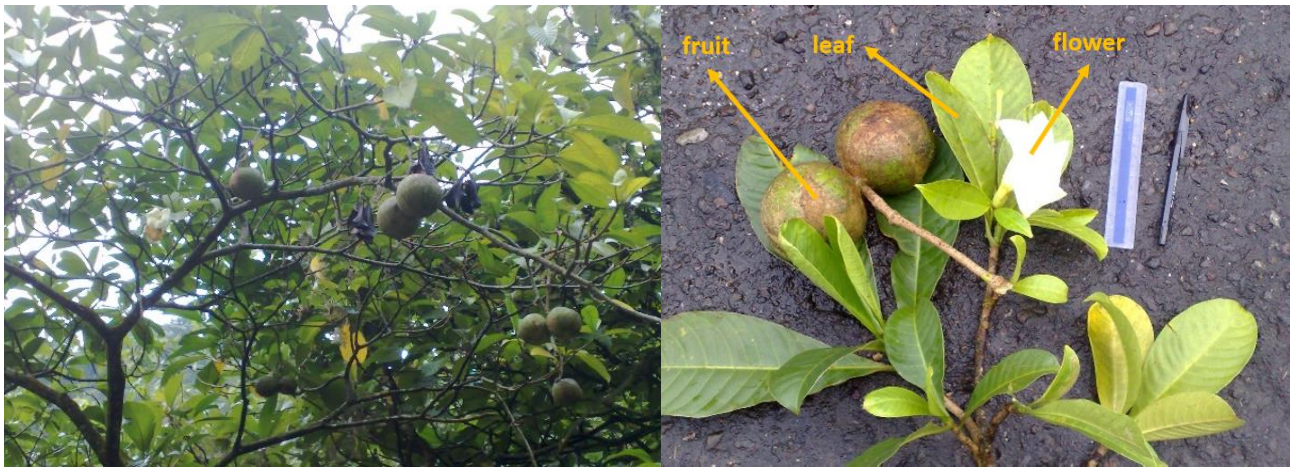
Materials

The materials used in this work included *n*-hexane, ethyl acetate, cysts/eggs *Artemia salina* leach (Supreme Plus), sea salt, T47D cell line, RPMI 1640 powder (Rosewell Park Memorial Institute), Penicillin-streptomycin, fungizone, Fetal Bovine serum (FBS), phosphate-buffered saline (PBS), MTT Trypsin-EDTA (trypsin-ethylenediaminetetraacetic acid) reagent, and dimethylsulfoxide (DMSO).

Methods

Sample collection and extraction

The plant sample, as shown in **Figure 1**, was collected from Biological Education and Research Forest Universitas Andalas, Padang, West Sumatra, Indonesia[14]. The identification document number 315/K-ID/ANDA/IX/2020 identified it as *Voacanga foetida* (Bl.) Rolfe. The leaves part of *V. foetida* was washed, dried, and sorted. As much as 2.15 kg of chopped simplicia was macerated using *n*-hexane for five days at room temperature and in triplicate. The *n*-hexane macerates were separated by filtration, and the residues were macerated using ethyl acetate for five days at room temperature, triplicate, and then filtered to afford ethyl acetate macerates. The ethyl acetate macerates were concentrated by a vacuum rotary evaporator to afford 70 g of ethyl acetate concentrated extract.



a **b**

Figure 1. (a) complete plant and (b) leaves, flowers, and fruits part of *V. foetida*

Brine Shrimp Lethality test (BSLT)

The BSLT is a preliminary screening to determine whether a plant has bioactive compounds that have the potential as anticancer. The advantages of the BSLT method are that it is fast, easy, and does not require expensive costs. The toxicity test against *A. salina* followed the standard protocol from previous literature[15,16]. The extract was tested in various concentrations (1000, 100, and 10 µg/mL), and the LC_{50} was calculated.

Cytotoxic test using MTT assay

The cytotoxic test was used to measure cancer cell viability after adding a sample solution. The advantages of this method are that the work is relatively fast, the results are accurate, the interpretation of the results is relatively easy, and the equipment used is simple. In this work, the cytotoxic test was performed using an MTT assay, a colorimetric assay. The principle of this assay is the reduction of the yellow tetrazolium salt to form purple formazan crystals that are insoluble in water by the succinate reductase enzyme present in the mitochondria of living cells. The addition of a stopper reagent will dissolve the formazan crystals, which are then measured for absorbance using an ELISA reader at a wavelength of 570 nm. The intensity of the purple color formed is proportional to the number of living cells. The greater the absorbance, the greater the number of living cells.

The cytotoxicity assay using MTT assay followed the standard protocol from the previous literature[17]. In this work, *in vitro* cytotoxic activity of the ethyl acetate extract at various concentrations (10, 1, and 0.1 µg/mL) and various incubation times were evaluated against the T47D breast cancer cell line, and the IC_{50} values were calculated. The test on the normal cell has not been conducted because the Vero cell was not available in the laboratory where we worked.

RESULTS AND DISCUSSION

Brine Shrimp Lethality test (BSLT)

The toxic effect of an extract based on the BSLT test was determined by determining the LC_{50} value. LC_{50} is the concentration of the tested extract that can cause the death of 50% of *A. salina*. The death of the larvae was caused by the tested compound, which acted as stomach poisoning. The test compound that entered the larval body interfered with the larval digestive system and inhibited taste receptors in the larval mouth area. This causes the larvae to fail to get a taste stimulus, so they cannot recognize their food, and as a result, the larvae starve to death[18]. The resulting toxic effect indicates the disruption of the cell formation process, which is assumed to be cancer cells[19].

In this test, a negative control was also used to see whether the response to the test animals' death was caused by the extract and not caused by the solvent used[18]. The standard criterion for measuring the mortality of *A. salina* larvae is if the larvae do not show movement during observation[20]. Based on the BSLT result presented in **Table I**, the ethyl acetate extract of *V. foetida* leaves exhibited an LC_{50} value of less than 10 µg/mL. Based on this result, the ethyl acetate extract can be categorized as very toxic against *A. salina*.

Table I. The BSLT result of ethyl acetate extract of *V. foetida* leaves in various concentrations

Concentrations (µg/mL)	Death cells (%)	Probit value	LC_{50} (µg/mL)
1000	100	8.7	8.6
100	86.7	6.1	
10	66.7	5.4	

Cytotoxic test using MTT assay

The MTT assay showed a consistent result with BSLT results, where the ethyl acetate extract of *V. foetida* leaves also exhibited an IC_{50} value of less than 10 µg/mL against T47D breast cancer cells, as presented in **Table II**. According to the literatures[21,22], an extract is categorized as highly cytotoxic if the IC_{50} value is ≤20 µg/mL, moderately cytotoxic if IC_{50} of 21-200 µg/mL, weak if IC_{50} of 201-500 µg/mL, and not cytotoxic if IC_{50} >500 µg/mL. Based on the categories, it can be concluded that the ethyl acetate extract of *V. foetida* leaves was highly cytotoxic against T47D breast cancer cells. It can also be observed that the IC_{50} value of the extract is not time-dependent. The incubation time of 24 hours caused the extract to have an IC_{50} value of 0.87 µg/mL. When the incubation time is extended to 48 hours, the IC_{50} value of the extract decreases to 0.66 µg/mL. The

decrease in this IC_{50} value from an incubation time of 24 to 48 hours might be caused by the tested extract still acting to kill the cancer cells, and the optimum action of the extract was achieved at an incubation time of 48 hours. In other word, the optimum incubation time was achieved at 48 hours.

Table II. IC_{50} value of ethyl acetate extract of *V. foetida* leaves against T47D breast cancer cells in various incubation times

Incubation time (hours)	IC_{50} (µg/mL)	Category
24	0.87	Highly cytotoxic
48	0.66	Highly cytotoxic
72	0.95	Highly cytotoxic

Based on **Figure 2**, generally, there is a decrease in the percentage of cell viability when the incubation time is extended from 24 to 48 hours. After 48 hours, the viability percentage tends to increase again, and it cause the IC_{50} value to rise to 0.95 µg/mL at the incubation time of 72 hours. This is a common phenomenon in cytotoxic assay against cancer cells, and previous researchers have also reported similar things, and the different plant extracts might show a different phenomenon[23]. However, the difference in incubation times from 24 to 72 hours in this work was observed not to cause a difference in the category of their cytotoxic activities. In other word, it can be observed that the variation in incubation time did not affect the percentage of cell viability. All extracts in various incubation times still exhibited the same cytotoxic category.

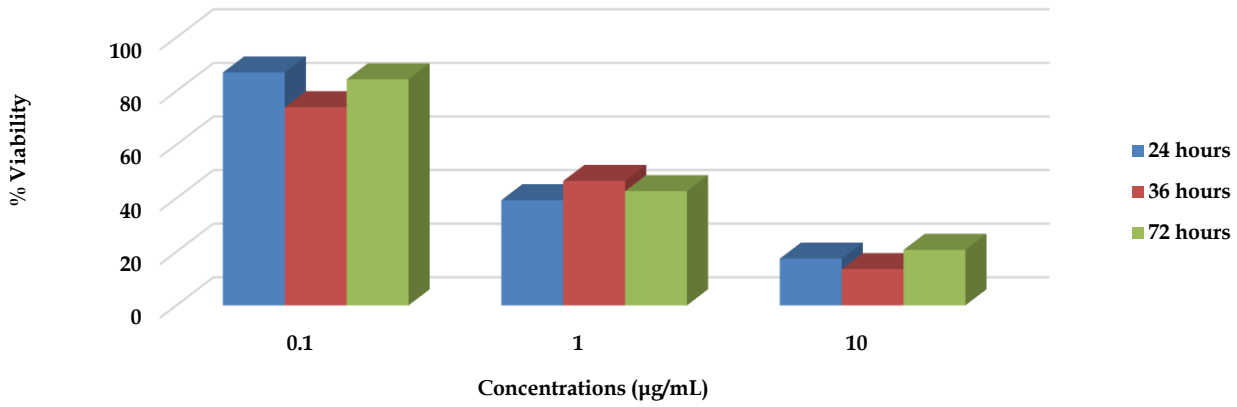


Figure 2. Comparison of % viability of T47D breast cancer cells after giving ethyl acetate extract in various concentrations after incubation for 24, 48, and 72 hours

To support this hypothesis, the data were analyzed using the two-way ANOVA statistical method to see the effect of the independent variables (concentration and time) on the dependent variable (percentage of cell viability). Furthermore, the Tukey test was also performed to see the difference between the concentration and time group variables on the percentage of cell viability. The result of ANOVA analysis gave a significant difference if $p < 0.05$. Based on the two-way ANOVA analysis, it was found that the variation in incubation times (24, 48, and 72 hours) did not show a significant effect on the percentage of cell viability that was indicated by a p-value of 0.606 ($p > 0.05$). While the variation in tested concentrations (0.1, 1.0, and 10 µg/mL) showed a significant effect on the percentage of cell viability that was indicated by the p-value of 0.000 ($p < 0.05$). Furthermore, the Tukey test was conducted to see the difference in concentration variables. Tukey's test is a further test that assesses significant differences in variables in a group. Based on this test, it is known that each concentration is significantly different, where the concentration of 0.1 µg/mL is significantly different from the concentration of 1.0 and 10 µg/mL and vice versa.

The cytotoxic effect of the ethyl acetate extract of *V. foetida* leaves is thought to be due to secondary metabolites such as alkaloids, steroids, and terpenoids[24]. Some alkaloids are used as cancer drugs and can induce apoptosis by binding to DNA by inhibiting the topoisomerase I enzyme in the DNA replication process so that it will cause permanent DNA double strands damage that triggers apoptosis[25]. Some steroids also have anticancer activity by occupying estrogen hormone

receptors on breast cancer cells so cell proliferation does not occur[26]. Some terpenoids were also reported to have anticancer activity by inducing DNA fragmentation through activating the DNase enzyme so that cell apoptosis occurs[27]. The result of the cytotoxicity assay in this work also showed a similar effect to our previous work. In previous work, we have reported that the ethyl acetate extract of *V. foetida* leaves also exhibited highly cytotoxic activity against HTB-38 colon cancer cells[13]. In addition, the other extracts, fractions, and isolates of this plant also showed highly cytotoxic activity against various cancer cell lines[10-12].

CONCLUSION

The cytotoxic activity of the ethyl acetate extract of *V. foetida* leaves collected from Padang, West Sumatra, Indonesia, in May has been conducted by BSLT and MTT assay. The result showed that the extract exhibited highly cytotoxic activity against T47D breast cancer cells with IC_{50} values of 0.66 µg/mL at optimum incubation times (48 hours). This result is consistent with the BSLT result, where the extract also showed a very toxic effect against *A. salina* with LC_{50} of 8.61 µg/mL.

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AUTHORS' CONTRIBUTION

Conceptualization: Adriani Susanty, Dachriyanus, and Fatma Sri Wahyuni. **Methodology**: Adriany Susanty and Mira Febrina. **Extraction and BSLT**: Dian Sanita Putri. **MTT assay**: Mira Febrina and Adriani Susanty. **Data analysis**: Adriani Susanty, Ihsan Ikhtiarudin, and Dian Sanita Putri. **Writing original draft preparation**: Adriani Susanty and Mira Febrina. **Review and editing**: Adriani Susanty and Ihsan Ikhtiarudin.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

All authors declare no conflict interest.

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