

Cytotoxicity differences from roots and leaves ethanol extracts of *Annona muricata* Linn on HSC-3



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Abstract

Objective: The aim of this study is to examine cytotoxicity differences between ethanol extract of *A. muricata* roots and leaves against HSC-3 cell line.

Material and Methods: HSC-3 cell lines were treated with ethanol extracts of *A. muricata* roots and leaves with 100, 30, 10, and 1 µg/mL concentrations for 24 hours. The percentage of cell viability was measured using MTT assay.

Results: The results showed significant differences ($p < 0.001$) of treatments group compared to negative control. Ethanol extract of *A. muricata* roots showed the highest cytotoxicity against HSC-3 cell lines.

Conclusion: There is difference in cytotoxicity between ethanol extracts of *A. muricata* roots and leaves against HSC-3 cell lines where root extracts showed higher cytotoxicity.

Keywords: *Annona muricata* Linn, Anticancer, Cytotoxicity, Ethanol, HSC-3
DOI : [10.15562/jdmfs.v8i2.1593](https://doi.org/10.15562/jdmfs.v8i2.1593)

Introduction

Oral cancer ranks eighth of the most common cancers worldwide.¹ In the 5-year survival rate, oral cancer is one of the most deadly cancers.² Tongue cancer is the most common malignancy represents 25% to 50% of all oral squamous cell carcinomas with a poor prognosis.¹ Based on a 2018 literature study using the Australian Cancer Incidence and Mortality database and information from the National Registry of Diseases Office, Singapore, it was found that tongue cancer cases experienced a significant increase especially in individuals aged 45 years and under in Australian and Singaporean populations.³ According to a study from World Health Report, the incidence of tongue cancer is most often found in men (48.2%) compared to women (20.5%).³ Tongue cancer is mostly located in the anterior two thirds of the tongue, generally on the lateral and lingual side of the tongue.¹ Smoking tobacco and alcohol consumption are major risk factors of tongue cancer.⁴ Some other risk factors are poor oral hygiene, bacterial infections, and viral infections such as Human Papillomavirus.^{5,6}

The main treatments that are often performed for tongue cancer include surgery, radiotherapy, and chemotherapy.⁷ However, these treatments are known to be less selective in killing cancer cells so that they have a negative impact to healthy cells in the body.⁸ Therefore, the development of a safe, effective, and selective alternative treatment for cancer is very impor-

tant to research. For several years, many Indonesians still rely on traditional medicine as medical treatment.⁸ One of the plants that is still and often used is *A. muricata* or commonly known as soursop.⁹

A. muricata is a family of annonaceae originating from central america and widely distributed throughout Asia to Australia.¹⁰ The leaves of *A. muricata* are very popularly used to treat inflammation, cancer, diabetes, and liver disease.¹¹ In addition to the leaves, previous studies have shown that the roots of *A. muricata* has a constituent that is highly toxic to lung cancer cell lines (A549) and liver cancer cell lines (HepG2) which called (+)-Xylopin.¹² Compounds that contained in *A. muricata* such as alkaloid, phenol/tannin, flavonoid, steroid, and terpenoid exhibit a range of bioactivities such as anticancer, antidiabetic, antimicrobial, anti-inflammatory and antioxidant.¹² Acetogenin which discovered in *A. muricata* showed active cytotoxicity against cancer cells but non-toxic to normal cells.¹³

Another previous study reported that *A. muricata* leaves extract was able to induce apoptosis in colon cancer cell lines (HT-29 and HCT-116) and lung cancer cell lines (A549) with significant inhibition of cancer cell migration.¹⁴ Moreover, the ethanol extracts of roots and leaves of *A. muricata* have been shown to kill leukemia cell lines (HL-60) more than 80%.¹⁵ Previous studies have proven that ethanol extract has selective cytotoxic properties on tumor cells

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Received 8 February 2022
Revised 25 October 2022
Accepted 2 January 2023
Available online 1 August 2023

Table 1. Qualitative phytochemical tests of ethanol extracts from *A. muricata* roots and leaves

Compound	Results
Alkaloid	+
Flavonoid	+
Steroid	+
Tannin/phenol	+
Terpenoid	+
Quinone	-

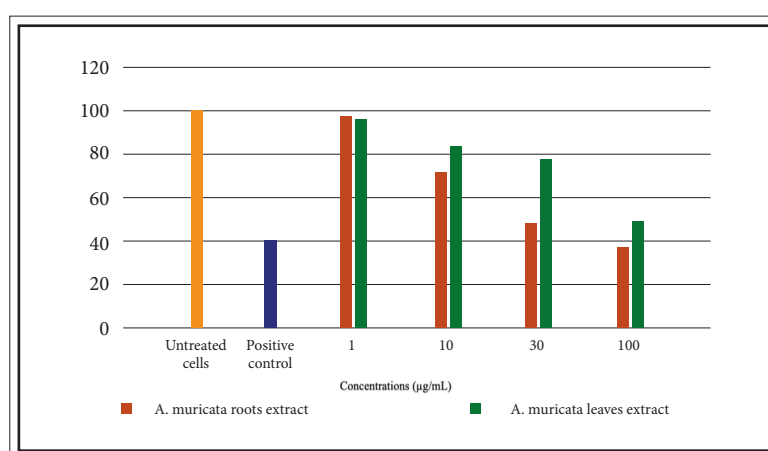


Figure 1. Cell viability after being treated with ethanol extracts of *A. muricata* roots and leaves at 100, 30, 10, and 1 µg/mL concentrations. Doxorubicin 3 µM served as a positive control. The data are expressed as mean ± SD (n = 3).

the ethanol extracts of roots and leaves of *A. muricata* have been shown to kill leukemia cell lines (HL-60) more than 80%.¹⁵ Previous studies have proven that ethanol extract has selective cytotoxic properties on tumor cells (ehrlich asciter carcinoma cell) and human breast cancer cell lines (SKBR3).¹¹ The aim of this study was to examine the cytotoxicity differences between ethanol extracts of *A. muricata* roots and leaves against HSC-3 cell lines.

Material and Methods

This research is an in vitro experimental laboratory with a post-test only control group design which was performed at BioCORE laboratory, Faculty of Dentistry, Trisakti University, Jakarta, Indonesia. This study used roots and leaves extracts of *A. muricata* which obtained from Indonesian Medicinal and Aromatic Crops Research Institute (IMACRI). HSC-3 cancer cell lines were obtained from YARSI University

Laboratory, Jakarta, Indonesia.

Plant Material Preparation

The roots and leaves of *A. muricata* which obtained from the soursop tree were washed and dried for two weeks. The dried samples were then turned into powder with a blender. The roots and leaves were then extracted by maceration technique using 6 L of 96% ethanol absolute (Merck, USA) for 1 kg of simplicia with 1:5 ratio at room temperature. After three days, the suspension was filtered using no.1 filter paper (Whatman Int. Ltd., Maidstone, UK) and the filtrate was evaporated with a rotary evaporator (Buchi, Switzerland) at 50-60°C. This procedure was repeated three times until a total color decolorization occurred. The procedure was performed at IMACRI.

Cell Culture

HSC-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 1% amphotericin B, and 1% penicillin-streptomycin at 37°C in a 5% CO₂ humidified incubator (Mettmert, Germany). The medium was changed regularly and HSC-3 cells were sub-cultured when cells reached 80-90% confluency in the culture flasks.

Cytotoxicity Test

Concisely 1 x 10⁴ HSC-3 cells/well were incubated for 24 hours in 96-well plates. Then the cells were washed with phosphate buffered saline (PBS). Hereafter, the cells were treated with various concentrations (100 µg/mL, 30 µg/mL, 10 µg/mL, dan 1 µg/mL) of *A. muricata* roots and leaves extracts for 24 hours. Doxorubicin 3 µM was used as a positive control. MTT (mitochondrial-dependent reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium-bromide). (Sigma Aldrich, USA) was added to a final concentration of 0.5 mg/mL and HSC-3 cells were incubated for four hours at 37°C, 5% CO₂. The medium was then removed and the formazan precipitate was dissolved in acidified isopropanol. Observe the condition of the cells under an inverted microscope and then measure the absorbance at 570 nm using a microplate reader (Tecan, Salzburg, Austria). Calculate and compare the cell viability (%) produced by the two extracts.

Statistical Analysis

Normality test was performed using Shapiro-Wilk method. Differences between treatment groups were analyzed using one-way ANOVA and Tukey's Post-Hoc. All tests were conducted using SPSS for Mac software version 25 (IBM, Armonk, NY, USA). A p-value (p<0.05) is considered statistically significant.

Results

Based on qualitative phytochemical tests, *A. muricata* contains alkaloid, flavonoid, steroid, tannin/phenol, and terpenoid [table 1](#). The results of the MTT test indicated that *A. muricata* roots extracts at 100µg/mL concentration ($38.44\% \pm 4.15$) and 30µg/mL concentration ($48.81\% \pm 9.10$), *A. muricata* leaves extracts 100 µg/mL concentration ($49.58\% \pm 6.93$) and positive control ($22.73\% \pm 10.34$) are significantly different compared to untreated cells. In other treatment groups, *A. muricata* roots extracts at 10µg/mL concentration ($72.72\% \pm 8.01$) and 1µg/mL concentration ($97.16\% \pm 3.12$), *A. muricata* leaves extracts 30µg/mL concentration ($78.63\% \pm 11.05$), 10µg/mL concentration ($83.61\% \pm 5.41$), and 1µg/mL concentration ($95.98\% \pm 3.70$) had no significant difference compared to untreated cells [figure 1](#). The results of shapiro-Wilk normality test obtained p-value ($p > 0.05$) which indicates the data is normally distributed. The results of one-way ANOVA test showed a significant difference with $p < 0.001$. Followed by Tukey's Post-Hoc test, the results were significantly different between groups with $p < 0.001$.

Discussion

Soursop (*A. muricata*) is a plant that is popularly used as an alternative cancer treatment.⁹ Several studies have shown that *A. muricata* has antimicrobial, anti-inflammatory, antioxidant and cytotoxic effects on tumor cells.¹⁵ Previous studies reported that *A. muricata* has anticancer activity against various cell lines such as HT-29,¹³ HCT-116,^{12,13} A549,¹⁶ HepG2,¹¹ A375,¹⁷ and HL-60.¹⁴

Based on MTT assay results, it was found that *A. muricata* roots extracts at 100µg/mL concentration ($38.44\% \pm 4.15$) had higher cytotoxic activity against HSC-3 cell lines proliferation than *A. muricata* leaves extracts 100µg/mL concentration ($49.58\% \pm 6.93$). The cell viability of *A. muricata* leaves extracts at 30µg/mL concentration ($78.63\% \pm 11.05$) while the cell viability of *A. muricata* roots extracts 30µg/mL concentration ($48.81\% \pm 9.10$), so it can be concluded that the roots extracts of *A. muricata* at 30µg/mL concentration still had better cytotoxicity on HSC-3 cell lines.

The results of this study are in line with a previous study which stated that the cytotoxicity produced by *A. muricata* roots extracts were higher than *A. muricata* leaves extracts.¹⁴ In a previous study, the ethanol extracts of *A. muricata* roots at 100µg/mL and 30µg/mL concentrations showed 10% and 70% cell viability, respec-

tively. Differences in results may occur due to different cell lines and incubation times of treatment. The previous study used leukemia cell lines (HL-60) with 48 hours incubation times while in the present study used 24 hours.¹⁴

Positive control showed $22.73\% \pm 10.34$ cell viability. It shows that *A. muricata* roots extracts at 100µg/mL concentration ($38.44\% \pm 4.15$), *A. muricata* roots extracts 30µg/mL concentration ($48.81\% \pm 9.10$), and *A. muricata* leaves extracts 100µg/mL concentration ($49.58\% \pm 6.93$) have cytotoxicity that is almost similar with positive control because there was no significant difference between the three extracts with positive control.

The compound which was found in phytochemical test of *A. muricata* has a mechanism in its role to inhibit the proliferation of cancer cells. Phenolic compounds consisting flavonoid and tannin can induce apoptosis through a mitochondrial mediated pathway by stopping the cell cycle in the G1/S and G2/M phases.¹⁶ Flavonoid also able to reduce the expression of the Bcl-2 gene. Alkaloid compounds called Annonaceous acetogenins exhibit active cytotoxicity against cancer cells, whereas acetogenins do not show toxicity to normal cells, but are highly toxic to cancer cells.⁹

Phytochemical research proves that Annonaceous acetogenins are the most dominant component contained in *A. muricata*.⁹ The mechanism of action of acetogenins includes inhibiting the performance of NADH in the respiratory chain which causes inhibition of ATP formation so that cancer cells division doesn't occur.¹⁸ In addition, acetogenin also induces apoptosis by affecting mitochondrial membrane permeability so that cytochrome increases from mitochondria to cytosol until cell apoptosis occurs.¹⁸ Steroid compounds work as anticancer by damaging the mitochondrial membrane permeability in cancer cells leading to necrosis and death.¹⁹ The high cytotoxic activity in *A. muricata* roots extracts can also caused by (+)-Xylopin compound that contained in the roots of *A. muricata*.¹¹

Based on the results obtained from this research, it has been proven that there is difference in cytotoxicity between roots and leaves ethanol extracts of *A. muricata* on HSC-3 cell lines where *A. muricata* roots extracts showed higher cytotoxic activity than *A. muricata* leaves extracts. It is supported by the percentage of cell viability of HSC-3 cell lines where ethanol extracts of *A. muricata* roots at 100µg/mL and 30 µg/mL concentrations can kill more than 50% HSC-3 cell lines while the ethanol extracts of *A.*

ethanol extracts of *A. muricata* on HSC-3 cell lines where *A. muricata* roots extracts showed higher cytotoxic activity than *A. muricata* leaves extracts. It is supported by the percentage of cell viability of HSC-3 cell lines where ethanol extracts of *A. muricata* roots at 100µg/mL and 30 µg/mL concentrations can kill more than 50% HSC-3 cell lines while the ethanol extracts of *A. muricata* leaves can kill more than 50% HSC-3 cell lines only at 100µg/mL concentration.

One limitation of this research was that the phytochemical test used was qualitative, so the number of active compounds in the extract of *A. muricata* could not be calculated. Another limitation was the selection of dose and incubation times of treatment. For future research, it is necessary to choose a wider range of concentrations and various incubation times of treatment to determine the optimal dose.

Conclusion

There is difference in cytotoxicity between ethanol extracts of *A. muricata* roots and leaves against HSC-3 cell lines where *A. muricata* roots extracts showed higher cytotoxicity. *A. muricata* roots extracts 100µg/mL concentration had the lowest percentage of cell viability, so it was concluded as the most toxic concentration in inhibiting the growth of HSC-3 cell lines. *A. muricata* roots extracts 100µg/mL concentration, *A. muricata* roots extracts 30µg/mL concentration, and *A. muricata* leaves extracts 100µg/mL concentration had no significant difference with Doxorubicin 3µM. For further research, it is recommended to perform quantitative phytochemical test and phytochemical screening on (+)-Xylopin compound in the roots of *A. muricata*.

Acknowledgment

None.

Conflict of Interest

The authors report no conflict of interest.

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