Trop J Nat Prod Res, March 2025; 921 - 924 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

In Vitro Evaluation of Soursop Leaf (*Annona muricata L*) Ethanol Extract against Development and Proliferation of 4T1 Breast Cancer Cell

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ARTICLE INFO

Article history:
Received 02 December 2024
Revised 08 February 2025
Accepted 12 February 2025
Published online 01 April 2025

ABSTRACT

Breast cancer, the most prevalent cancer among women, is on the rise, with many cases being diagnosed at advanced stages with metastasis. Traditional treatments, such as surgery, chemotherapy, and radiation, are associated with significant side effects, leading many patients to seek alternative treatments using natural ingredients. Soursop leaves contain annonaceous acetogenin, which are compounds reported to inhibit cancer cell growth and development. While previous studies have demonstrated their potential as an anticancer agent, their role in metastasis remains unclear. This study aims to examine the effects of ethanol extract from soursop leaves on breast cancer metastasis using molecular biomarkers including MMP-9, VEGF, HIF-1 expression, and angiogenesis. Soursop leaves were extracted with 96% ethanol and tested on 4T1 breast cancer cells using the MTT assay. The results showed an IC_{50} value of 117.87 µg/ml, with VEGF and HIF-1 levels decreasing at higher extract concentrations ($R^2 = 0.834$). This study concluded that ethanol extract from soursop leaves effectively reduces 4T1 breast cancer cell viability and acts as an antioxidant. These findings support the potential of soursop leaves as a beneficial alternative treatment for managing breast cancer.

Keywords: Breast cancer, Ethanol extract, Soursop leaf, 4T1 cell-line, MTT assay.

Introduction

Cancer is a leading cause of death worldwide, second only to cardiovascular diseases. The three most prevalent types of cancer are lung cancer, breast cancer, and colorectal cancer. Traditional treatments for breast cancer, such as surgery, radiation therapy, and chemotherapy, are effective but come with significant side effects, high costs, and discomfort.[1] Consequently, many patients turn to alternative therapies that use natural ingredients, which are often more affordable and have fewer side effects.[2] Herbal medicine has become an important consideration for health, particularly in developing countries. Extensive research efforts have been made to explore the potential of various plant parts as therapeutic agents or complementary treatments to conventional therapies. Given the global concern about cancer and the unique characteristics of many plants, there is a need to innovate and develop new approaches to manage cancer growth by targeting specific mechanisms.[3,4] Research indicates that soursop leaves contain annonaceous acetogenins, compounds with therapeutic and cytotoxic effects on various cancer cell lines in humans and other mammals.[5]

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Citation: Tianing NW, Sumardika IW, Wiryanthini IAD, Indrayani AW, Fatmawati D, Thongiratama GW, Widyasanti W. *In Vitro* Evaluation of Soursop Leaf (*Annona muricata L*) Ethanol Extract against Development and Proliferation of 4T1 Breast Cancer Cell. Trop J Nat Prod Res. 2025; 9(3): 921 – 924 https://doi.org/10.26538/tjnpr/v9i3.5

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Annonaceous acetogenins inhibit cancer cell proliferation by disrupting the cell cycle. Specifically, these compounds halt the cell cycle in the G1 phase and reduce the number of cells in the S phase by downregulating cyclin D1, a crucial regulatory protein in the cell cycle.[6] Annonaceous acetogenins, derivatives of C32 or C34 fatty acids, exhibit cytotoxicity by inhibiting mitochondrial complex I and cytoplasmic ATP production. These actions deplete ATP and nutrients, leading to programmed cell death (apoptosis). Annonaceous acetogenins may also exert bi-phasic effects, including agonist/antagonist properties, reversal of karyokinesis and cytokinesis, and influence on Cdk1-cyclin B and Cdk2-cyclin A, causing cell cycle arrest in G1 and G2 phases for genetic lesion correction or cell death. Additionally, they inhibit b1 integrin expression (anoikis) and possess potent antioxidant capacity.[7]The 4T1 breast cancer cell line is widely used for advanced-stage cancer analysis in *in vitro* research due to its close resemblance to human breast cancer with metastasis.[8] This adherent continuous cell line is isolated from the mammary gland of BALB/cfC3H mice. With a doubling time of 22.9 hours, it is considered one of the most reliable models for syngeneic xenograft studies in mice, making it highly valuable for cancer research.[9]

Given the potential of soursop leaf extract as a remedy for breast cancer, this study aims to examine and validate the activity of soursop leaf fractions as a therapeutic agent *in vitro*. This study also investigates the proposed mechanisms involving cytotoxicity and angiogenesis inhibition through the downregulation of HIF-1α in cancer cell line within a dose dependent manner.

Material and Methods

Breast Cancer Cell Culture

The 4T1 breast cancer cell line was obtained from the Cancer Chemoprevention Research Center at Universitas Gadjah Mada (CCRC

UGM), Yogyakarta. Subsequent intervention and evaluation were conducted at Sultan Agung Islamic University, in accordance with the collaboration agreement filed under B/96-96/UN14.4.A/PT.01.05/2021. The 4T1 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and were incubated in a CO2 incubator until 80% confluence was achieved. The cells were then seeded into 96-well plates and divided into seven groups: media control (medium only), cell control (cells with medium), and various concentrations of ethanol extracts (200 µL, 125 µL, 62.5 µL, 31.25 µL, and 15.625 µL).
The MTT assay method was used to assess cell viability, based on the reduction of yellow tetrazolium salts (MTT) to purple formazan crystals by the reductase enzyme. A stopper reagent was added to dissolve the crystals, and the absorbance was measured using an ELISA reader to determine cell viability or proliferation.

Cancer Cell Preparation
The 4T1 breast cancer cell line was prepared by removing the medium from the flask, washing the cells twice with 500 µL of PBS, and adding 450 µL of trypsin-EDTA. The cells were incubated for three minutes. Subsequently, 4 mL of culture medium was added, and the cell suspension was transferred to a 14-mL tube and centrifuged at 4,000 rpm for three minutes. The supernatant was removed, and the pellet was resuspended in 4 mL of culture medium. The obtained cell concentration was calculated at 150 x 103 cells/mL. A total of 100 µL of the cell suspension was added to each well at concentrations ranging from 1,000 to 15 µg/mL. The cells were then incubated in a 5% CO2 incubator at 37°C for 24 hours.

Plant Collection and Identification
Soursop leaf samples were sourced by the Bali Provincial Department of Agriculture and Food Sustainability, Udayana University, at GPS coordinates 8°47'59.4096" S, 115°10'3.7704" E. The extracted samples were then sent to the Biology Laboratory, Faculty of Medicine, Sultan Agung Islamic University (coordinates 6°57'23.2164" S, 110°27'28.7208" E). The extracts were identified by the Head of the Biology Laboratory in August 2021, receiving voucher numbers E0210Ra for HIF-1α investigation and E940Ra for VEGF examination.

Extraction and Dose Assignment
The research utilized 3 kg of soursop leaves, extracted using 96% ethanol at a ratio of 100 milliliters (ml) of ethanol per 10 grams of plant material. The intervention was prepared at a maximum concentration of 1000 µg/mL and diluted in a series of concentrations: 1000, 500, 250, 125, 62.5, 31.25, and 15.63 µg/mL. A stock solution was created by dissolving 1 mg of the extract in 1 mL of culture medium with 900 µL of DMSO, resulting in a 10 mg/mL solution.

Cytotoxicity Assessment
The cytotoxic potential of the leaf extract was evaluated by calculating the IC_{50} value. A maximum of 100 µL of MTT solution was added to each well, followed by incubation in a CO2 incubator for three to six hours. The solution was then removed, and 100 µL of 10% SDS stopper was added, followed by incubation without CO_2 for 24 hours. Finally, absorbance was measured using an ELISA reader at a wavelength of 550-600 nm.
The tests were performed in triplicate for both cell control and media control groups. The percentage of cell inhibition was determined by the absorbance data. Cytotoxic tests to evaluate viability and determine the IC_{50} value were conducted on the 4T1 breast cancer cell line. The cells were cultured and treated with various concentrations of the leaf extract to identify the optimal concentration for 50% inhibition. The relationship between extract concentration and cell viability was analyzed to determine the IC_{50} value using Microsoft Excel or SPSS through linear regression analysis.

Cancer Cell Absorbance Evaluation and Analysis
Cancer cell absorbance was assessed using the MTT and SDS assays. Initially, the culture medium was removed, and TX was added to the culture plate. Subsequently, 100 µL of MTT solution (5 mg/mL) was added to each well, followed by incubation for four hours. After incubation, 100 µL of SDS was added, and the plates were covered with aluminum foil and incubated overnight. The absorbance data were used to calculate the percentage of cell inhibition, and the IC_{50} value was determined using linear regression analysis.

Results and Discussion

This experimental study investigates the effect of ethanol leaf extract on the 4T1 breast cancer cell line (**Figure 1**). The objective is to measure the effectiveness of the extract by determining IC_{50} values and assessing the correlation between cell viability and extract concentration.

Phytochemical Evaluation
Further phytochemical analysis was carried out to identify the compounds present in the soursop leaves. The analysis revealed that the tested soursop leaf extract exhibits strong antioxidant activity with an IC_{50} value of 101.594 ppm (>100 ppm) (**Table 1**).

Cytotoxicity Assessment
The cytotoxic test to determine the viability and IC_{50} value of the ethanol leaf extract against the 4T1 breast cancer cell line was carried out *in vitro*. The test involved treating the cells with ethanol extract at an IC_{50} concentration of 116.677 µg/mL. Based on the IC_{50} value, three additional concentrations were prepared, resulting in four treatment groups, including a control group. The concentrations used for the cytotoxic test were as follows: 1.5x the IC_{50} (175.05 µg/mL), 1x the IC_{50} (116.677 µg/mL), 0.75x the IC_{50} (87.50 µg/mL), 0.5x the IC_{50} (58.33 µg/mL) (Figure 2).
Cytotoxicity tests using the ethanol extract from soursop leaves on the 4T1 cell line showed the highest effective treatment concentration at 1,000 µg/mL. The IC_{50} value for cytotoxicity was determined to be 116.677 µg/mL, indicating that this concentration of soursop leaf extract is required to inhibit 50% of 4T1 cancer cell growth.

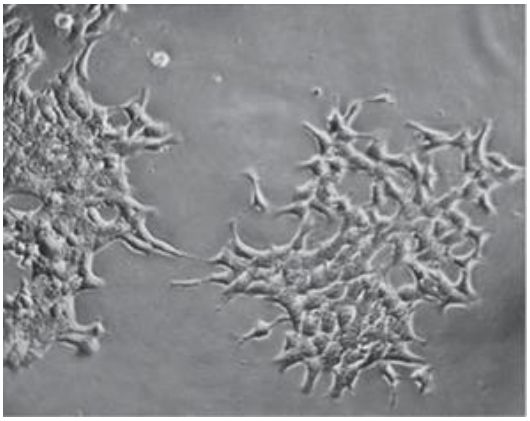

Figure 1: Electron microscopic view of the morphology of the 4T1 breast cancer cell line[12]

Cell Viability Evaluation
Cell viability decreased with increasing concentrations of ethanol leaf extract, showing a strong correlation (R^2 = 0.834). Higher concentrations of the extract resulted in lower levels of VEGF and HIF-1, demonstrating an effect on cell absorbance and viability. HIF-1 levels treated with ethanol leaf extract showed the highest absorption in the cell control group and the lowest at a concentration of 58.33 µg/mL (**Table 2**). These findings suggested that cells without leaf extract lack the antioxidant capacity to reduce HIF-1 levels. In contrast, higher concentrations of the extract led to reduced HIF-1 levels, indicating strong antioxidant capacity with an IC_{50} value of less than 1000 µg/mL.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

The relationship curve between extract concentration and cell viability showed that the higher the extract concentration, the lower the viability of the 4T1 cell line.

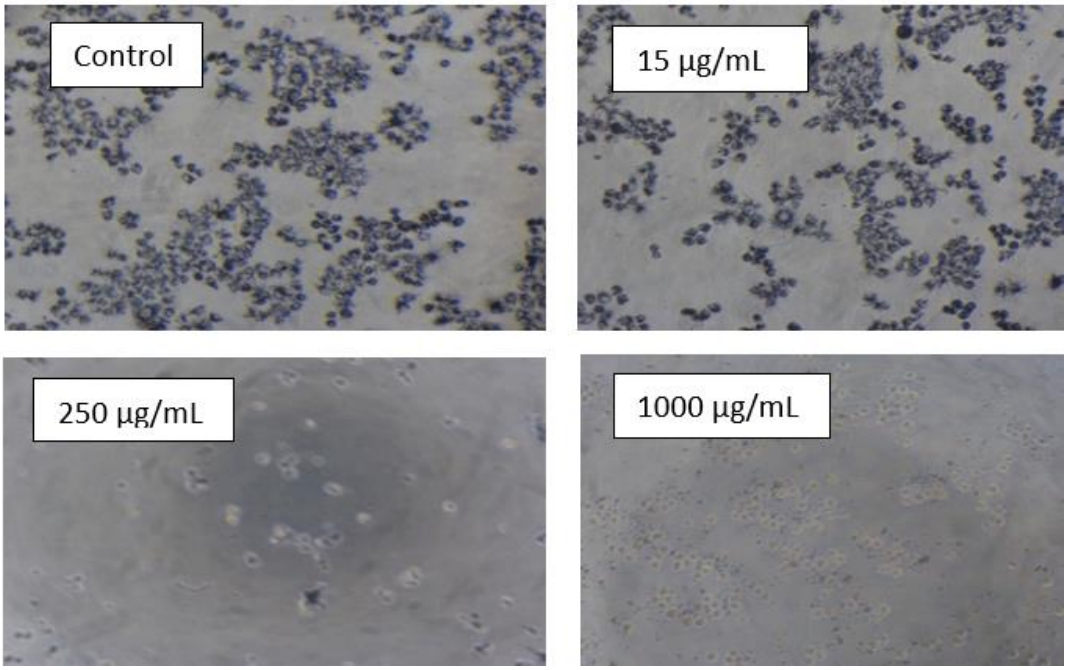

Figure 2: Cultivation Results After Treatment at Various Concentrations: (1) Control Cells; (2) 15 µg/mL; (3) 250 µg/mL; (4) concentration 1000 µg/mL

Research indicates that an extract is considered to have cytotoxic activity if the IC_{50} value is less than 1000 µg/mL after 24 hours of contact with cancer cells.[10] The IC_{50} value of the ethanol extract of soursop leaves was determined to be 116.677 µg/mL, indicating cytotoxicity to 4T1 breast cancer cells.

Table 1: Results of phytochemical analysis of Soursop leaf extract

Sample	IC_{50} (ppm)	Antioxidant Capacity (mg/L GAEAC)	Phenol (mg/100g GAE)	Flavonoid (mg/100g QE)	Tanin (mg/100g TAE)
Soursop Leaf Extract	101.5940	1,3691.68	1,652.01	4,496.31	1,042.57

GAEAC = gallic acid equivalent antioxidant capacity, GAE = gallic acid equivalent, QE = quercetin equivalent, TAE = tannin acid equivalent

Table 2: Results of HIF-1 absorption upon evaluation on breast cancer cell line after intervention with Soursop leaf extract

Concentration (µg/mL)	HIF-1 Absorption		Absorption on PBS
	Cell Line	Supernatant	
175	0.111	0.108	175
116	0.199	0.221	116
87.5	0.398	0.369	87.5
58.33	0.439	0.397	58.33
Control	0.458	0.507	Control

This toxicity is attributed to the antioxidant compounds in the ethanol extract, particularly annonaceous acetogenins, which exhibit anti-proliferative and anticancer properties. Annonaceous acetogenins in soursop leaves inhibit mitochondrial complex I due to their bis-THF structure, leading to the blockade of oxidative phosphorylation and decreased ATP production. This inhibition induces cell death through apoptosis and autophagy, while also affecting other metabolic pathways, such as inhibition of lactate dehydrogenase A, antioxidant activity, and cell cycle arrest.[11] The activity of acetogenins in inhibiting cancer viability is supported by a study by Agu et al. (2018),[7] which demonstrated that cancer cells exhibited significantly diminished integrin adhesive particles and increased dispersion after intervention, involving mechanisms of apoptosis and cell cycle inhibition.[7]

Further research is required to clarify the mechanisms by which ethanol leaf extract inhibits breast cancer cell proliferation and metastasis. A sample is considered a potent antioxidant alternative if its IC_{50} value is equal or close to that of a positive control. A test substance has strong antioxidant activity if its IC_{50} value is less than 200 µg/mL, while pure compounds must have an IC_{50} of less than 100 µg/mL. IC_{50} values between 200 and 1,000 ppm are less active but still have potential antioxidant properties.[10]

Conclusion

The ethanol extract of soursop leaf (*Annona muricata L.*) has demonstrated the ability to inhibit the growth and development of 4T1 breast cancer cells *in vitro*, as demonstrated using the MTT assay with an IC_{50} value of 117.87 µg/mL. The extract is an effective antioxidant and inhibits angiogenesis, reducing the risk of breast cancer metastasis. With an IC_{50} value of 117.87 µg/mL, which is below the 200 µg/mL threshold, it holds significant potential as a natural anticancer therapy option for breast cancer.

Conflict of Interest

The authors declare no conflict of interest

Acknowledgements

We thank the LPPM Udayana University for the funding grant number 303/UN14/HK/2021 and Mrs Dina Fatimah as head of the Biochemistry Laboratory of FK UNISSULA for supporting facilities provided during the research.

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