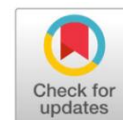




Original Research

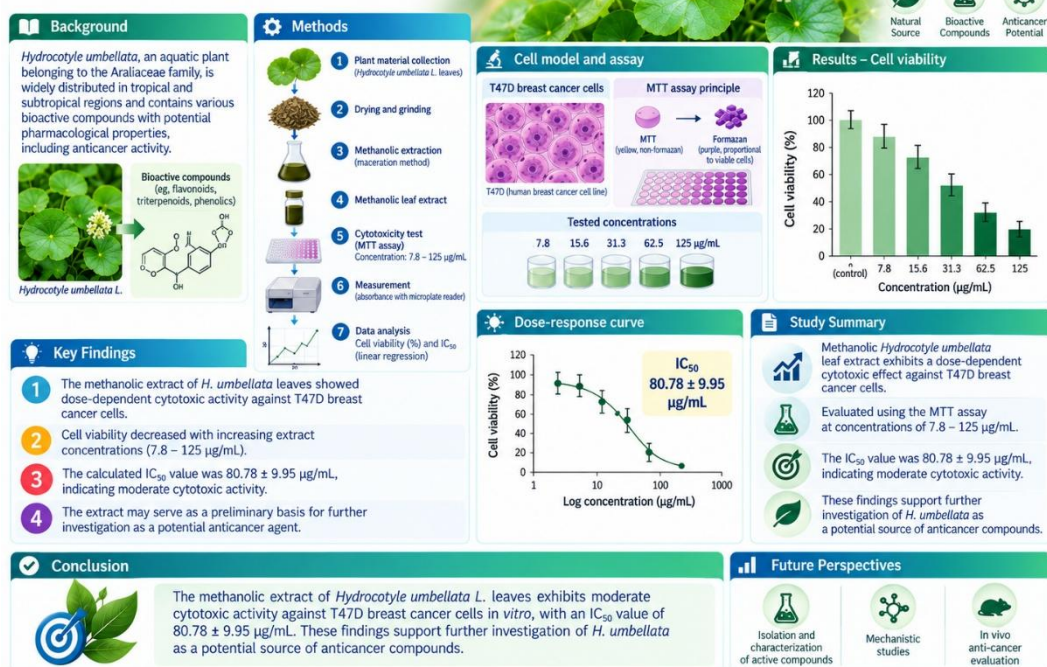
Cytotoxic effect of methanolic extract of *Hydrocotyle umbellata* L. leaves on T47D breast cancer cellsFairuz Hammad Albahi¹, Teguh Hari Sucipto², Radinal Kautsar³, Davina Gladys Javanisha⁴, Hariyono Hariyono^{5*}

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Graphical Abstract

Hydrocotyle umbellata L. Leaf Extract Exhibits Cytotoxic Activity Against T47D Breast Cancer Cells

In vitro evaluation using the MTT assay



Abstract: *Hydrocotyle umbellata*, an aquatic plant belonging to the Araliaceae family, is widely distributed in tropical and subtropical regions and is known to contain various bioactive compounds with potential pharmacological properties, including anticancer activity. This study aimed to evaluate the in vitro cytotoxic activity of methanolic *Hydrocotyle*

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umbellata L. leaf extract against T47D breast cancer cells using the MTT assay. The plant material was collected and processed through drying and grinding prior to extraction using the maceration method with methanol as the solvent. The resulting extract was then subjected to cytotoxicity testing using the MTT assay at a concentration range of 7.8-125 µg/ml. T47D cells were cultured under standard conditions and treated with different extract concentrations, followed by measurement of cell viability based on absorbance values obtained from a microplate reader. The percentage of viable cells was calculated and analyzed using linear regression to determine the IC₅₀ value. The results demonstrated that the extract exhibited a dose-dependent cytotoxic effect, with decreasing cell viability observed at higher concentrations. The calculated IC₅₀ value was 80.78±9.95 µg/ml, indicating moderate cytotoxic activity. These findings suggest that the methanolic extract of *H. umbellata* L. leaves possesses cytotoxic properties against T47D breast cancer cells and may serve as a preliminary basis for further investigation.

Keywords: Breast cancer; cytotoxicity; MTT assay; *Hydrocotyle umbellata*; IC₅₀; T47D cells.

INTRODUCTION

Breast cancer is a heterogeneous disease characterized by the uncontrolled proliferation of abnormal cells in breast tissue.¹ Numerous studies have shown that its development is influenced by a combination of internal and external factors. These include lifestyle and environmental exposures, such as diet, tobacco use, chemical exposure, and artificial light at night, as well as psychosocial factors. In addition, genetic mutations and family history play a significant role, while approximately 20% to 30% of cases are associated with potentially modifiable risk factors.^{2,3} Breast cancer is the most frequently diagnosed cancer in women worldwide with more than 2 million new cases occurred in 2020.⁴ This disease is also the most prevalent cancer in Indonesia, about 19.2% out of all cancers.⁵

Pathologically, breast cancer can be classified into molecular subtypes based on immunohistochemical (IHC) expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu). These subtypes consist of luminal A (ER+, PR+, and HER2 negative), luminal B (ER+, PR-, and any HER2), HER2 (ER-, PR-, and HER2 positive), and triple negative (ER-, PR-, and HER negative).^{6,7} Among these cancer subtypes, luminal A is considered the least aggressive and the most prevalent, accounting for approximately 50-60% of cases worldwide.^{8,9} This subtype is also the most commonly observed in Indonesia.¹⁰⁻¹³

Several therapeutic approaches, including surgery, chemotherapy, radiotherapy, endocrine therapy, and targeted therapy, are commonly employed in the management of breast cancer.² Among these modalities, chemotherapy remains one of the most frequently utilized treatment.¹⁴ Chemotherapy is the use of the pharmacological agents (such as cyclophosphamide, docetaxel, and paclitaxel) to eliminate cancer cells or inhibit their growth. In systemic chemotherapy, these drugs absorbed into the bloodstream, distributed throughout the body, and primarily target rapidly dividing cancer cells across various organs and tissues.^{15,16}

Chemotherapy is designed to target cancer cells or malignant tumors. However, its limited specificity also affects normal cells, leading to various off-target effects that can significantly impair patients' quality of life.¹⁷ The most commonly reported side effects of chemotherapy include nausea, fatigue, vomiting, and a reduction in white blood cell count. Additionally, many patients experience hair loss and dermatological issues, which represent major challenges in the management of chemotherapy treatment.¹⁸ In addition, the development of drug resistance and inter-individual variability in treatment response further limit the overall effectiveness of chemotherapy.^{19,20} These challenges highlight the urgent need for the development of novel anticancer agents that are more

selective, less toxic, and capable of overcoming resistance mechanisms while maintaining therapeutic efficacy.

In recent years, natural products have gained considerable attention as promising sources for anticancer drug discovery. This is largely attributed to their diverse chemical structures and pleiotropic biological activities, which enable them to modulate multiple molecular targets involved in cancer development and progression.^{21,22} Numerous studies have explored the anticancer potential of natural products using a wide range of natural sources, diverse compound isolation techniques, and various breast cancer cell line models, with the MCF-7 cell line being the most commonly utilized experimental model.²³ These in vitro studies have provided valuable insights into the cytotoxic and antiproliferative effects of natural compounds, demonstrating their ability to suppress breast cancer progression through multiple mechanisms, including inhibition of cell proliferation, induction of apoptosis, and regulation of key signaling pathways associated with tumor growth and metastasis.²³⁻³⁰

Hydrocotyle umbellata L. (*H. umbellata* L.), popularly known as Acaricoba, is a medicinal plant belonging to the family Araliaceae and genus *Hydrocotyle*. It is known to contain a variety of bioactive compounds, including triterpenoids, flavonoids, and phenolic constituents, which have been associated with potential anticancer activities.³¹⁻³⁴ These compounds are reported to exert antiproliferative and pro-apoptotic effects through modulation of multiple molecular pathways.^{35,36} However, despite its pharmacological potential, the anticancer activity of *H. umbellata* L. against breast cancer remains insufficiently explored. Therefore, this study aimed to evaluate the in vitro cytotoxic activity of methanolic *Hydrocotyle umbellata* L. leaf extract against T47D breast cancer cells using the MTT assay. T47D cells were selected as an experimental model due to their representation of estrogen receptor-positive (ER+) luminal A-like breast cancer, which is the most prevalent molecular subtype worldwide, including in Indonesia. The use of this model is expected to provide relevant insights into the potential of natural compounds for breast cancer.

MATERIAL AND METHOD

Sample Collection

Samples were taken from the waters of Sungelebak Village, Karanggeneng District, Lamongan Regency, East Java, Indonesia. The sampling was carried out in April 2024. The plant specimen was authenticated by a botanist, and a voucher specimen was deposited at Genbnesia under voucher number BT-0425162. A simple method to distinguish between *H. umbellata* L. and other species is to examine the leaf structure, *H. umbellata* L. is a herbaceous plant characterized by a single, round leaf borne on a long, slender petiole that attaches at the center of the leaf blade. The leaves are green and typically measure approximately 3-5 cm in diameter.³⁷

Extraction of *H. umbellata* L. Leaves

A total 1.5 kg of *H. umbellata* L. leaves were sun-dried for 2 days until they became completely dry and brittle. The dried leaves were then ground into powder. Approximately 150 g of the powdered material was weighed using an Ohaus analytical balance, transferred into a container, and macerated with 1.5 L of methanol for 3 days. Following the maceration, the mixture was filtered using Whatman filter paper to separate the filtrate from the residue. The filtrate was then concentrated by removing the solvent using a rotary evaporator at 40°C, resulting in 10.23 g of thick extract with a yield value of 6.82%. The extract was subsequently stored at room temperature (approximately 25°C) until further use.

Cell Line

The T47D human breast cancer cell line was obtained from the Institute of Tropical Disease, Universitas Airlangga, Surabaya, Indonesia. Cells were cultured in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin. The cells were seeded in 96-well plates and incubated in a humidified incubator at 37°C with 5% CO₂ for 24 hours prior to treatment to allow cell attachment.

Cytotoxicity Assay (MTT Assay)

The cytotoxic activity of the extract against T47D breast cancer cells was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay following Mosmann (1983)³⁸ protocol. T47D human breast cancer cells were harvested at a density of 5×10^3 cells/well. Cells were then washed with PBS (3 times) and treated with RPMI medium containing 0.1% DMSO (control) or methanol extract of *H. umbellata* L. leaves at the various concentrations (7.8-125 µg/ml), followed by 20 hours incubation. The final concentration of DMSO was maintained at 0.1% in all treatment groups to ensure consistency across experimental conditions. Each treatment concentration was evaluated in three independent biological replicates (n=3), with each replicate representing an independent experiment. A negative control consisting of untreated cells (cell control) cultured in complete medium was included to represent 100% cell viability. After treatment, wells were washed with PBS and incubated for 4 hours with 100 µL RPMI containing 5 mg/ml MTT. Formazan crystals formed by viable cells were solubilized using 50 µL DMSO after PBS wash, and plates were shaken for 10 minutes.

Calculation of Cell Viability and IC₅₀

Absorbance was measured at a wavelength of 595 nm using a microplate reader after the formation and solubilization of formazan crystals. The percentage of cell viability was calculated based on the absorbance values using the following formula:

$$\text{Cell Viability (\%)} = \frac{A_{\text{treatment}} - A_{\text{blank}}}{A_{\text{cell control}} - A_{\text{blank}}} \times 100\%$$

Where $A_{\text{treatment}}$ represents the absorbance of cells exposed to the extract, $A_{\text{cell control}}$ represents the absorbance of untreated cells, and A_{blank} represents the absorbance of blank well containing medium only.

The percentage of cell viability obtained at each concentration was used to construct a dose-response curve. Linear regression analysis was performed using the equation $y = Bx + A$, where y represents the percentage of cell viability and x represents the concentration of the extract. The half-maximal inhibitory concentration (IC₅₀) was determined by substituting $y = 50$ into the regression equation and solving for x . The standard deviation from each treatment was calculated using Microsoft Excel with the formula: (=STDEV.P).

RESULTS AND DISCUSSION

Cytotoxicity and Cell Viability of Methanolic Extract of *H. umbellata* L. Leaves

The cytotoxic activity of the methanolic extract of *H. umbellata* L. leaves against T47D breast cancer cells was evaluated using the MTT assay. The MTT assay was initially described by Mosmann (1983)³⁸ and became the most commonly used protocol in cell viability assay.³⁹ It is performed to measure metabolic activity in living cells. The underlying principle of this test is based on the enzymatic reduction of the water-soluble, yellowish tetrazolium salt 3-(4,5-dimethylthiazol)-2,5-diphenyl-tetrazolium bromide (MTT) to purple formazan by

introducing metabolically active cells. The succinate dehydrogenase system of the active mitochondria is responsible for this conversion.^{40,41}

Table 1. Cytotoxic Effect of Methanolic Extract of *H. umbellata* L. Leaves on T47D Cells Based on MTT Assay

Concentration ($\mu\text{g/ml}$)	Treatment Absorbance	Standard Deviation	% Cell Viability
7.8	0.2186	0.01	125.56
15.7	0.2126	0.04	119.96
31.25	0.184	0.01	93.28
62.5	0.1446	0.02	56.53
125	0.0976	0.01	12.69
Cell Control	0.1912	0.07	-
Media Control	0.084	0.001	-

The data presented in Table 1 show that treatment with the extract resulted in a concentration-dependent decrease in cell viability. These results are consistent with the Beer-Lambert Law, which states that absorbance is directly proportional to the concentration of a solute in a solution. Accordingly, a decrease in absorbance may reflect a reduction in the number of viable cells.^{42,43} This relationship is further supported by the IC_{50} analysis presented in Figure 1, where increasing extract concentrations correspond to a lower percentage of cell viability.

At lower concentrations (7.8 $\mu\text{g/ml}$), the extract exhibited minimal inhibitory effects, with cell viability remaining relatively high. However, as the concentration increased, a marked reduction in cell viability was observed, indicating enhanced cytotoxic activity of the extract. According to ISO 10993-5, the compound that is tested in cell culture and resulting the cell viability above 80% is considered non-cytotoxic, between 80-60% weak, 60-40% moderate, and below 40% strong cytotoxicity.^{44,45} Out of the five concentrations tested in this study, three concentrations (7.8 $\mu\text{g/mL}$, 15.7 $\mu\text{g/mL}$, and 31.25 $\mu\text{g/mL}$) exhibited no cytotoxic effects on T47D breast cancer cells, as indicated by viability values exceeding 80%, suggesting that the extract is well tolerated at these concentrations.⁴⁶

At the highest concentration (125 $\mu\text{g/ml}$), the extract demonstrated the greatest cytotoxic effect, reflected by the lowest percentage of viable cells compared to untreated control (untreated control). The absorbance values decreased proportionally with increasing concentration of the extract, reflecting reduced cellular metabolic activity and decreased cell viability.^{47,48} A study by de Oliveira et al. (2020)⁴⁹ reported different results, showing that spray-dried *H. umbellata* L. extract exhibited no cytotoxic effects in RAW macrophage cells, even at the highest concentration tested (500 $\mu\text{g/ml}$). These differences in results may be attributed to variations in the extraction solvent and the type of cell line used. Different cell types can respond differently to the same compound due to differences in metabolism, receptor expression, and genetic background. In addition, the choice of solvent may influence cellular responses, further contributing to the observed variability.^{50,51}

IC_{50} Determination of Methanolic Extract of *H. umbellata* L. Leaves

A compound is categorized as having anticancer activity, according to the IC_{50} values. The IC_{50} value is conventionally used as a measure of sensitivity or resistance to anticancer agents. It is defined as the concentration at which the ratio of viable treated cells to viable control (untreated) cells is reduced to 50%.⁵² According to the value of IC_{50} , cytotoxic activity can be categorized into four categories, ≤ 20 $\mu\text{g/ml}$ (active), >20 -100 $\mu\text{g/ml}$ (moderately active), >100 -1000 $\mu\text{g/ml}$ (weakly active), and >1000 $\mu\text{g/ml}$ (inactive).^{53,54} Based on linear regression analysis, the IC_{50} value of the methanolic extract of *H. umbellata* L. leaves against T47D breast cancer cells was calculated to be 80.78 ± 9.95 $\mu\text{g/mL}$, expressed as the mean $\pm$ standard deviation (SD) of three independent biological experiments.

This value falls within the moderately active category, indicating a moderate cytotoxic effect of the extract against T47D cells under the experimental conditions used (Figure 1).

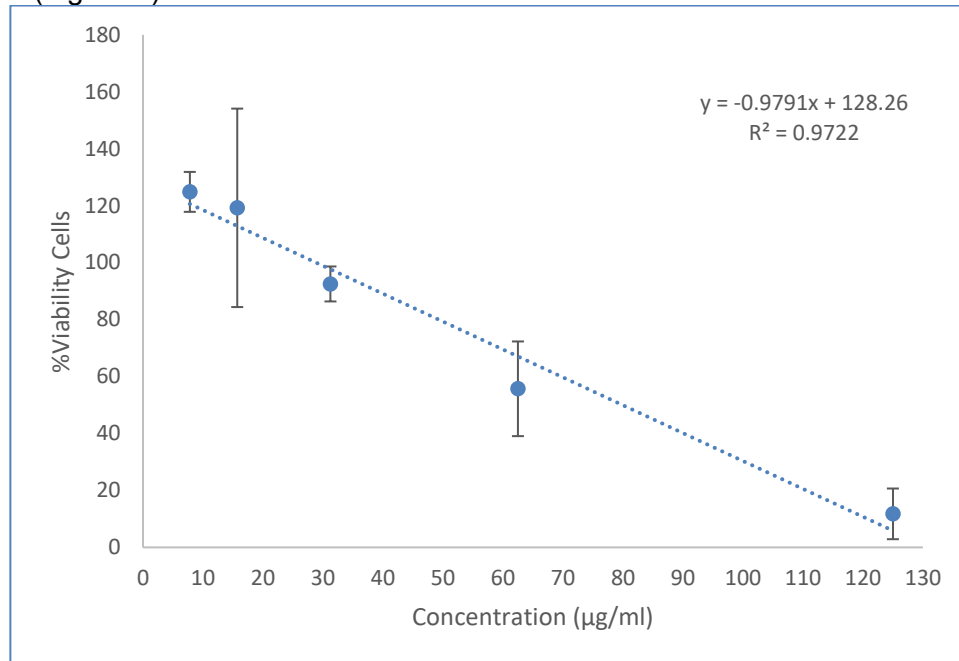


Figure 1. Dose-Response Curve and IC₅₀ Determination of Methanolic Extract of *H. umbellata* L. Leaves in T47D Breast Cancer Cells

H. umbellata L. has been widely used as medicinal herb due to its therapeutic effects such as anti-inflammation, antioxidant, antihypertensive, antidiabetic, and antimicrobial activity.^{55–57} These biological activities are closely associated with its rich phytochemical composition, which includes various bioactive compounds such as alkaloids, coumarins, protein, carbohydrates, tannin, saponins, glycosides, anthocyanins, phenolic, terpenoids, and flavonoids, known to contribute to its pharmacological properties.^{34,58}

In the context of anticancer activity, *H. umbellata* L. extract reported to exert cytotoxic effect through multiple mechanisms. A prior study by Kim et al. (2024)⁵⁹ reported that *H. umbellata* L. extract exhibited inhibitory activity against various tumor cell lines, including B16F10, while showing no detectable effects on non-cancerous cells. The extract was found to inhibit cellular respiration, thereby suppressing tumor cell proliferation. The genus *Hydrocotyle* has attracted growing attention for its potential anticancer properties due to its diverse phytochemical composition and broad range of biological activities. The essential oil extract of *Hydrocotyle bonariensis* demonstrated inhibitory activity against the CA-431 cell line, with an IC₅₀ value of 15.5 µg/mL.⁶⁰ The extract of *Hydrocotyle sibthorpioides* has also been reported to exhibit anticancer activity by inhibiting tumor cell growth, while simultaneously promoting the development and function of the thymus and spleen in tumor-bearing mice.⁶¹

Previous studies have suggested that phenolic compounds and flavonoids may contribute to cytotoxic effects through their ability to modulate oxidative stress and cellular signaling. Their hydroxyl groups can act as electron or hydrogen donors and may participate in free-radical scavenging and peroxide decomposition. However, depending on the cellular context and concentration, flavonoids may also exert pro-oxidant effects in cancer cells, potentially contributing to the modulation of apoptosis-related pathways and inflammatory signaling.^{62,63} Similarly, alkaloids have been reported to exhibit cytotoxic effects in various cancer cell models through mechanisms involving the regulation of apoptosis-related proteins, including increased expression of p53 and BAX and

reduced expression of Bcl-2. These changes may affect mitochondrial integrity and promote cytochrome c release, followed by activation of caspase-9 and caspase-3, which are involved in the execution of apoptosis. Saponins have also been reported in previous studies to exert cytotoxic effects through mechanisms associated with cell-cycle arrest and apoptosis.^{64,65}

The moderate cytotoxic activity observed in the study suggests that the methanolic extract of *H. umbellata* L. leaves may act through a combination of these mechanisms, leading to reduced viability of T47D breast cancer cells. Furthermore, the dose-dependent decrease in cell viability observed in the MTT assay supports the potential antiproliferative effect of the extract. Although the activity is categorized as moderate, these findings indicate that *H. umbellata* possesses promising bioactive constituents that may contribute to anticancer activity. However, several limitations should be acknowledged. This study did not include a positive control, which limits the ability to directly compare the efficacy of the extract with established anticancer agents. In addition, further studies are required to isolate specific active compounds and elucidate the underlying molecular mechanisms responsible for its cytotoxic effects. Future investigations should also include appropriate positive controls to enable comparative evaluation of the extract's cytotoxic activity against established anticancer agents.

CONCLUSION

Based on the findings of this study, the methanolic extract of *Hydrocotyle umbellata* L. leaves demonstrated in vitro cytotoxic activity against T47D breast cancer cells, with an IC_{50} value of $80.78 \pm 9.95 \mu\text{g/mL}$, indicating moderate cytotoxic activity. These findings provide preliminary evidence of the cytotoxic potential of *H. umbellata* L. leave extract against T47D cells and support its further investigation. However, the findings should be interpreted within the scope of this in vitro study. Further studies involving appropriate positive controls, normal cell models, additional cancer models, and in vivo evaluations are required to further characterize its biological activity. Moreover, detailed investigations of the underlying molecular mechanisms and identification of the active compounds are necessary to better understand the basis of its cytotoxic effects.

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AUTHORS' CONTRIBUTIONS

Teguh Hari Sucipto and Hariyono Hariyono: Conceptualization, Methodology. Davina Gladys Javanisha: Resources. Radinal Kautsar and Fairuz Hammad Albahi: Writing - Original Draft. Radinal Kautsar, Fairuz Hammad Albahi, and Teguh Hari Sucipto: Writing - Review & Editing

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DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request

AI STATEMENT

Generative artificial intelligence was used to support language. No AI tool was used to generate original research data, perform experiments, or alter study results.

DISCLOSURE STATEMENT

The authors declare no conflict of interest. The data are original and have not been published in another journal.

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