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Cytotoxic Activity of Ethanol Extract of *Piper aduncum* L. on T47D Breast Cancer Cell Line using the MTT Method

Rezlie Bellatasie[1], Sri Oktavia[1], Hanaya Fathiha R.[1], Hendri Satria Kamal Uyun[2]*

[1] Departement of Pharmacology and Clinical Pharmacy, Sekolah Tinggi Ilmu Farmasi Padang, Padang, West Sumatra, Indonesia

[2] Departement of Biology Pharmacy, Sekolah Tinggi Ilmu Farmasi Padang, Padang, West Sumatra, Indonesia

ABSTRACT

Cancer treatment is known to cause side effects ranging from mild to severe, which can also affect the quality of the patient's health. Natural ingredients are an alternative source of cytotoxic substances with good anticancer activity and minimum side effects. Sirih hutan (*Piper aduncum* L.), also known as spiked pepper, is a medicinal plant with potential cytotoxic activity. This study aims to determine the cytotoxic activity of the ethanol extract of *P. aduncum* L. leaves with the Microculture Tetrazolium Test method. Phytochemical screening showed that *P. aduncum* L. contains alkaloids, flavonoids, saponin, phenol, and steroid/terpenoid compounds. The cytotoxic activity test was carried out on T47D cells with a concentration of 100 µg/mL, 10 µg/mL, 1 µg/mL, and 0.1 µg/mL test solution. The obtained IC_{50} value of the ethanol extract of *P. aduncum* L. was 171.2 µg/mL, belonging to the moderate toxic category. From this study, it is concluded that *P. aduncum* L holds potential as an anticancer agent.

Keywords: cancer; cytotoxic activity; IC_{50}; *Piper aduncum* L.; spiked pepper

INTRODUCTION

Breast cancer ranks as the second leading cause of death by cancer among women. By the end of 2020, approximately 7.8 million women had received a cancer diagnosis in the last five years, establishing breast cancer as the most widespread cancer (Giaquinto et al., 2022). Based on data from the West Sumatra Health Office in 2020, cancer cases in West Sumatra from 2017-2019 have increased. The incidence of breast cancer was 303 in 2017, 422 in 2018, and 479 in 2019 (Dinas Kesehatan Kota Padang, 2020).

Although included as one of the standard treatments for patients with cancer, chemotherapy has low tumor specificity and high toxicity. Chemotherapy is often associated with many side effects found in patients, ranging from mild to severe in intensity (Schirrmacher, 2019). Some side effects of chemotherapy can worsen the patient's condition. Among them are conditions of sepsis and infection due to systemic chemotherapy. This condition can increase the risk of death in patients within the first year after diagnosis (Afifi et al., 2020). Long-term side effects from chemotherapy can also affect a patient's quality of life (Joly et al., 2019). In addition, high drug resistance also continues to encourage researchers to find new drugs that can improve patient outcomes (Ling et al., 2019).

***Corresponding author : Hendri Satria Kamal Uyun**
Email : hendrisatria@stifarm-padang.ac.id

Compounds from natural ingredients are an alternative in anticancer treatment. Apart from being abundant in quantity, compounds derived from nature have diverse activities, efficacy, biodegradability, and good biocompatibility (Abdalla et al., 2022; Cragg & Pezzuto, 2016; Naeem et al., 2022). Natural products also have fewer side effects due to their similarity to ingredients commonly consumed in the diet (Abdalla et al., 2022). A review article by Newman & Cragg (2020) states that almost 79% of cancer drugs developed since 1946 have come from nature or inspired by natural products. Anticancer originating from natural ingredients such as vinca alkaloids (vincristine, vinblastine), taxanes (paclitaxel, docetaxel), campotechin (topotecan, irinotecan), and podophyllotoxin (etoposide) are evidence of the potential for drug development from natural ingredients and used in chemotherapy regiment (Cragg & Pezzuto, 2016).

The spiked pepper plant (*Piper aduncum* L.) is one of the plants in the Piper genus belonging to the Piperaceae family (Syamsuhidayat & Hutapea, 1991). This plant is easy to grow, especially in tropical areas. *P. aduncum* L. contains several metabolites like flavonoids, monoterpenes, sesquiterpenes, and benzoic acid derivatives. This plant also has pharmacological activities such as antiparasitic, antimicrobial, insecticidal, antitumor, and anticancer (Taher et al., 2020). Research by Arroyo-Avecedo et al. (2015) showed that flavonoids were the most abundant

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metabolites in the ethanol extract of *P. aduncum* L., followed by phenols and alkaloids.

Previous studies indicate the potential of *P. aduncum* L. to be developed as an anticancer agent. Research by Shandy (2018) obtained an LC_{50} value of 12.603 µg/mL (high cytotoxic) using the Brine Shrimp Lethality Test (BSLT) method. Research on rats induced by breast cancer showed that *P. aduncum* L. had antitumorigenic, hypolipidemic, anti-inflammatory, and antioxidant activity (Arroyo-Acevedo et al., 2015). This study aims to assess the cytotoxic activity of the ethanol extract of *P. aduncum* L. on T47D breast cancer cells using the MTT method.

MATERIALS AND METHODS

Sample collection and identification

P. aduncum L. leaves were obtained from the Khatib Sulaiman area, Padang, West Sumatra. Samples were identified at Herbarium Universitas Andalas (ANDA), Department of Biology, Faculty of Mathematics and Sciences, Universitas Andalas, Padang.

Extract Preparation and Characterization

Simplisia of *P. aduncum* L. was prepared through the stages of sample collection, wet sorting, washing, chopping, drying, dry sorting, and preparation of simplisia powder following the procedures established by the Ministry of Health of the Republic of Indonesia (Departemen Kesehatan RI, 1985). A total of 300 g of dry *P. aduncum* L. simplisia powder was soaked with 3 L of solvent in the macerators. Stirring is carried out for the first 6 hours of soaking and then left at room temperature for the next 18 hours. Separate the macerate by filtration using filter paper. This process is repeated three times (Departemen Kesehatan RI, 2008). Characterization includes organoleptic examination for specific extract characterization. It consists of shape, color, smell, and taste. Meanwhile, the non-specific extract characterization includes determining drying losses, total ash content, and acid-insoluble ash content following established procedures (Departemen Kesehatan RI, 2000).

Thin Layer Chromatography

The test solution is applied between 1.5 to 2 cm from the bottom edge of the chromatography plate and left to dry. Subsequently, the chromatography plate is placed into the chromatography vessel and kept within the system until the mobile phase reaches the maximum migration distance. Afterwards, the chromatography plates are taken out and air-dried, and the spots are inspected under visible light UV (254 nm & 366 nm). The distance of each spot recorded from the spotting point is then measured to determine the Rf value (Hanani, 2017).

Phytochemical screening

Phytochemical screening tests were carried out using the standard method to determine the presence of alkaloids, flavonoids, saponins, phenols, steroids, and terpenoids (Harborne, 1987).

Cell Preparation

T47D cells were taken from the liquid nitrogen tank and thawed at room temperature. The cell suspension was transferred into a sterile conical tube containing RPMI media. The cell suspension was centrifuged for 10 minutes at 1200 rpm, discarding the supernatant. Add new growth medium, centrifuge, and resuspend slowly until homogeneous. T47D cells were grown in a tissue culture flask and incubated for 1 hour at 37 °C in a 5% CO_2 incubator. The medium is then replaced, and the cells are grown until the number is sufficient as material for treatment.

Cells were harvested when the number of cells was 80% confluent. The cell was harvested by removing the medium and washing the cells with PBS. Add trypsin-EDTA solution, incubate, and inactivate using media. Cells are observed using a microscope, and if cells are clustered together, then resuspension. The cells were transferred into a new sterile conical tube one by one. To count the number of cells, take 10 µL of the cell harvest and pipette it into a hemacytometer. Count cells under an inverted microscope with a counter.

Cytotoxic Activity

Distribute $1x10^4$ of T47D breast cancer cells/ well into 96 well plates, incubate for 24 hours in the CO_2 incubator, and discard the media. Add 100 µL of PBS to all wells containing cells and then discard. Add 100 µL of media containing test extract with the concentration series (0.1; 1; 10; 100 µg/mL) into the wells. The plates were then re-incubated for 24 hours. Remove the plates from the CO_2 incubator, then discard the cell media. Wash using 100 µL PBS, and add 100 µL MTT reagent to each well, including the media control. The cells were then incubated for 4 hours. Stop the MTT reaction by adding 10 µL DMSO. The plates were incubated for half an hour and inserted into the ELISA reader. Measure the absorbance value of each well with an ELISA reader at a wavelength of 550 nm. Calculate the percentage of viable cells using cell absorbance data by constructing a curve between log concentration and the percentage of

Figure 1. *P. aduncum* L (personal collection)

live cells. Calculate the IC_{50} value (Cancer Chemoprevention Research Center, 2019).

Data Analysis

The IC_{50} values were determined using probit regression analysis of the percentage of viability cells to log concentrations of ethanol extract of *P. aduncum* L. The percentage of viable cells can be calculated based on the absorbance results using the equation below:

$$\%\text{Viabilitas Sel} = \frac{AT-AM}{AK-AM} \text{ X } 100\%$$

AT: Absorbance of treatment (ethanol extract of *P. aduncum* L. leaves); AM: Absorbance of culture media; AK: Absorbance control cell

RESULTS

Sample Identification, extraction and extract characteristics

A total of 1 kg of sample produces 300 g of simplicia, which is then extracted with the maceration method using 96% ethanol. From maceration, a thick extract of 111.0264 grams was obtained with a yield value of 37%. The result from organoleptic observation showed the colour is green-black, with a characteristic odour and bitter taste. The non-specific extract characterization is as follows: the mean drying shrinkage is 2.1171 % ± 0.0050; the mean total ash content is 5.7382 % ± 0.0045; the mean acid insoluble ash content is 3.7550 % ± 0.0015.

Phytochemical Screening and Thin Layer Chromatography

The phytochemical evaluation of the ethanol extract of *P. aduncum* L. using different reagent for alkaloids, flavonoids, saponin, phenol dan steroid/terpenoid presented with notable positive phytochemical result (Table I).

The thin-layer chromatography showed eight spots (eight Rf values). The Rf3 is the closest value to the quercetin as a comparison (Figure 2).

Cytotoxic activity

The percentage of cell viability is 50.29%, 75.82%, 90.25%, and 102.72% with a concentration of 100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL, respectively (Table II). From the regression equation, the IC_{50} of ethanol extract of *P.aduncum* L. is 171.2 µg/mL.

DISCUSSION

This study aims to determine the cytotoxic activity of *P. aduncum* against T47D breast cancer cells. The initial stages of research are plant identification, characterization and phytochemical screening. Secondary metabolites were identified with the appropriate reagent and showed positive results for alkaloids, flavonoids, saponins, phenol, and steroids/terpenoids The phytochemical screening intends to determine the groups of compounds contained in plants (Insanu et al., 2017). The leaves of *P. aduncum* L. contain many phytochemical compounds, including flavonoids, monoterpenes, sesquiterpenes, chalcones, and benzoic acid derivatives (Taher *et al*., 2020). The results of the phytochemical screening and GC-MS analysis by Arroyo-Acevedo (2015) showed that *P. aduncum* L. contains large amounts of flavonoids, followed by moderate amounts of alkaloids, phenols, saponins, and tannins. Furthermore, anthraquinones, steroids/terpenes, fats, and oils

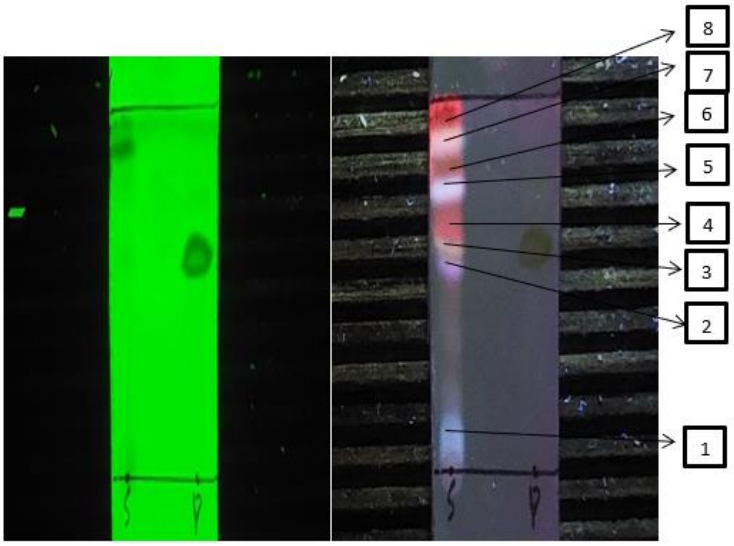

Figure 2. The thin-layer chromatogram of ethanol extract of *P. aduncum* L. observed by UV light (Camag®): 254 nm (left) and 366 nm (right). There are eight spots with Rf value as follows: (1) 0.15; (2) 0.53; (3) 0.6; (4) 0.65; (5) 0.73; (6) 0.83; (7) 0.93; (8) 0.98

Table I. Phytochemical screening of the ethanol extract of *P. aduncum* L leaves

Secondary metabolite compound	Reagent/Methods	Result
Alkaloids	Mayer Dragendroff Wagner	+
Flavonoids	Mg (powder) + HCl	+
Saponin	Water + HCl (concentrated)	+
Phenol	FeCl3	+
Steroid/Terpenoid	$CH_3COOH + H_2SO_4$	+

Table II. Viability of T47D breast cancer cell lines

Repetition	Absorbance			
	0,1 µg/mL	**1 µg/mL**	**10 µg/mL**	**100 µg/mL**
1	0.621	0.558	0.474	0.341
2	0.624	0.561	0.493	0.363
3	0.631	0.564	0.495	0.364
Mean ± SD	0.625 ± 0.005	0.561± 0.003	0.487 ± 0.011	0.356 ± 0.013
Cell viability (%)	102.72 %	90.25 %	75.82 %	50.29 %

were also detected in small numbers (Arroyo-Acevedo *et al*., 2015). In another study, the results of the phytochemical screening of the ethanol extract of *P. aduncum* L. contained flavonoids, saponins, tannins, quinones, and steroids/terpenoids (Insanu *et al*., 2017).

Piper plants have been used as a source of medicinal ingredients. *P. aduncum* L. is a plant often found in South and Central America. In the 1860s, this plant was introduced to Indonesia and is now found in many Southeast Asia areas, especially Indonesia and Malaysia (De Almeida et al., 2009). The effects of the growing environment can influence the type and levels of secondary metabolites and may impact the pharmacological activity of plants (De Almeida et al., 2009).

The cytotoxic activity test of the ethanol extract of *P. aduncum* L. was carried out using the Microculture Tetrazolium Test (MTT) method on the T47D breast cancer cell line. According to the National Cancer Institute (NCI), the cytotoxicity of a compound is deemed as high cytotoxic activity if the IC_{50} is less than 20 µg/mL, moderate activity falling within the range of 21-200 µg/mL, weak cytotoxic activity between 201-500 µg/mL, and absent (no cytotoxic activity) if IC_{50} exceeds 500

µg/mL. The results showed that the ethanol extract of *P. aduncum* L. leaves had an IC_{50} value of 171.2 µg/mL against T47D breast cancer cells. This value is classified in the moderate cytotoxic category based on NCI.

Several studies regarding the cytotoxic activity of *P. aduncum* L. have been carried out in various parts of the world. Research by Orjala (1994) using CH_2Cl_2 extract of *P. aduncum* L. originating from Papua New Guinea found that *P. aduncum* L. had moderate category cytotoxic activity towards KB nasopharyngeal carcinoma cells (Orjala *et al*., 1994). Another study using CH_2Cl_2 extract from *P. aduncum* L. leaves from tropical forests from 7 countries tested cytotoxic activity against MCF-7, H-460, and SF-268. The research showed 27, 25, and 25 µg/ml EC values for each cancer cell line (Calderón *et al*., 2006). The ethanol extract of *P. aduncum* L. leaves also has cytotoxic activity against HeLa cells with IC_{50} values of 3.91 and 0.53 µg/ml.

Flavonoids are abundant, especially in fruit and aromatic herbs, including *P. aduncum* L. (Al-Jumaili & Al hdeethi, 2021). The ethanol of *P. aduncum* L. leaves contain a large amount of flavonoid and acts as an antioxidant. This antioxidant property is estimated to have an anticancer effect by scavenging free radicals (Taher et al., 2020). Research by Insanu et al. (2017) stated that *P. aduncum* L. ethanol extract had a total flavonoid content of 8.3 ± 0.01 mg QE/100 mg extract. This antioxidant activity is better than the ethyl acetate and n-hexane extracts (Insanu et al., 2017). An in vivo study of *P. aduncum* L. showed antioxidant, anti-inflammatory, and antigenotoxic properties. This finding suggests that *P. aduncum* L. exhibit a protective effect on DMBA-induced breast cancer in rats (Arroyo-Acevedo et al., 2015)

Several studies have shown the potential of flavonoids as an anticancer. The anticancer effect arises because flavonoids can suppress the activation of carcinogenic substances by blocking cell cycle regulators such as cyclin-dependent kinase and inhibiting vascular endothelial growth factor (VEGF) (Al-Jumaili & Al hdeethi, 2021). Some of the effects of flavonoids as an anticancer include inducing apoptosis and autophagy, suppressing the proliferation and invasion of cancer cells, and also playing a role in stopping the cell cycle. Regarding ROS homeostasis, flavonoids can act as antioxidants with their activity to counteract ROS. Apart from that, flavonoids are also pro-oxidants in cancer cells, thereby triggering the apoptotic pathway. This occurs through the suppression on cell proliferation by inhibiting several epidermal growth factor receptor/mitogen-activated protein kinase (EGFR/MAPK), phosphatidylinositide 3-kinases (PI3K), protein kinase B (Akt) as well as the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Kopustinskiene et al., 2020). In addition, flavonoids can reduce the risk of side effects from chemotherapy drugs by increasing tumor sensitivity to chemotherapy (Li et al., 2022).

CONCLUSION

In conclusion, the ethanol extract of sirih hutan leaves (*Piper aduncum L*.) has moderate cytotoxic activity against T47D breast cancer cells using the MTT method.

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