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Cytotoxic Activities of Bioactive Fraction of *Uncaria gambir* (Hunter) Roxb on MCF-7 Human Breast Cancer Cell Line

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ABSTRACT

Breast cancer is a disease with the highest mortality rate. The first line of breast cancer treatment uses chemotherapy, but it has serious side effects on patients. Gambir (*Uncaria gambir* (Hunter) Roxb.) contains catechin compounds that show cytotoxic activity. This study aims to evaluate the antioxidant activity and cytotoxic effects on MCF-7 human breast cancer cells from the bioactive fraction of gambir leaves. Gambir extract was fractionated with water, ethyl acetate, and n-hexane solvents. Determination of catechin levels in the fractions used high-performance thin-layer chromatography. Antioxidant activity was tested using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and cytotoxicity tests on MCF-7 cells were carried out using MTT assay with inhibition of protein expression anti-apoptosis was determined by immunocytochemistry assay. The ethyl acetate fraction had the highest catechin content, namely 93.05%. The results of the antioxidant activity test showed that the IC50 values were consecutively for the water fraction 42.48 µg/mL, ethyl acetate 14.86 µg/mL, and n-hexane 49.63 µg/mL. In the MTT test, the ethyl acetate fraction had more potent cytotoxic activity with an IC50 value of 87.52 µg/mL. This study shows that the ethyl acetate fraction of gambir leaves has strong antioxidant and cytotoxic activity. The higher catechin content relates to antioxidant and cytotoxic activity against MCF-7 cells.

***Keywords*:** Breast cancer; Cytotoxic; Catechin; MCF-7; *Uncaria gambir*

INTRODUCTION

Cancer is a very complex disease and is one of the leading causes of death worldwide. According to Globocan 2020 data, there are 19.3 million cases of cancer in the world with a death rate of up to 10 million people. Of the many types of cancer, breast cancer is suffered by women with 2,261,419 million cases (11.7%).[1] In Indonesia, data from the Indonesian Ministry of Health in 2023, the incidence of cancer in Indonesia was 136 people per 100,000 population and ranked 8th in Southeast Asia.[2] In poor and developing countries, the increase in the incidence of cancer is in line with the increasing prevalence of risk factors such as smoking, weight, lack of physical activity, and reproductive changes associated with urbanization and economic growth.[3]

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In the treatment of breast cancer, many methods are used such as chemotherapy, radiotherapy, and surgery. The first line of breast cancer treatment currently used is chemotherapy, but it has several limitations, such as inadequate efficacy, side effects, and resistance events. In chemotherapy treatment, many side effects appear such as diarrhea, nausea, vomiting, stomatitis, alopecia, neuropathy, thrombocytopenia to myalgia.[4] Chemotherapy also has side effects in the form of metastasis of the cancer cells themselves.[5] This results in inefficiency of therapy, so it is necessary to develop more effective and efficient chemopreventive agents. Chemoprevention is an agent that can inhibit the development of cancer cells, suppress the growth of abnormal cells into cancer, and reverse the stages of the carcinogenesis process.[6] Chemopreventive agents can reduce the risk of cancer by inhibiting the initiation of preneoplastic lesions by carcinogens or reversing cancer progression. One approach to finding chemopreventive compounds is through the exploration of natural materials, especially plants.[7]

Exploration of plants that have high secondary metabolites with abundant raw material availability is a fairly potential strategy in the development of breast cancer therapy. Several plants are known to have different mechanisms in producing cytotoxic effects. One plant that has the potential as a chemotherapy agent is gambir (*Uncaria gambir* (Hunter) Roxb). Gambir leaf extract has a catechin compound marker with a very high content of around 50-70%. Catechins have been shown to have antioxidant, antibacterial, and antihyperlipidemic activities.[8] Research conducted Bakhtra shows that gambir leaf extract and fractions also have cytotoxic activity. Antioxidants have the potential as anticancer agents that work selectively and have great potential in the development of raw materials for cancer drugs.[9]

One type of cancer cell that can be used in drug testing for breast cancer treatment is MCF-7 cells. MCF-7 cells are classified as adherent cell lines. These cells are classified as adherent cell lines and have properties such as resistance to chemotherapy agents. MCF-7 cells express estrogen alpha (ER-α), overexpress Bcl-2, do not express caspase-3.[10] Proof of the activity of fractions from gambir leaves as a breast cancer drug has not been available. This study aims to examine the antioxidant and cytotoxic activity of MCF-7 cells from gambir leaf fractions. The data obtained from this study are expected to be scientific evidence supporting the development of gambir leaves as an Indonesian natural medicine in breast cancer therapy.

METHODS

Equipment and materials

The equipment used in this study were Rotary evaporator (Buchi), spectrophotometer (Spectroquant Pharo 300), High-performance thin layer chromatography (Camag), oven (Memert), water bath (Memert), grinding insert (Fritsch), muffle furnace (Thermo Scientific), moisture balance (Sartorius), analytical balance (Mettler toledo), sonicator (GB-928 Ultrasonic Cleaner), ELISA reader (Accuris MR9600).

MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC), 10% Fetal Bovine Serum (FBS) (Gibco), 1% penicillin-streptomycin (Gibco), Catechin Standard ≥98 (Sigma), Ethyl acetate (Merck), and n-Hexane (Merck). Gambir leaf extract (Uncaria gambir (Hunter) Roxb.) obtained from Mungka, Limapuluh Kota District, West Sumatra Province.

Preparation of the bioactive fraction

The process of making water and ethyl acetate fractionation in a separating funnel by taking 200 g of fine powder and dissolving it in 1000 ml of distilled water and 2000 ml of ethyl acetate, then shaking it until homogeneous and letting it stand for 8 hours until 2 layers are formed. After letting it stand for 8 hours and there has been a separation between water and ethyl acetate, then the water is released into the

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Erlenmeyer and also the ethyl acetate into the Erlenmeyer separately so that the water fraction and the ethyl acetate fraction are obtained. The water fraction is dried in an oven at a temperature of 60°C for 72 hours until a dry fraction is obtained. If the ethyl acetate in the funnel is clear yellowish, it means that the fractionation is complete, and then the ethyl acetate is released little by little into the Erlenmeyer. The evaporation process is rotary using a rotary evaporator until separation occurs and a thick extract is obtained. After that, leave it for a while in a water bath and dry it in an oven at a temperature of 40°C for 48 hours.[11,12]

Determination of catechin content

Determination of catechin content in gambir bioactive fractions was carried out using a High-Performance Thin Layer Chromatography (HPTLC) tool. The stationary phase used a silica gel GF254 plate and the mobile phase was a mixture of chloroform: ethyl acetate: formic acid (5:4:1). Observations were made at a wavelength of 254 nm. Analysis was carried out by comparing the Rf values between the catechin standard and the gambir extract fraction. Weighed 50 mg of catechin standard, dissolved using a pro-analysis methanol solvent (p.a) up to 50 mL in a 50 mL measuring flask to obtain a pure catechin solution concentration of 1000 µg/mL. After that, to make a standard series with concentrations of 50, 100, 150, 200, 250, and 300 µg/mL, pipettes were taken from the stock solution (1000 µg/mL) as much as 0.25; 0.5; 0.75; 1; 1.25; 1.5 mL and diluted with methanol solvent p.a in a 5 mL flask to the boundary mark. Then the standard solution was filtered with a 0.45µm syringe filter. Put the sample solution into the vial and measure it with an HPTLC device.[13]

Antioxidant activity

Each sample was filtered using a syringe filter, and then a stock solution of 100 µg/mL samples was made in a 100 mL measuring flask. The sample solution was made into a series of dilutions to obtain a series of concentrations of 10, 20, 40, 60, and 80 µg/mL in a 5 mL measuring flask. Next, 1 mL of sample solution was taken at all concentrations and added with 2 mL of 50 µg/mL DPPH solution. The mixture was then incubated for 30 minutes and its absorbance was measured at a wavelength of 514 nm. Methanol was used as a blank, 50 µg/mL DPPH solution as a control, and ascorbic acid as a comparison.

$$\%\ \text{inhibisi} = \frac{(abs\ blank - abs\ sample)}{abs\ blank} \text{ x } 100\ \%$$

To obtain the IC_{50} value, the % inhibition value was entered into the linear regression equation. The IC_{50} value is obtained from the intersection of the line between the inhibitory power & concentration axis, then entered into the equation: Y = ax + b. The IC50 value is calculated by entering the 50% value into the regression equation as the y value and then calculating the x value as the IC_{50} concentration.[14]

Cytotoxicity assay

Cytotoxicity test was performed using the MTT assay method on MCF-7 cells. Confluent cells in the dish were harvested with 0.25% EDTA trypsin (Sigma). Cells were counted using a hemocytometer. A total of 103 cells/well were distributed into 96-well plates. Cells were incubated for 48 hours in a 5% CO_2 incubator at 37°C. Extract 10 mg in 100 µl DMSO, diluted in culture media with gambir concentrations of 20, 40, 80, 150, 200,250 µg/ml. The extract was put into the wells, each treatment as many as 3 wells (triplicate). Cells were incubated for 24 hours in a CO_2 incubator, at the end of incubation, the media was discarded, cells were washed with phosphate buffer saline (PBS) 100 µL/well, MTT 100 µL/well was added, incubated in a CO_2 incubator for 3 hours. Then a sodium dodecyl (SDS, Sigma) 10 stopper in 0.01N HCl was added. The plate was left overnight at room temperature protected from light, read with an ELISA reader Wavelength 596 nm.[15]

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RESULTS AND DISCUSSION

Determination of catechin content

Gambir contains polyphenols and catechins that work as antioxidants and inhibit free radicals as one of the causes of cancer. Catechin is a secondary metabolite produced naturally by plants, which is included in the flavonoid group containing polyphenols.[16] catechin compounds were used in High-Performance Thin Layer Chromatography (HPTLC). Determination of catechin levels using HPTLC equipment begins with making a linearity graph. The linearity graph was made with a standard catechin solution at 50, 100, 150, 200, 250, and 300 µg/mL concentration.

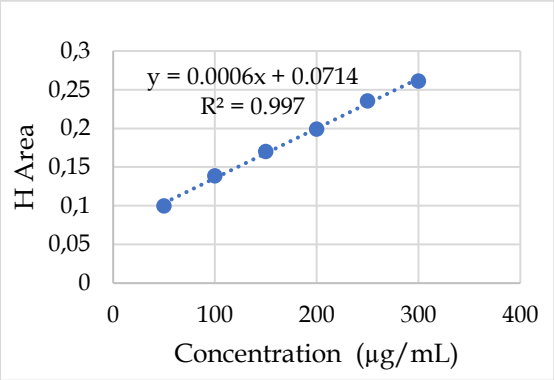

Figure 1. Catechin standard curve

The method in this study was declared linear because it had a correlation coefficient (r) value of 0.997 (Figure 1). The correlation coefficient value approaching 1 proves an increasingly linear correlation between concentration and the height of the chromatogram area. Based on AOAC (2023), this value meets the specified requirements, namely 0.9900.[17] A high correlation coefficient value indicates a linear relationship between the measured detector signal and the catechin content.[14]

In observing the elution results in thin layer chromatography, all spots in all standards and fractions were tested. This shows that in the test samples of the water fraction, gambir ethyl acetate fraction and gambir n-hexane fraction there are catechin compounds with an average Rf value of 0.3. However, it is necessary to calculate the determination of catechin levels in each fraction.[14]

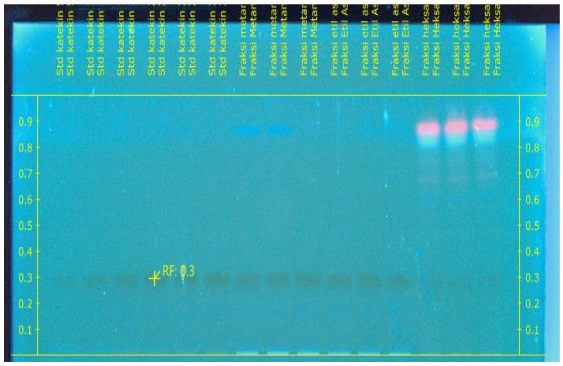

Figure 2. HPTLC result of bioactive fraction in gambir leaves

Table 1. Determination of catechin content using HPTLC

Testing	Water fraction (µg/mL)	Ethyl acetate fraction (µg/mL)	n-Hexane fraction (µg/mL)
1	48.35	92.86	2.29
2	45.65	92.53	0,98
3	41.65	93.75	1.14
Mean	**45.22±3.37**	**93.05±0.63**	**1.47±0.71**

Measurement of catechin levels in fractions was carried out in triplicate. By using the calibration curve equation in the validation of the method above, the highest catechin levels were obtained in the ethyl acetate fraction of 93.05 ± 0.63% (Table 1) which is under the requirements of the Herbal Pharmacopoeia of catechin levels of not less than 90%.[18] These results are in line with the research conducted by Anova et al. (2018), the determination of catechin levels from fractionation in the ethyl acetate fraction of gambir leaves had catechin levels of 94.42% and 93.31%.[19] In the HPTLC chromatogram (Figure 2), catechin is seen as a major compound although not a single compound, because other compounds appear even though the area looks very small.

Antioxidant activity of bioactive fraction in gambir leaves

In this study, quantitative antioxidant activity testing was carried out using the DPPH method, the comparison used as a positive control was vitamin C. The results of antioxidant activity testing on water, ethyl acetate, and n-hexane fractions are

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Table 2. Antioxidant activity of bioactive fraction in gambir leaves

Fraction	Konsentrasi (µg/mL)	Absorbansi	% inhibsi	Persamaan Kurva Baku	IC50 (µg/mL)
Water	20	0,982	39.31	y = 0.4632x + 30.352 R^2 = 0.9962	42.48
	40	0.823	49.13		
	60	0.685	57.66		
	80	0.504	68.85		
	100	0.392	75.77		
Ethyl acetate	20	0.775	52.10	y = 0.496x + 42.627 R^2 = 0.9968	14.86
	40	0.614	62.05		
	60	0.422	73.92		
	80	0.287	82.26		
	100	0.136	91.59		
n-Hexane	20	1.086	32.88	y = 0.3813x + 31.075 R^2 = 0.9974	49.63
	40	0.992	38.69		
	60	0.884	45.36		
	80	0.792	51.05		
	100	0.687	57.54		
Vitamin C	5	1.186	26.7	y = 2.7083x + 13.294 R^2 = 0.9997	13.55
	10	0.967	40.2		
	15	0.742	54.1		
	20	0.518	68.0		
	25	0.315	80.5		

shown in Table 2. The absorbance value obtained can then be calculated for the DPPH radical inhibition value (% inhibition), the linear regression equation is searched for and a curve of the relationship between concentration and absorbance is made. The purpose of making this curve is to help determine the levels of IC_{50} antioxidant compounds in samples through a linear regression equation from the antioxidant standard curve.[20]

The working mechanism of the DPPH method is that antioxidant compounds will react with DPPH radicals through a hydrogen atom donation mechanism and cause the DPPH color to decay from purple to yellow which is then measured at a wavelength of 517 nm. The decrease in the color intensity of the DPPH solution can indicate that a reaction occurs between the hydrogen atoms released by the test material and the DPPH radical molecules to form a yellow 1,1-diphenyl-2-picrylhydrazine compound.[21]

Antioxidant activity in the bioactive fraction of gambir leaves is influenced by the presence of the main flavonoid content. namely catechin. The results of the antioxidant activity test showed that the ethyl acetate fraction had the strongest antioxidant activity. The higher the level of catechin in flavonoids is a phenolic compound that has antioxidant potential because it can ward off free radicals in the body while repairing damaged body cells and can prevent aging by inhibiting factors that can accelerate the aging process.[22] The structure that allows the radical scavenging activity of flavonoids is the presence of 3,4-dihydroxyl. for example, dihydroxyl (catechol structure) in ring B, acts as an electron donor and becomes a radical target. The 3-OH structure of ring C is also beneficial for the antioxidant activity of flavonoids. The conjugation of the double bond at C2-C3 with the 4-keto group plays a role in delocalizing electrons from ring B, thereby increasing the radical scavenging

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capacity. The presence of 3-OH and 5-OH groups in combination with the 4-carbonyl function and the C2-C3 double bond increases radical activity. In the absence of the o-dihydroxy structure in ring B, the hydroxyl substituent in ring A can be compensated and increase the antiradical activity of flavonoids.[23]

Cytotoxic assay bioactive fraction of gambir leaves in MCF-7 cells

A test to detect the presence of antineoplastic activity of a compound can be done by cytotoxic testing. This test is to test toxicity using cell culture. IC_{50} is a sample concentration that shows 50% inhibition of cell proliferation, so this IC50 value is usually used as a cytotoxic test parameter. The cytotoxic test that is commonly used is using the MTT assay method. This method measures the presence of formazan crystals.[24] The results of the cytotoxic test of the bioactive fraction of gambir leaves on MCF-7 cells are shown in Table 3. These formazan crystals will form due to the reaction between MTT salt and the succinate reductase enzyme owned by living cells found in the mitochondrial organelles of the cell so that the more purple the color produced, the more living cells there are.

Table 3. Effect of the bioactive fraction of gambir leaf on MCF-7 viability

Fraction	Concentration (µg/mL)	Absorbance	% viability	Curve equation	IC_{50} (µg/mL)
Water	20	0.194	89.36	y = -0.27x + 92.404 R^2 = 0.9941	157.05
	40	0.354	80.59		
	80	0.538	70.50		
	150	0.917	49.73		
	200	1.145	37.23		
	250	1.327	27.25		
Ethyl acetate	20	0.621	65.95	y = -0.2037x + 67.827 R^2 = 0.9968	87.52
	40	0.757	58.50		
	80	0.964	47.15		
	150	1.077	40.95		
	200	1.286	29.50		
	250	1.565	14.20		
n-Hexane	20	0.064	96.49	y = -0.1602x + 99.961 R^2 = 0.9974	311.87
	40	0.115	93.70		
	80	0.238	86.95		
	150	0.417	77.14		
	200	0.605	66.83		
	250	0.727	60.14		
Doxorubicin	20	0.872	52.2	y = -0.2079x + 52.544 R^2 = 0.9859	12.24
	40	1.053	42.3		
	80	1.204	34.0		
	150	1.457	20.1		
	200	1.628	10.7		
	250	1.785	2.1		

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The cytotoxic activity test showed a smaller % of cell viability along with the addition of the fraction concentration series. The IC_{50} value in the best test sample was in the ethyl acetate fraction test sample because it had an IC_{50} of 87.52 µg/mL, although its potential was less when compared to the positive control doxorubicin. This indicates that the higher the level of catechin in the fraction correlates with the cytotoxic potential in MCF-7 cells. These results are in line with a study conducted by (Alshatwi. 2010). which proved that catechins are able to kill MCF-7 cells through apoptosis induction.[25]

The data presented in this study showed inhibition of MCF-7 breast cancer cell proliferation with increasing doses of catechins. The mechanism of apoptosis is known through the mitochondrial apoptosis pathway and the Mitochondrial death receptor pathway has a central role in regulating the caspase cascade and apoptosis. Caspases have a central role in the apoptosis process because they trigger a series of apoptosis pathways. The release of cytochrome C from mitochondria causes the activation of procaspase-9 and then caspase-3. Caspase-3 activation is an important downstream step in the apoptosis pathway of MCF-7 cells.[26] The ability as an antioxidant compound in the bioactive fraction of gambir leaves can be used as a free radical scavenger, which shows the chemopreventive role of the fraction. Catechins in gambir leaf fractions can be used as effective cancer chemopreventive agents, inhibitory agents that prevent mutagenic initiation of the carcinogenic process, and suppressive agents that prevent further development or progression of existing lesions.[27]

CONCLUSION

The bioactive fraction of gambir leaves has very potential as an antioxidant. The ethyl acetate fraction has the strongest antioxidant activity and shows good cytotoxic power. The higher the catechin content, the greater the cytotoxic activity against MCF-7 cells. The results obtained can provide support for the potential of gambir leaves as a chemopreventive agent and as a promising candidate for the development of breast cancer drugs.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that they will bear any liability for claims relating to the content of this article.

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