Advances in Health Sciences Research, volume 40
2nd International Conference on Contemporary Science and Clinical Pharmacy 2021 (ICCSCP 2021)

Anticancer and Antioxidant Activity of Asam Kandis (*Garcinia cowa* Roxb) Leaf Extract and Fraction

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ABSTRACT

Breast cancer is the second-highest cancer case in the world. One of the causes of cancer is oxidative stress by free radicals. Antioxidants are free radical neutralizing compounds that can prevent cancer. Leaves extract of asam kandis (*Garcinia cowa* Roxb) has been shown to have breast cancer and antioxidant activity. Therefore, further research is needed regarding the anticancer and antioxidant activity of *G.cowa* leaf. *G.cowa* leaves powder was macerated with ethanol. The ethanol extract was concentrated and fractionated with n-hexane and ethyl acetate. Ethanol extract, n-hexane, and ethyl acetate fraction of *G.cowa* leaves were tested for anticancer and antioxidant activity. Anticancer activity was determined using the MTT (Microtetrazolium) method against cell line T47D and antioxidant activity was carried out using the DPPH (2-2-diphenyl-1-picrylhydrazyl) assay. The ethanol extract had strong cytotoxic activity and very strong antioxidant of IC_{50} 8.8 ± 1.97µg/ml and 41.36 ± 1.25 µg/ml. The n-hexane fraction had moderate cytotoxic activity and strong antioxidant of IC_{50} 139.9 ± 3.67 µg/ml and 71.84 ± 1.88 µg/ml. The ethyl acetate fraction had moderate cytotoxic activity and very strong antioxidant of IC_{50} 132.9 ± 1.77 µg/ml and IC_{50} 29.36 ± 1.25 µg/ml. the ethanol extract, n-hexane fraction and ethyl acetate fraction of *Garcinia cowa* leaf had potential as a source of herbal medicine for cancer and antioxidants.

Keywords: *Anticancer, breast cancer, antioxidant, Garcinia cowa Roxb, MTT, DPPH*

1. INTRODUCTION

Cancer is a genetic failure of cells that grow uncontrollably and abnormally [1]. According to the World Health Organization (WHO), cancer is the second leading cause of death in the world [2]. Breast cancer is a malignancy that occurs in breast tissue originating from the ductal epithelium or its lobules [3], not only experienced by women but also men with a prevalence of 1% [4]. One of the causes of cancer is oxidative stress resulting from the balance between the formation and neutralization of prooxidants. Oxidative stress is generated by free radicals [5]. Antioxidants can neutralize free radicals and prevent cell damage caused by free radicals [6].

Garcinia cowa Roxb or known in Indonesia as Asam Kandis grows scattered in undulating areas and evergreen forests in tropical and subtropical countries, Southeast Asia, West Africa, and East Africa [7,8]. The leaves and fruit of this plant are used as vegetables and food, while in West Sumatra the dried fruit is used as a cooking spice [9–11]. In traditional medicine, the bark of *G. cowa* is used to treat for microbial infections and to treat fever [12–14]. The leaves and fruit are used for blood circulation, expectorant and laxative for the treatment of coughs and indigestion, and as a laxative, while the roots are used to treat fever [15].

The leaves of the *G. cowa* plant have also been known to contain various compounds from the xanthone group, namely cowanin, cowanol, mangosteen, β-mangosteen, cratoxylone, 3-4-dihydrojacareubin, and mangostinone [13].

Ethanol extract from this plant has anticancer activity [16]. And the content of xanthones and benzophenones contained in various parts of the *G.cowa* plant has anti-

inflammatory [17], antioxidant [15], antidiabetic [7], and antibacterial activities [18].

This study is a further study from previous studies and there are no studies related to the anticancer activity of the *G.cowa* leaf fraction.

2. METHODS

2.1. Plant Material and Extraction

The leaves of *Garcinia cowa* Roxb were obtained from Padang, Indonesia. The fresh leaves were sorted wet and dried in a greenhouse for five days, the dried leaves were then sorted dry and ground with a grinder. 1.1 kg of dry leaf powder was macerated using 11 L of ethanol. The extract was separated by pulp and concentrated using a rotary evaporator.

2.2 Fractionation

Fractionation was carried out from 100 grams of ethanol extract using a separating funnel. First, the thick extract was added with 0.5 L of warm distilled water and then fractionated using 2 L of n-hexane. Fractionation was then continued with 2 L of ethyl acetate. The n-hexane and ethyl acetate fractions were concentrated using a rotary evaporator.

2.3 Anticancer Activity Testing

Anticancer activity testing was carried out using the MTT (microtetrazolium) method against T47D breast cancer cells.

2.3.1 Cell Preparation

The cells used were T47D breast cancer cells which were collected from the Cancer Chemoprevention Research Center (CCRC) Faculty of Pharmacy UGM. Cancer cells in cryotubes were removed from the freezer (-80^{0}C) and then warmed in a water bath at 37°C for 2-3 minutes. After that, the cancer cells from the cryotube were transferred to a colonial falcon centrifuge tube containing 9 ml of complete medium. Then the colonial falcon centrifuge tube was centrifuged at 2000 rpm for 10 minutes. The supernatant from the centrifuge was discarded and the cancer cell pellet was added ±1-2 ml of complete DMEM (Dubelccoâs Modified Eagle Medium), then homogenized and put into the flask and added 3-4 ml of complete DMEM, after that the flask was incubated in a 5% CO_2 incubator, then viewed under a microscope to see if the cells stick to the flask layer forming a monolayer. The growth medium is changed once a day and when the pumpkin cell count has reached 70-85%, culture the cells until the cell count is sufficient.

2.3.2 Cell Laying (Cell Viability Test)

Enter as much as 180 µL of the cell suspension that has been made and the number of cells previously counted with a hemacytometer into each plate 96 well, except for the blank wells which will only contain a medium. Then the plate was incubated for 24 hours at 37^{0}C 5% CO_2.

2.3.3 MTT

The MTT method is a colorimetric method that measures the mitochondrial dehydrogenase activity of living cells, living cells can convert yellow MTT into purple formazan crystals [19].

The test plate contains cells that have been incubated for 24 hours, the test solution was made in concentrations of multiples of ten, namely 100, 10, 1, and 0.1 µg/ml. A total of 20 µL of the test solution was pipetted into the test well except for the control. Into the control well 200 µL of media was added and 180 µL of the test age was added. The plate was again incubated for 48 hours in an incubator at 37^{0}C 5% CO_2. Then added 0.5 mg/ml MTT solution as much as 100 µg/ml and incubated again for 4 hours in an incubator at 37^{0}C 5% CO_2. Then the supernatant was removed and added with 100 µL of DMSO and the absorbance of the formazan crystals formed at a wavelength of 550 nm was measured [19].

Cell viability percent calculation is done manually with the formula:

$$\text{Viability (\%)} = \frac{\text{average absorbance of duplicate extract well}}{\text{average absorbance of duplicate of controll well}} x100\%$$

2.4 Antioxidant Activity

The antioxidant activity test was carried out using the DPPH (2,2-Diphenyl-1-1-picrylhydrazyl) method. DPPH is a reactive free radical that acts as an electron acceptor (oxidant/oxidizing agent) and causes the oxidation of other substances. While antioxidants act as electron donors that neutralize DPPH [20]. The neutralization reaction of DPPH by antioxidant compounds is indicated by a purple color that changes to pale yellow or colorless [20] and a decrease in DPPH absorbance [6].

The DPPH solution used was 100 µg/ml in methanol. Extracts and fractions were made in test solutions with concentrations of 100, 50, 25, 12.5, and 6.25 µg/ml and Vitamin C was prepared in concentrations of 20, 10, 5, 2.5, and 1.25 µg/ml. Into well plate 96, 100 µg/ml was added and the control test was added 100 µg/ml methanol, then 100 µg/ml DPPH solution was added. Then were incubated at room temperature and dark for 30 minutes.

After incubation with DPPH, the absorbance was calculated at a wavelength of 516 nm.

Percent inhibition of antioxidant compounds against DPPH is calculated by the formula:

$$\%inhibition = \frac{Ablank - Asample}{Ablank} \; x \; 100\ \%$$

2.5 Statistical Analysis

Statistical analysis was performed with SPSS 23.0 and GraphPad Prism 9 for Windows software packages. The results are expressed as mean±SD. One-way ANOVA and Duncan's multiple-distance test were used to determine the difference between each concentration variation on the percentage of cell viability and the percentage of inhibition.

3. RESULTS AND DISCUSSION

3.1 Result

3.1.1 Anticancer Activity

The percentage of cell viability of the extract and fraction of *G.cowa* leaves is different, where at the same concentration the lowest percentage of viability is the ethanol extract, then the ethyl acetate and n-hexane fractions, but for a concentration of 100 the n-hexane fraction has a lower percentage of cell viability. compared to the ethyl acetate fraction. The $IC5_0$ results from small to large are ethanol extract (8.8 ± 1.97µg/ml), ethyl acetate fraction (132.9 ± 1.77 µg/ml), and n-hexane fraction (139.9 ± 3.67 µg/ml). When compared with doxorubicin, doxorubicin has a lower percentage of cell viability and IC_{50} than the extract and leaf fraction of *G.cowa.*

Table 1. Percentage of cell viability of ethanol extract, N-hexane fraction and ethyl acetat fraction of *G. cowa* leaves against T47D breast cancer cells

Concentration (µg/ml)	Percentage of cell viability (%)								
	Ethanol extract			N-hexane fraction			Ethyl acetat fraction		
100	5.27	5.62	5.72	48.96	49.06	50.59	52.19	55.6	52.47
10	34.77	34.21	39.89	82.68	69.72	87.29	74.36	75.59	75.76
1	58.55	62.94	63.72	83.83	73.24	89.18	91.22	92.96	95.06
0.1	69.54	70.44	67.12	91.45	84.04	94.12	90.99	93.43	96

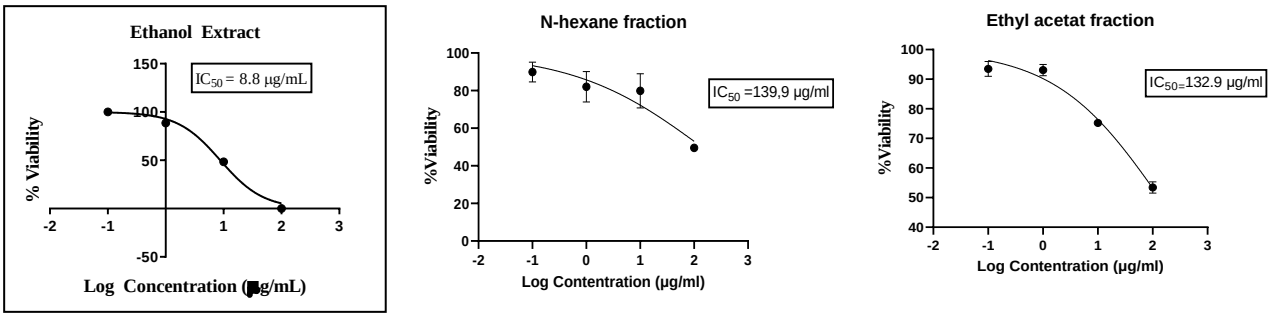

Figure 1. The dose-response relationship curve of ethanol extract, n-hexane fraction and ethyl acetat fraction of *G. cowa* leaves against T47D breast cancer cell

Table 2. Percentage of cell viability of doxorubicin against T47D breast cancer cells

Concentration (µg/ml)	Percentage of cell viability (%)		
100	21.42	21	21.20
10	24.49	22.38	22.79
1	24.65	23.54	23.25
0.1	36.22	35.31	34.65

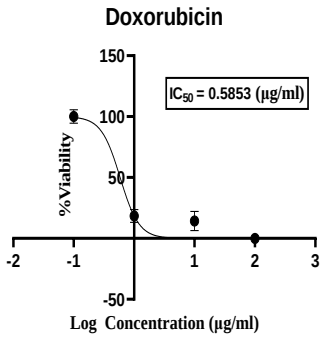

Figure 2. The dose-response relationship curve of doxorubicin against T47D breast cancer cells

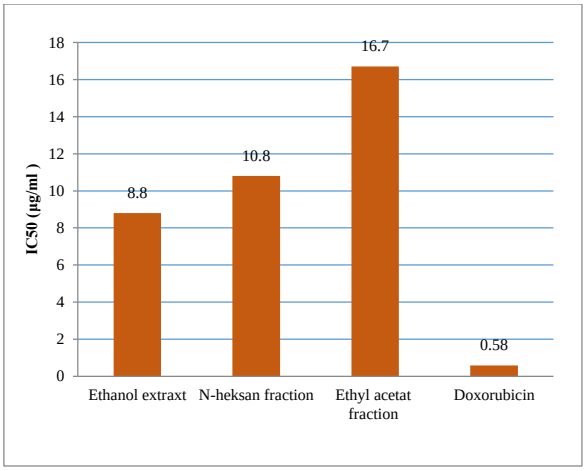

Figure 3. Bar chart of anticancer activity of *Garcinia cowa* Roxb leaf extracts and fraction and doxorubicin

3.2 Antioxidant Activity

The results of testing the antioxidant activity using the DPPH method obtained the percentage of inhibition and IC_{50} values. At the same concentration, the ethyl acetate fraction had a higher percentage of inhibition than the ethanol extract and the n-hexane fraction. As the concentration decreases, the percentage of DPPH inhibition by extracts and fractions decreases. And the highest IC_{50} was indicated by the ethyl acetate fraction (29.36 ± 1.25 µg/ml), ethanol extract (41.36 ± 1.25 µg/ml), and the n-hexane fraction (71.84 ± 1.88 µg/ml).

Table 3. Percentage inhibition of ethanol extract, N- hexane fraction and ethyl acetat fraction of *G. cowa* leaves

Concentration (µg/ml)	Percentage inhibition (%)								
	Ethanol extract			N- hexane fractions			Ethyl acetate fraction		
50	58.92	59.20	58.57	34.12	34.47	34.34	81.02	77.41	84.69
25	36.1	31.73	30.62	17.53	17.24	16.95	41.86	43.33	46.20
12.5	21.85	18.13	14.04	6.85	4.51	6.93	27.19	24.97	27.56
6.25	10.65	9.07	6.74	3.01	1.50	3.19	13.32	15.72	13.71
3.125	6.92	2.75	1.26	0.27	0.27	1.16	6.99	8.06	7.46

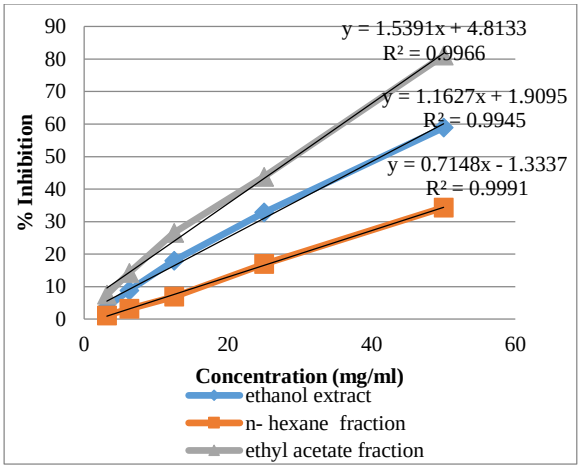

Figure 4. Graph of Relationship Between Extract Concentration and Fraction With Percent Antioxidant Activity

Table 4. Percentage of DPPH inhibition by vitamin C

Concentration (µg/ml)	Percentage inhibition (%)		
10	86.24	87.75	87.13
5	37.47	37.41	38.08
2.5	18.94	19.73	19.11
1.25	8.04	10.48	10.16
0.625	3.68	3.81	5.01

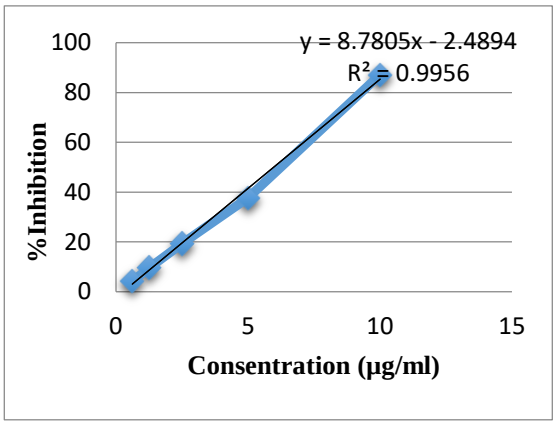

Figure 5. Graph of Relationship Between Vitamin C With Percent Antioxidant Activity

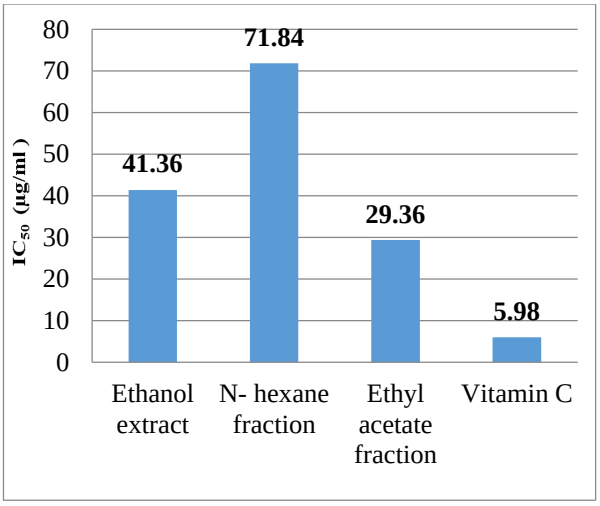

Figure 6. Bar chart of antioxidant activity of *Garcinia cowa* Roxb leaf extract and fraction and vitamin C

4.2 Discussion

Breast anticancer assays were performed on T47D cells, a breast cancer cell line isolated from the ductal breast tumor of a 54-year-old woman [21]. The T47D cell line has been widely used in in vitro studies because it is sensitive to chemotherapeutic agents and has rapid replication capability which is suitable for cytotoxic assays [16].

Cytotoxic test with MTT assay using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Dipenyltetrazolium Bromide salt which is yellow. This method has been widely used for cell viability testing in cell proliferation and cytotoxic assays [22]. living cells will change yellow MTT into purple formazan crystals, formazan crystals are the product of the MTT reduction process by the oxidoreductase enzyme produced by the mitochondria of cancer cells [22–24].

The amount of live cells is directly proportional to the number of formazan crystals, absorbance, and percent cell viability. Percent cell viability is an illustration of the percentage of cells that are alive after the MTT test. The higher the percentage of cell viability, the greater the IC50 and the smaller the anticancer activity.

The IC_{50} value indicates the concentration value that inhibits 50% of cell proliferation. Based on the IC_{50} value, the extract can be categorized into four, namely strong for compounds having $IC_{50} \leq 20$ µg/ml, moderate for compounds having IC_{50} = 21-200 µg/ml, weak for compounds having IC_{50} = 201-500 µg/ml and not cytotoxic for compounds having $IC_{50} \geq 500$ µg/ml [24]. From this category, the ethanol extract (8.8 ± 1.97 µg/ml) is a strong

anticancer, the n-hexane (139.9 ± 3.67) fraction and the ethyl acetate (132.9 ± 1.77 µg/ml) fraction are included in the moderate cytotoxic group. As a positive control in this test, doxorubicin was used which has proven efficacy as an anticancer of the breast [25]. Doxorubicin has a smaller IC_{50} than the extract and leaf fraction of *G.cowa*, which is 0.585 µg/ml.

Data processing using One Way ANOVA followed by Duncan Advanced Test. The results of the One Way ANOVA test (α=0.05) showed that the ethanol extract, ethyl acetate fraction, and n-hexane fraction of asam kandis leaves gave significantly different barriers to the growth of T47D breast cancer cells. The results obtained in the One Way ANOVA test were significantly different, then continued with Duncan's test at a significance of 5% level. Duncan then tested the data obtained that the ethanol extract at the concentration of 100 µg/ml was significantly different from the concentration of 10 µg/ml, the concentration of 10 µg/ml is significantly different from the concentration of 1 µg/ml and 1 µg/ml were significantly different from the concentration of 0.1 µg/ml. Duncan then tested the data, it was found that the n-hexane fraction at a concentration of 100 µg/ml was significantly different from a concentration of 10 µg/ml, the concentration of 10 µg/ml was not significantly different at a concentration of 1 µg/ml and 1 µg/ml was not significantly different from a concentration of 0.1 µg/ml. Duncan then tested the data, it was found that the ethyl acetate fraction at a concentration of 100 µg/ml was significantly different from a concentration of 10 µg/ml, a concentration of 10 µg/ml was significantly different at a concentration of 1 µg/ml and 1 µg/ml was not significantly different from a concentration of 0.1 µg/ml.

Antioxidant testing was carried out using the DPPH method. Purple DPPH acts as a free radical. The results of the DPPH test are the change of DPPH from purple to yellow diphenylpicrylhydrazyl which is not free radicals. This color change occurs because the purple DPPH is reduced by antioxidant compounds. The results of the DPPH test are the percentage of inhibition and IC_{50}.

The percentage of inhibition was used to determine the percentage of inhibition of the sample against free radicals (DPPH). The results of the percentage inhibition of the extract and the fraction at the same concentration showed that the ethyl acetate fraction had a higher percentage of inhibition than the ethanol extract and the n-hexane fraction, indicating that the ethyl acetate fraction had better free radical inhibitory activity than the ethanol extract and n-hexane fraction.

The strength of antioxidant activity is grouped based on the IC_{50} value into 4, namely very strong if IC_{50} <50 µg/ml, strong if IC_{50} value is 50-100 µg/ml, moderate at 101-250 µg/ml, and weak if IC_{50} 250-500 µg/ml, and classified as inactive if IC_{50} > 500 µg/ml [26].Based on this category, the ethanol extract and ethyl acetate fraction are classified as very strong antioxidants and the n-hexane fraction is classified as a strong antioxidant. As a comparison, Vitamin C is used which is an antioxidant, seen from the IC_{50} value of the extract and the fraction of *G.cowa* leaves which have weaker antioxidant activity.

Data processing using One Way ANOVA followed by Duncan Advanced Test. The results of the One Way ANOVA test (α=0.05) showed that the ethanol extract, ethyl acetate fraction, and n-hexane fraction of asam kandis leaves gave significantly different barriers to the growth of T47D breast cancer cells. The results obtained in the One Way ANOVA test were significantly different, then continued with Duncan's test at a significance of 5% level. Duncan then tested the data obtained that the ethanol extract, n-hexane fraction, and ethyl acetate fraction at a concentration of 50 µg/ml was significantly different from the concentration of 25 µg/ml, the concentration of 25 µg/ml is significantly different from the concentration of 12.5 µg/ml, 12.5 µg/ml were significantly different from the concentration of 6.25 µg/ml, and 6.25 µg/ml were significantly different from the concentration of 3.125 µg/ml. The results showed that the ethanol extract, n-hexane fraction, and ethyl acetate fraction of *Garcinia cowa* leaf had potential as a source of herbal medicine for cancer and antioxidants.

4. CONCLUSION

The results showed that the ethanol extract, n-hexane fraction and ethyl acetate fraction of *Garcinia cowa* leaf had potential as a source of herbal medicine for cancer and antioxidants.

ACKNOWLEDGMENT

This work was supported by Research and Community Service, Ministry of Research and Technology and national innovation through World Class Research with contract no 104/SP2H/LT/DRPM/2021 and SP DIPA-042.06.1.401517/2021

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