Chloroform Fraction of Cucumis melo L. 'Gama Melon Parfum' Cytotoxicity against Breast Cancer Cell T47D and MCF7

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Abstract. Nature is the source of various types of phytochemicals. Some of them are unique chemical compounds only found in certain plants. For example, Cucurbitacin is found in the family Cucurbitaceae. Cucumis melo 'Gama Melon Parfum' (GMP) is a member of the Cucurbitaceae family. Unlike melons in general, GMP melons have a bitter taste. The taste is caused by the high Cucurbitacin content. These compounds are known as anti-cancer. This study aims to identify Cucurbitacin in GMP melons and determine their effects on T47D and MCF7 breast cancer cells. GMP melon fruit medium phase (20DAA) was macerated by methanol and continued by fractionation with chloroform. Cucurbitacin B compounds in the chloroform fraction were identified using HPLC. A cell cytotoxicity test was carried out using an MTT assay with five concentration variations of the chloroform fraction. They were 7.8125, 15,625, 31.25, 62.5, and 125µg/ml. The apoptosis test was carried out using the flow cytometry method with five concentration variations of the chloroform fraction. They were 1, ½, ¼, 1/10, and 1/100 IC50. The results showed that Cucurbitacin B was found in the chloroform fraction with a concentration of 15.69 µg/mL per 1 mg of the chloroform fraction. The IC50 values of T47D and MCF7 cells, respectively, were 98 and 10 µg/ml. The apoptotic test on MCF7 cells showed that at a concentration of 1/100 IC50, the chloroform fraction was able to induce cells to undergo apoptosis.

1 Introduction

Plants are a source of natural bioactive compounds that have the role of medication. The effect of bioactive compounds on various diseases has been widely studied. Various phytochemicals in plants have the ability as antidiabetic, antioxidant, anti-inflammatory, and even anti-cancer. Certain phytochemicals cannot be found in all types of plants, such as Cucurbitacin, commonly found in the Cucurbitaceae family, and several other families such as Schopulariaceae, Begoniaceae, Primulaceae, Liliaceae, and Tropaeolaceae[1]. Cucurbitacin is a compound that causes a bitter taste in fruit. The bitter the fruit taste, the higher the Cucurbitacin concentration [2].

Cucurbitacin is well-known as an anti-cancer compound. These compounds are divided into 16 types based on their different structure. Cucurbitacin types often used in research include B, D, E and I [1]. The four types are known to inhibit the growth of cancer cells and even induce apoptosis. Cucurbitacin B can inhibit breast cancer cells by blocking the G2/M cycle [3]. Cucurbitacin D is able to inhibit the expression of PI3K/AKT and STAT3 genes, which are genes that trigger the proliferation of cervical cancer cells [4]. Cucurbitacin E induces prostate cancer cell apoptosis by induction of p53 gene expression [5]. Meanwhile, Cucurbitacin I induces P-STAT3 gene expression and inhibits STAT3 expression, which causes Sz cells to undergo apoptosis [6].

C. melo 'Gama Melon Parfum' is a melon fruit from breeding between NO3 and MR5. This breeding produces fruit with a distinctive character, the fruit size that is only a fist with a turbine at the end. In addition, the fruit has a fragrant aroma and a bitter taste [7]. The bitter taste is caused by the high Cucurbitacin content compared to other types of melon [8]. The antioxidant activity of the fruit has been studied. The antioxidant activity of GMP melons increases as the fruit ripens. The highest antioxidant activity occurred in ripe fruit. The antioxidant activity of the fruit is influenced by the content of vitamin C and flavonoids. The concentration of these two phytochemicals increased with increasing fruit maturity, and the highest concentration was found in ripe fruit [9]. Meanwhile, the anti-cancer activity of this melon has not been explored.

In this study, an anti-cancer exploration was carried out on the chloroform fraction of GMP melon fruit. The anti-cancer activity was carried out in vitro using T47D and MCF7 breast cancer cells. Both breast cancer cells have differences in the expression of apoptosis-inducing genes. In T47D, the gene is mutated, while in MCF7, it is not [10].

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2 Materials and Methods

2.1 Material preparation

GMP melons were obtained from the Agricultural Education, Research and Development Garden (KP4), Faculty of Biology, Kalitirto, Berbah, Sleman. Melon fruit is harvested at the age of 20 days after pollination (medium phase). A total of 10 kg of fruit was cut into thin slices and baked at 50°C to reduce the water content. After the water content is reduced, the fruit is dried in the sun. The dried simplicia was blended until a powder was obtained.

2.2 Extraction and Fractionation

Extraction using maceration method. The powder was dissolved in methanol in a ratio of 1:10 (g/v) and shaken for 48 hours in the dark. The solution was filtered to separate the pellet and supernatant. The supernatant was thickened using a rotary evaporator (temperature 50°C, pressure 15Pa). The remaining methanol was evaporated in an oven at 50°C. The extract was dissolved with distilled water in a ratio of 1:25 (g/v). The solution was put into a separating funnel, and chloroform was added in a ratio of 1:1 (v/v). The mixture was homogenized until homogeneous, then allowed to stand until separated. The process was repeated three times. The chloroform fraction (bottom layer) was accommodated in Erlenmeyer. The fraction was thickened using a rotary evaporator (temperature 50°C, pressure 15Pa). The remaining chloroform was evaporated in an oven at 50°C (chloroform can evaporate at 20 °C ((ICSC 0027, 2000)).

2.3 HPLC Mobile Phase Preparation

The mobile phase consisted of 0.1% formic acid in 500 ml of distilled water (solution A) and 0.1% formic acid in 500 ml of acetonitrile (Ul Haq et al., 2019). The column used was C-18, a PDA detector and a wavelength of 190-340 nm. The flow rate is 1ml/min with an injected volume of 10µl.

HPLC. Preparation of Cucurbitacin standard solution. The standard solution used was Cucurbitacin B with concentrations of 10, 20, 60, 120, and 240μg/ml'. Each concentration was filtered using a syringe filter (Filtstar) 0.45µm. The standard solution was put into an HPLC vial and labelled. The vial was wrapped using parafilm and sonicated for 5 minutes. After that, the standard solution was injected into the HPLC device (Shimadzu). The peak area of the standard solution is used to make the HPLC standard curve, and the equation Y=aX+b will be obtained.

HPLC. Measurement of Cucurbitacin Levels in Total Chloroform Fraction. The chloroform fraction (10mg) was dissolved into methanol (1ml). The solution was vortexed until homogeneous then centrifuged at 10,000 rpm for 10 minutes. The supernatant was taken and filtered using a syringe filter. The sample was put into an HPLC vial, labelled, wrapped in parafilm, and sonicated for 5 min. The peak area at RT, which is close to the standard solution is entered into the equation as Y. The X value obtained is the concentration of the Cucurbitacin compound in the fraction.

2.4 Cell Culture

T47D and MCF7 cells were obtained from ECACC with catalogue numbers 86012803 and 85102201, respectively. Cells were cultured in DMEM HG medium (catalogue no. D7777) with 10% FBS and 1% Pen-Strep. Cells were thawed in flask T25 and incubated in an incubator at 37°C, with 95% humidity and 5% CO2. After confluent, cells were subcultured two times. The subcultured cells were used for cytotoxicity and flowcytometry assays.

MTT Assay. A total of 10 mg of the chloroform fraction was dissolved in 10 L of DMSO. The solution was homogenized and variations in concentration were 7,8125, 15,625, 31.25, 62.5, and 125μg/mL. Cells (5x104/well) were cultured on 96 wellplates and incubated for 24 hours. Each concentration was given in triples and incubated for 24 hours. After that, the cells were washed using PBS, given an MTT reagent (0.5 mg/ml), and incubated for 4 hours to form formazan crystals. After the formation of formazan crystals, the reaction was stopped using 10% SDS in 0.01 N HCl and left overnight in the dark. The absorbance value of the test results was measured using an Elisa reader with a wavelength of 595nm.

2.5 Flow cytometry

The flowcytometry method was used to determine the chloroform fraction's ability to induce cell apoptosis. The concentration variations were IC50, I, 1/10, and 1/100 IC50. Cells were cultured in a T25 flask and waited until 70% confluent. After that, the cells were treated for 24 hours. Cells were harvested using the trypsin enzyme (300μL). Cells were resuspended using a complete medium and centrifuged at 1200 rpm. The supernatant was discarded, and the cell pellet was resuspended using 1× cold PBS (4°C). The cell suspension was centrifuged at 200 rpm for 5 minutes. PBS was removed, and the cell pellet was resuspended with ANNEXIN-V-FLUOS labelling solution. Cells were incubated for 10 to 15 min at 10 to 25°C. Cell suspensions were analyzed on a flow cytometer.

2.6 Statistical analysis

The data obtained were tabulated in Ms. Excel. The concentration of the total Cucurbitacin compound was analyzed using a linear regression method. The relationship between concentration and percentage of cell survival was analyzed using a correlation test in SPSS software. The IC50 value was analyzed using the probit analysis method. Cell apoptosis was analyzed by observing the cell population using a microscope and the distribution of the cell population in a flow cytometry diagram.

3 Results and Discussion

3.1 Results

Cucurbitacin B concentration in chloroform fraction. HPLC is a method of detecting the presence of a single compound in a sample. The output of the detection is the concentration of the compound, which is calculated using a linear regression equation. In the detection of compounds, a standard solution is needed as a reference. In this study, the standard solution of Cucurbitacin B injected with five variations of concentration was used. From the variation in concentration, the peak area appears at 18.4 <RT<18.6 and the equation y = 5249.3x – 31430 with R2 = 0.9972 is obtained. A high linearity value indicates the standard solution used is still pure. The detection of the chloroform fraction showed that the peak area appeared at RT 18.453. The peak area value was used to calculate the concentration of Cucurbitacin B and obtained a concentration of 15.69 g/mL per 1mg of the chloroform fraction.

Cytotoxicity of chloroform fraction against T47D and MCF7 breast cancer cells. A cytotoxicity test was carried out using the MTT Assay method. The test results are absorbance values which are then analyzed using probit analysis. The analysis showed an increase in concentration which led to a decrease in the percentage of life. The higher the concentration of the fraction used, the lower the percentage of life. This relationship occurred in both T47D cells and MCF7 cells. The cell survival value of T47D was higher than that of MCF7.

The percentage of viable cells can be used to calculate the IC50 value using a linear regression equation. The R2 value in the equation in T47D and MCF7 cells is close to 1. IC50 is a concentration value that can cause half of the population to die. The IC50 value of the T47D cell was 98 μg/ml. If seen in Table 1, these values are appropriate. In T47D cells, the percentage of cell survival of 50% was between 62.5 and 125 μg/ml concentrations. However, statistical analysis showed no correlation between the increase in concentration and the percentage of cell survival.

Table 1. The survival cell presentation after 24 hours of treatment by 5 chloroform fraction concentration

Concentration ($\mu g/mL$)	Cell Survive (%)	
	T47D	MCF7
7.8125	91.04±1.64	108.01±1.12+,*
15.625	107.81±3.43	43.06±0.88+,*
31.25	108.75±11.28	33.02±9.69+,*
62.5	57.20±36.84	29.20±8.93+,*
125	41.59±32.06	11.07±0.92+,*
R^2	0.872	0.93
IC50	98 $\mu g/mL$	10 $\mu g/mL$

Note. +: There is a correlation between concentration and percentage of cell survival. *: correlation between concentration and percentage of cell survival is significant

The IC50 value of MCF7 cells was much lower than that of T47D, which was 10 μg/ml. MCF7 cells showed a higher level of sensitivity. The percentage of viable cells from a concentration of 15.525 g/mL to 125 showed a much lower number than T47D cells. The very low IC50 values of MCF7 cells can also be observed in Table 1. The

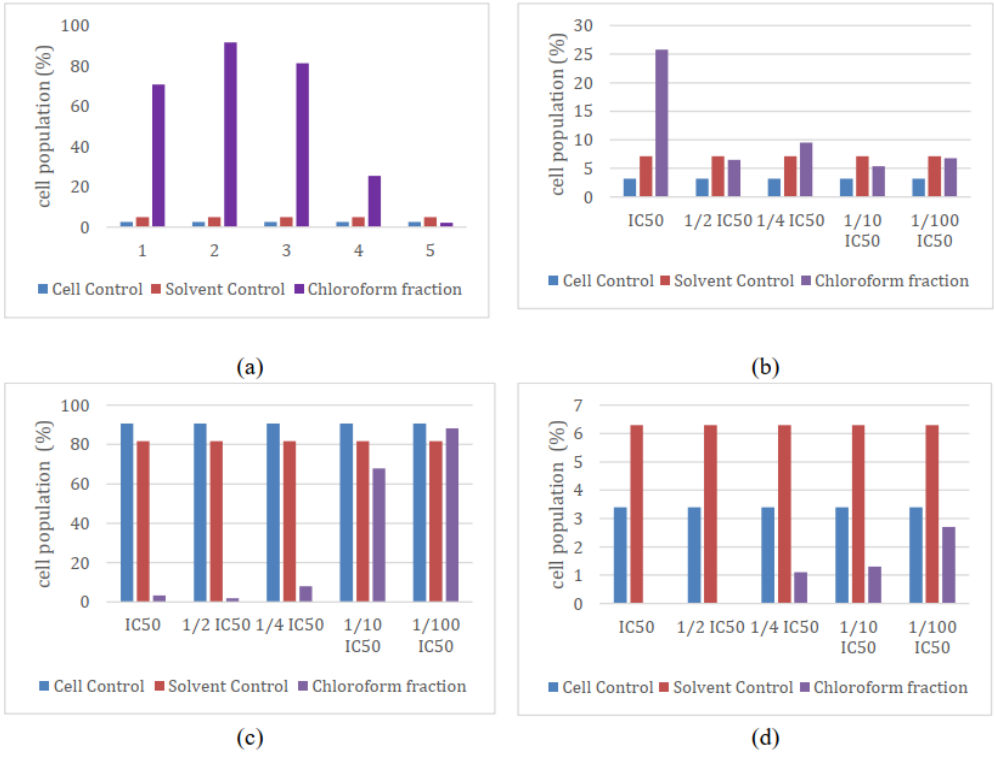

Fig 1.MCF7 cell distribution cycle after being treated with chloroform fraction for 24 hours. (a) Q1 = cells undergo necrosis, (b) Q2 = cells undergo early apoptosis, (c) Q3 = viable cells, and (d) Q4 = cells undergo late apoptosis.

50% cell survival percentage was between 15.625 and 7.8125 μg/ml concentrations.

Induction of chloroform fraction apoptosis on MCF7 cells. The concentration variations used include 1, ½, ¼, 1/10, and 1/100 IC50. The results of flow cytometry are the distribution of cells in 4 quadrants. At IC50 to ¼ IC50 concentrations, the number of necrotic cells (Q1) was more than 60%, and the value of early apoptosis (Q2) was highest at IC50 concentrations. At a concentration of 1/100 IC50, the number of cells undergoing early apoptosis (Q2) was almost equivalent to ½ and ¼ IC50. Meanwhile, in late apoptosis (Q4), the highest number of cells was found in the treatment with a concentration of 1/100 IC50 (Figure 1).

3.2 Discussions

Cancer is a disease caused by gene mutations in cells. One of the effects is uncontrolled cell growth which causes malignancy. The main treatments for people with cancer are surgery and chemotherapy. Surgery does not promise a cure for cancer patients because cancer cells have metastasized to other body organs. Therefore, chemotherapy is chosen as the primary treatment for cancer patients. Treatment with chemotherapy uses chemicals and can cause harmful side effects. Scientists have explored many natural ingredients as a combination for chemotherapy. Using natural ingredients combined with chemicals can reduce these side effects [11].

One of the natural ingredients known as anti-cancer is Cucurbitacin B. This compound is found in GMP melon. In this study, fractionation using chloroform was carried out to obtain high concentrations of CuB, and the concentration of CuB was 15.69μg/ml per 1 mg of chloroform fraction. This fraction showed toxic properties to both T47D and MCF7 cells. According to the US National Cancer Institute, cell cytotoxicity can be categorized into 4, including very toxic (IC50≤20μg/ml), toxic (21≤IC50≤200μg/ml), low toxicity if the IC50 value is between (201≤IC50≤500μg/ml), and non-toxic (IC50≥500μg/ml) [1]. The IC50 value of T47D is in the toxic category, while the IC50 value of MCF7 is in the very poisonous category.

The IC50 value indicates a concentration capable of causing 50% of the cell population to die. MCF7 cells showed significantly better sensitivity than T47D. Both cells are breast cancer cells with different characteristics, especially differences in protein expression. T47D cells expressed more growth regulatory proteins than MCF7 cells. Besides that, apoptosis regulatory proteins were not expressed much. Meanwhile, MCF7 cells expressed more apoptotic regulatory proteins than T47D cells, while growth regulatory proteins expressed less [12].

The MTT assay method can only detect cells that have died. Meanwhile, there are two cell death mechanisms, including necrosis and apoptosis. Necrosis is cell death because of environmental changes both physically and chemically. Apoptosis is a program of cell death caused by changes in DNA so that cells regulate their own death based on compounds that affect their DNA [13].

In this study, the expected cell death is apoptosis. To find out, this is followed by the flow cytometry method. One of the principles of this method is detecting changes in the structure of the cell surface [14]. In apoptosis, a flow cytometer can see a series of changes. Changes in cell structure begin with the cleavage of effector caspases, namely caspase-3/-6/-7. These events lead to DNA fragmentation, reorganization of the cytoskeleton, cytoplasmic condensation, and formation of apoptotic bodies [2]

Apoptosis is only carried out on cells considered potential, namely MCF7. This is based on a very low IC50 value (10μg/ml). At a 10μg/ml concentration, as much as 70% of the cell population underwent necrosis. This value indicates that the concentration is too high. Cells are poisoned and eventually die. The concentration test must be chosen carefully because the concentration determined will deal with healthy cells in the human body. So, when a concentration causes cancer cells to undergo necrosis, it can also cause the same in healthy cells. The concentration capable of inducing apoptosis with a minimum number of necrotic cells was 1/100 IC50 or 0.1μg/ml. At this concentration, cells undergoing apoptosis were higher than cells undergoing necrosis.

4 Conclusion

Cucurbitacin B was detected in the chloroform fraction of GMP melon fruit at 15.69μg/mL per 1mg fraction. This fraction was highly toxic to MCF7 cells with an IC50 value of 10 g/ml. The chloroform fraction was able to induce apoptosis at a concentration of 1/100 IC50.

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