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RESEARCH ARTICLE

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n-Hexane Fraction of *Cucumis melo* L. Cultivar Gama Melon *Parfum*: An *in vitro* Study in MCF7 and T47D Cells Line

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Background: *Cucumis melo* a melon species, typically has a sweet taste. Some cultivars are known for their distinctive bitter flesh due to its higher levels of cucurbitacin. Cucurbitacin is semipolar compound that has anticancer properties. However, the anticancer effects of cucurbitacin from gama melon parfum (GMP) have not been widely studied. The use of n-Hexane as a non-polar solvent in GMP melon fractionation is to dissolve the non-polar parts of the plant. However, Cucurbitacin was found in the n-hexane fraction of *Cucurbita pepo* L. Therefore, this study will investigate the presence of Cucurbitacin in the n-Hexane fraction and its effects on breast cancer cells T47D and MCF7.
Materials and methods: Dry simplicia of GMP melon fruit were macerated using methanol and fractionated using n-hexane. The presence of cucurbitacin was detected using the high-performance liquid chromatography (HPLC) method. Cell cytotoxicity tests were assessed using the MTT assay, with concentrations of 7.8125, 15.625, 31.25, 62.5, and 125 µg/mL
Results: Cucurbitacin compounds were detected in the n-hexane fraction at a concentration of 7.6 µg/mL per 10 mg of n-hexane fraction. MCF7 cell viability was lower than that of T47D cells across all concentrations tested. MCF7 cell viability was below 50% at a concentration of 62.5 µg/mL. In contrast, T47D cell viability remained at 100% even at the highest concentration of 125 µg/mL. The IC_{50} value of MCF7 cells was 43.5 µg/mL.
Conclusion: The cucurbitacin content in the n-Hexane fraction was 7.6 µg/mL per 10mg fraction. At this concentration, it moderately inhibits the proliferation of MCF7 cells.

Keywords: *gama melon parfum, cucurbitacin, HPLC, T47D, MCF7*

Introduction

Melon is a fruit that is consumed because of its sweet taste and high-water content. In addition, melon also contains bioactive compounds that are beneficial for health.[1] Cucumis melo var. gama melon *parfum* (GMP) is a melon variety that has unique characteristics, including its small size, very strong fragrance, and bitter taste.[2] The bitter taste of the fruit

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makes the fruit inedible. The bitter taste of Cucurbitaceae is caused by the cucurbitacin content in it.[3]

Cucurbitacin is included in triterpene hydrocarbons with a framework in the form of a cucurbitane ring that has a group composed of oxygen and double bonds. Cucurbitacin is a semi-polar compound that can dissolve in methanol, ethanol, petroleum ether, chloroform, benzene, and ethyl acetate.[4] Purification of cucurbitacin compounds is done through fractionation using solvents with increasingly high polarity levels, including n-hexane, chloroform, ethyl acetate, and n-butanol. Chloroform is the best solvent to obtain cucurbitacin in optimal concentration. This solvent has a high affinity for cucurbitacin.[5] The concentration of cucurbitacin chloroform fraction in melon var. GMP is 15 g/mL per 1 mg of chloroform fraction.[6]

Cucurbitacin is a compound known as an anti-cancer agent. The pure compound cucurbitacin B can inhibit the proliferation of breast cancer cells (MDB-MB-231) at concentrations below 12.01±0.75 µM.[7] Cucurbitacin D inhibits the proliferation of breast cancer cells (MCF7) at a concentration of 19.2 µg/mL.[8] Meanwhile, cucurbitacin in the chloroform fraction of melon var. GMP can inhibit the proliferation of T47D and MCF7 cells at concentrations of 98 and 10 µg/mL.[6]

In this study, two types of cell lines were used, these were T47D and MCF7. Both cells maintain the specific characteristics of the mammary epithelium, one of which is hormone dependence. T47D and MCF7 cells are equally sensitive to the hormone oestrogen, so they are referred to as estrogen receptor-positive (ER+). Meanwhile, the sensitivity of the two cells to the hormone progesterone is different. T47D cells are progesterone receptor-positive (PR+) and MCF7 are progesterone receptor-negative (PR-).[9] T47D cells are breast cancer cells that have good stress tolerance. Therefore, T47D cells are more invasive than MCF7 because they can survive in stressful conditions, one of which is by suppressing mitochondrial activity.[10]

Breast cancer is the type of cancer with the second highest incidence and the fourth highest mortality rate in the world.[11] Research on compounds that can inhibit, or cure has been widely conducted. One of these compounds is cucurbitacin, which has been widely isolated from various plants. This is hoped that a low concentration can be obtained that inhibits the proliferation of breast cancer cells. So, its use in the treatment process does not interfere with another healthy cell.

In this study, the cucurbitacin was macerated from plant material. The optimum concentration of cucurbitacin can be obtained by multilevel fractionation. The n-hexane solvent is a solvent with the lowest polarity in the cucurbitacin purification process. The aim of using n-hexane is to remove the non-polar compound of plant cell. In previous research, triterpene compounds were found in the n-Hexane fraction of *Cucurbita pepo* L.[12] However, the concentration and its effects on breast cancer cells have not yet been determined. *C. melo* var. GMP is a cultivar melon whose anticancer are not widely studied yet. The previous study showed that cucurbitacin fraction using chloroform to T47D and MCF7 cells.[6] In this study, cucurbitacin was found in the n-Hexane fraction, the non-polar solvent. Therefore, this study was conducted to determine the amount of cucurbitacin in the n-Hexane fraction and its effect on T47D and MCF7 cells.

Materials and methods

Sample Preparation

GMP is a hybrid between melon ♀ NO3 and ♂ MR5.[2] The fruit was grown in the Agricultural Education, Research, and Development Garden, Universitas Gadjah Mada. The fruit was harvested at the medium phase (20 DAA). The fruit was cut into thin slices and placed in an oven at 50˚C to reduce the water content. Then it was dried under the sun. The dried fruit was blended into powder. Extraction was carried out by maceration. Fruit powder (1gr) was dissolved in methanol (Merck, Darmstadt, Germany) (10mL), and shaken for 48 hours in the dark, then filtered (Whatman filter paper #1, China) to separate the pellet and supernatant. The supernatant obtained was thickened using a rotary evaporator (Stuart RE3022) (temperature 50˚C, pressure 15Pa). The remaining methanol (Merck, Darmstadt, Germany) in the extract was evaporated in an oven (Memmert In55, Germany) at a 50˚C.

The extract, which was already in the form of a paste, was then fractionated using n-Hexane. A total of 1 gram of extract was dissolved in 25 mL of distilled water. The extract solution was put into a separating funnel (IWAKI, Jawa Barat, Indonesia) and 25 mL of n-Hexane (Merck, Darmstadt, Germany) was added. The mixture was shaken until mixed and allowed to separate. The process was repeated three times. The n-Hexane (Merck, Darmstadt, Germany) fraction in the upper layer was collected into an Erlenmeyer flask (IWAKI, Jawa Barat, Indonesia) to be thickened using a rotary evaporator (Stuart RE3022,

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Staffordshire, UK) (temperature 50˚C, pressure 15Pa). The remaining n-Hexane solvent was evaporated in an oven at a temperature of 50˚C.

HPLC Examination

High-pressure liquid chromatography (HPLC) is a method of separating compounds using columns and liquid chromatography as a separator, quantification, characterization, and investigation of the active groups of a compound. In this study, the mobile phase used 0.1% formic acid in 500 mL of distilled water as solution A, and 0.1% formic acid in 500 mL methanol as solution B. The mobile phase consisted of solution A, namely 0.1% formic acid in 500 mL of distilled water and solution B, namely 0.1% formic acid in 500 mL methanol (Merck, Darmstadt, Germany).[13] The column in HPLC can be different for each target compound, in this study the column used was C-18 with a flow rate of 1 mL/minute.[14] The detector used is PDA, and the wavelength is 190-340 nm.

Cucurbitacin B standard solutions were prepared with concentrations of 10, 20, 60, 120, and 240 µg/mL. Each concentration solution was filtered using a 0.45 µm syringe filter (Filtstar, Jawa Barat, Indonesia). The filtrate is put into an HPLC vial and labeled. Then, the vial was wrapped using parafilm and sonicated for 5 minutes. After that, the standard solution was injected into the HPLC device (Shimadzu, Japan). The peak area of the standard solution is used to create an HPLC standard curve, and the y=ax+b will be obtained.

The n-hexane fraction (10mg) was dissolved in methanol (Merck, Darmstadt, Germany) (1 mL). The n-Hexane fraction solution was vortexed until homogeneous, then centrifuged at a speed of 10,000 rpm for 10 minutes. The supernatant was taken and filtered using a syringe filter (Filtstar, Jawa Barat, Indonesia). The same process as the standard solution is carried out for the sample. The peak area at the retention time (RT) which is close to the standard solution is entered into the equation as Y. The X value obtained is the level of the cucurbitacin compound in the fraction.

Cell Culture

T47D and MCF7 cells (Cat. No. 86012803 and 85102201 ECACC, UK) were thawed in a T25 flask (IWAKI, Jawa Barat, Indonesia) and incubated at 37°C, 95% humidity and 5% CO_2 concentration.14 The culture medium used was DMEM HG (Cat. No. D7777, Merck, Darmstadt, Germany). Medium was supplemented with 10% FBS (Gibco, Grand Island, New York) and 1% Pen-Strep (Biowest, Nuaillé, France). Before being used for treatment, cells were subcultured twice.

MTT Assay

The n-Hexane fraction (1 mg) was dissolved in 10 mL of DMSO (Merck, Darmstadt, Germany). The concentrations of the fractions used were 7.8125, 15.625, 31.25, 62.5, and 125 µg/mL. Cells (5×10^4/well) were seeded in 96 well plates for 24 hours. The treatment was given in triplicate and incubated at 37˚C, 95% humidity, and 5% CO^2 concentration for 24 hours. After 24 hours, the cells were washed with PBS (Merck, Darmstadt, Germany) and treated with MTT (Merck, Darmstadt, Germany) at a concentration of 0.5 mg/mL, using 100 µL/well. The plate was re-incubated in a CO_2 incubator for 4 hours or until formazan crystals were formed. The reaction was stopped using 10% SDS (Merck, Darmstadt, Germany) in 0.01 M HCl (Merck, Darmstadt, Germany). The plate was incubated at room temperature in the dark overnight, and then the treatment results were measured using an ELISA reader (Thermo Fisher, Massachusetts, AS) at a wavelength of 595 nm.

Data Analysis

Cucurbitacin concentration data in n-hexane fraction was calculated using linear regression. Meanwhile, IC_{50} MTT Assay was calculated using probit analysis. Both were performed using Microsoft Excel. The viability cells in each concentration were analyzed using one way ANOVA in SPSS software.

Results

Cucurbitacin Compounds Detected in n-Hexane Fraction by HPLC

HPLC measurements of samples extracted with non-polar solvents revealed the presence of cucurbitacin compounds (Figure 1). The peak area can be used to calculate the concentration of cucurbitacin in the n-hexane fraction.

The concentration of the n-Hexane fraction was calculated using the cucurbitacin B standard curve, with peak area values (8,606) entered as Y in the equation y=5249.3x-31430 (Figure 2). The results showed that the concentration in the n-Hexane fraction was 7.6 µg/mL per 10 mg of fraction.

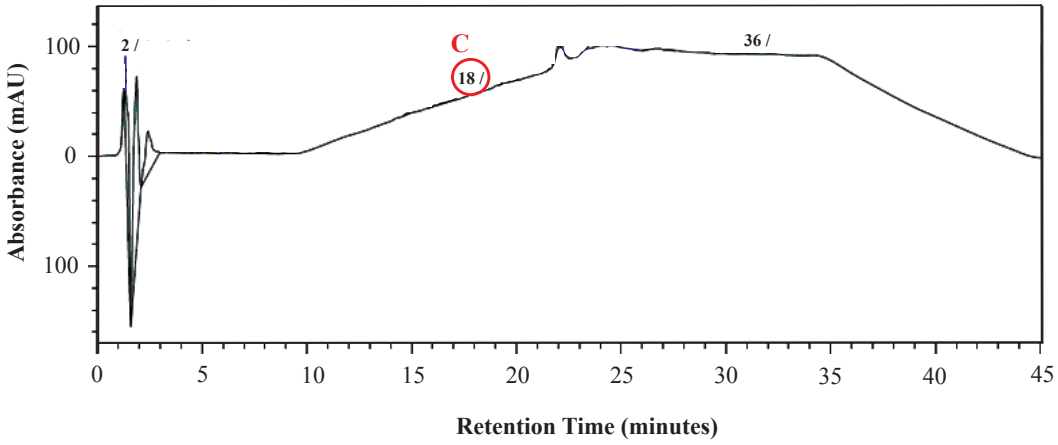

Figure 1. The Cucurbitacin peak was detected at a retention time of 18.144 minutes, with a peak area of 8,606. C: Cucurbiatcin.

GMP n-Hexane Fraction Inhibited MCF7 Cells but Not T47D Cells

The cytotoxicity test of the n-hexane fraction of GMP fruit showed differences in cell viability (Figure 3). The n-hexane fraction did not affect to the cell viability of T47D cells. At the highest concentration, cell viability remained at 100%, and the IC_{50} value was 216.72 µg/ml. This results suggest that Cucurbitacin in the n-hexane fraction did not inhibit the cell proliferation after 24 hours of treatment.

Cell viability in MCF7 cells was 27.6% at a concentration of 62.5µg/mL. At lower concentrations, the n-hexane fraction did not affect cell viability. These results suggested that cucurbitacin in the n-hexane fraction is ineffective at inhibiting breast cancer cell proliferation at lower concentrations. The concentration of the n-hexane fraction that inhibited 50% of MCF7 cell growth was 43.55µg/mL.

Discussion

In this study, the concentration of cucurbitacin in the n-hexane fraction was 7.6 µg/mL per 10 mg of fraction. In comparison, the cocentration in the chloroform fraction from a previous study was higher, at 15 µg/mL per 1 mg of fraction.[6] In this study, the concentration of Cucurbitacin is 7.6 µg/mL per 10 mg. Compared to the chloroform fraction from a previous study, its concentration is lower, which was 15 µg/mL per 1 mg. Many studies mention that n-hexane, a non-polar solvent, is used for the isolation of cucurbitacin.[5,8,15] The use of non-polar solvents aims to dissolve the non-polar compound within the cells, allowing the other polar compound inside the cells to be optimally isolated.[16] Typically, non-polar solvents are not continued to subsequent research stages because target compounds may not be found or are found in low concentrations. However, non-polar solvents are not carried over to subsequent steps, as the target compounds may either be absent or present in low concentrations.

This study reports on the n-hexane fraction analysis previously conducted by others. As with earlier research, the identification of terpene compounds, including Cucurbitacin, in the n-hexane fraction of *Cucurbita pepo*

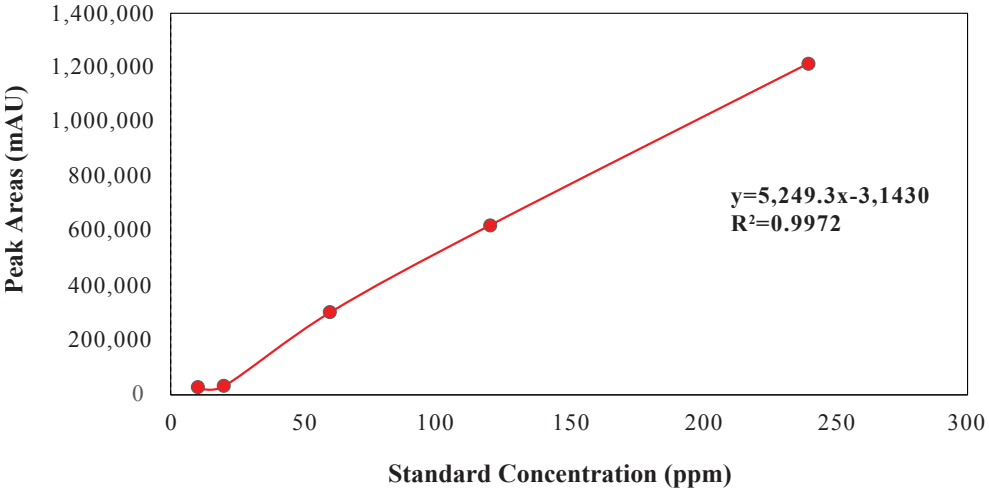

Figure 2. Standard curve of Cucurbitacin B with concentrations of 10, 20, 60, 120, and 240 µg/mL.

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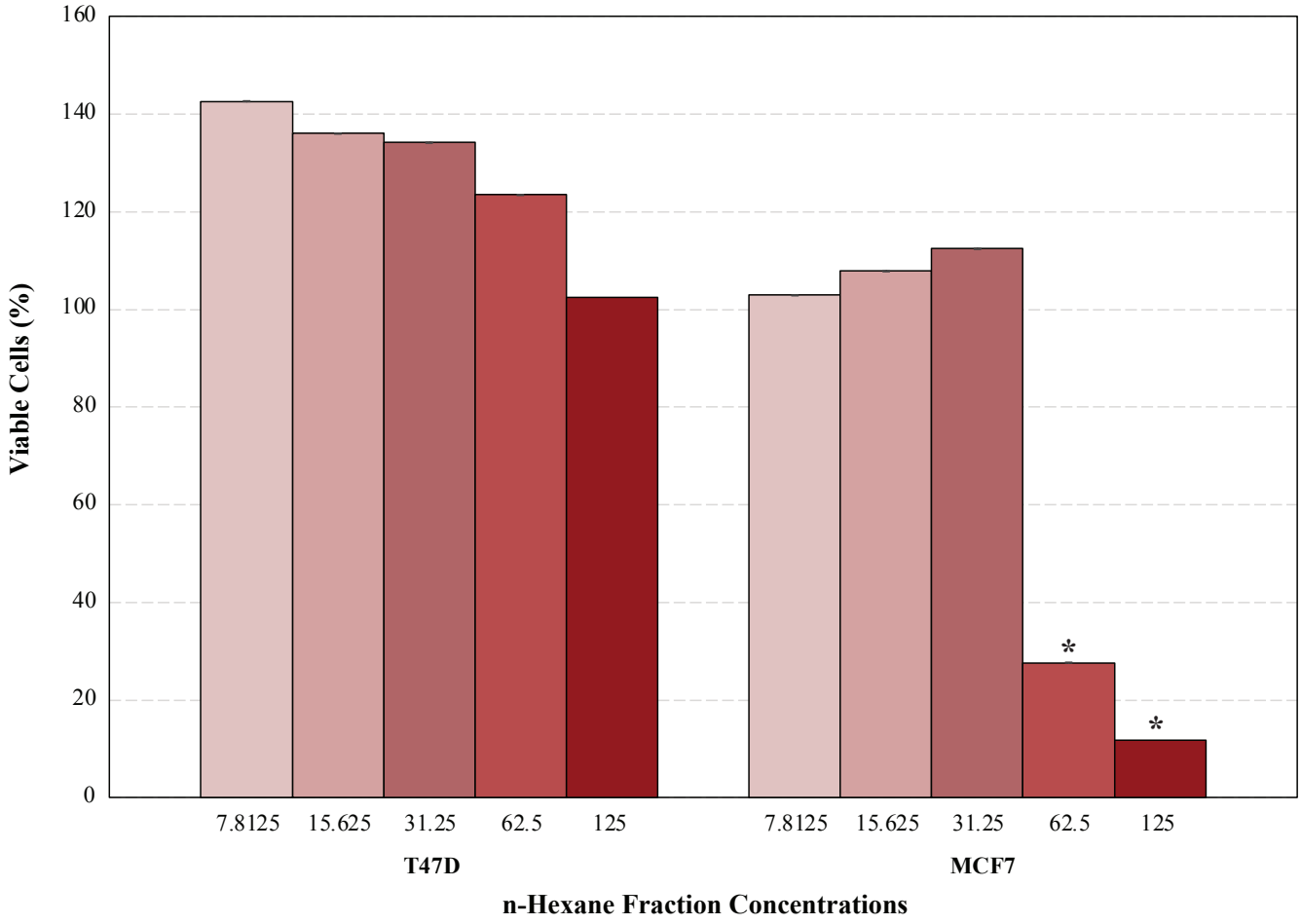

Figure 3. Cell viability after treatment with the n-hexane fraction for 24 hours. The n-hexane fraction concentrations were 7.8125;15.625;31.25;62.5; and 125 µg/mL. The MTT assay was used to measure the viability cell. The histogram showed the mean ± standard deviation. *Significant difference among concentrations (*post hoc* DMRT test).

led researchers to conduct HPLC tests on the n-hexane fraction of GMP melon.[12]

The standard compound used in this study was cucurbitacin B. The appearance of cucurbitacin compounds at RT of 18.144 closely matches the RT range of the standard compounds. There are 12 types of cucurbitacin, and almost all are soluble in non-polar solvents.[17] Therefore, these compounds were detected in the n-hexane fraction. The low concentration observed may be due to the dissolution of other cell components, which are also non-polar, in the n-Hexane solvent.

The low concentration of cucurbitacin in the n-hexane fraction resulted in an IC_{50} values of 216.72 µg/mL for T47D cells and 43.5 µg/mL for MCF7 cells. These IC_{50} values suggested that the n-Hexane fraction is not toxic to T47D cells but is toxic to MCF7 cells. Compared to the IC_{50} values of the chloroform fraction in the previous study, which were 98 µg/mL on T47D and 10 µg/mL on MCF7,[6] the IC_{50} values in both fractions showed higher for T47D cells than for MCF7 cells, indicating that MCF7 cells are more sensitive than T47D cells.

This sensitivity is due to the expression of the p53 gene.[18] The p53 gene encodes the p53 protein, which plays a role in cell cycle arest, cellular senesence, and apoptosis. Activation of the p53 protein is primarily triggered by DNA damage. Enzymes that detect DNA damage transmit signals that activate the p53 protein. The response of damaged cells is then adjusted to the type of stress received.[19] In T47D cells, the p53 gene carries a missense mutation, which inhibits apoptosis and cell cycle arrest, thereby rendering p53 nonfunctional. In contrast, MCF7 cells do not have a mutation in p53 gene and can induce apoptosis in response to stress stimuli.[20]

The presence of multiple peak areas in the HPLC results indicates that other compounds are also present in the GMP melon variety. The cytotoxicity observed in MCF7

cells, in addition to being caused by cucurbitacin, may also be attributed to these compounds. Identification of the compounds present in the fruit is necessary.

The limitations of this study include a small sample size and short exposure duration to the n-hexane fraction, which may not fully capture long-term cytotoxic effects. The n-hexane fraction might also contain unidentified compounds contributing to the observed toxicity, requiring further analysis. Future research should focus on testing different solvent fractions to identify the most potent cucurbitacin and conduct *in vivo* studies on its pharmacokinetics and pharmacodynamics. Expanding the range of cancer cell lines and exploring molecular mechanisms, particularly apoptosis and cell cycle arrest, would offer valuable insights into the compound's therapeutic potential.

Conclusion

The cucurbitacin content in the n-hexane fraction was 7.6 µg/mL per 10 mg of fraction from 10 kg of fresh GMP melon fruit. Cucurbiatcin moderately inhibits the proliferation of MCF7 cells but does not affect T47D cells.

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Authors' Contributions

NW and SW were involved in conceptualizing and planning the research. RS performed the data acquisition and collection, calculated the experimental data, and conducted the analysis. RS, NW, and SW drafted the manuscript and designed the figures. RS and NW assisted in interpreting the results. RS, NW, and SW contributed to the critical revision of the manuscript.

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