In Vitro Evaluation of Cytotoxicity Effect of Ihau Fruit Extract
(*Dimocarpus longan var. Malesianus* Leenh.) on MCF-7 Breast Cancer Cell Line

Irma Sarita Rahmawati¹, Ghina Putri Dyanti¹, Rahma Micho Widyananto¹*, Annisa Rizky Maulidiana¹, Wyna Nabila¹, Ratna Chrismiari Purwestri²

¹† Department of Nutrition, Faculty of Health Sciences, Universitas Brawijaya, Malang, Indonesia
2 Czech University of Life Sciences, Prague, Czech Republic

*Correspondence Address: micho@ub.ac.id

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ABSTRACT
Cancer prevalence is steadily increasing each year and becomes the second leading cause of death worldwide. In Indonesia, breast cancer had a prevalence of 16.7% in 2018. Free radicals contribute to the occurrence of breast cancer, while antioxidants play a vital role in protecting cells and repairing the damage caused by free radicals. Ihau, an endemic fruit in Kalimantan Island, contains phytochemical compounds with potential antioxidant and anticancer properties. Utilizing local food as natural antioxidants could serve as an alternative for breast cancer prevention and treatment. This study aims to assess the anticancer potential of Ihau fruit extract on the MCF-7 breast cancer cell line. A post-test-only control group design method using the MTT assay was used. The two treatment groups were water and 96% ethanol extract, with four different concentrations (125, 250, 500, and 1000 ppm), and each was replicated three times. Statistical analysis using the ANOVA test found no significant difference among all concentrations. The IC₅₀ values of the cytotoxic activity of water and ethanol extracts were 1,197.7 ppm and 1,148 ppm, respectively. It can be concluded that both water and ethanol extract of Ihau fruit exhibited very weak cytotoxic activity.

Keywords: antioxidant, cancer, cytotoxicity, Ihau fruit, MTT assay

ABSTRAK
Setiap tahun, prevalensi kanker terus meningkat dan menjadi faktor penyebab kematian nomor dua di dunia. Pada tahun 2018, prevalensi kanker payudara di Indonesia sebanyak 16,7%. Radikal bebas merupakan salah satu penyebab terjadinya kanker payudara. Antioksidan merupakan suatu senyawa yang mampu menangkal radikal bebas. Buah Ihau merupakan buah endemik khas Kalimantan yang diketahui memiliki kandungan senyawa fitokimia yang berpotensi sebagai antioksidan dan antikanker. Pemanfaatan bahan pangan lokal sebagai antioksidan alami dapat menjadi suatu alternatif pencegahan dan penanganan kanker payudara. Penelitian ini bertujuan untuk mengetahui potensi ekstrak daging buah Ihau sebagai agen antikanker pada sel kanker payudara MCF-7. Metode *post-test only control group design* dengan MTT assay dilakukan pada dua kelompok perlakuan ekstrak air dan etanol (konsentrasi 125, 250, 500, dan 1000 ppm) dengan tiga kali pengulangan. Analisis data

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menggunakan One-Way ANOVA menunjukkan bahwa tidak ada perbedaan pada seluruh konsentrasi. Nilai IC50 aktivitas sitotoksik ekstrak air dan etanol masing-masing sebesar 1.197,7 ppm dan 1.148 ppm. Dapat disimpulkan baik ekstrak air maupun etanol daging buah Ihau memiliki aktivitas sitotoksik yang sangat lemah.

Kata kunci: antioksidan, kanker, sitotoksisisitas, buah Ihau, MTT assay

INTRODUCTION

Cancer is a major health problem and the second leading cause of death after cardiovascular disease in the world, accounting for around 10 million deaths in 2020 (1). In Indonesia, the prevalence of cancer in people of all ages exceeds one million people (2). Breast cancer is one of the most common cancers. Breast cancer is a type of cancer, originating from the ductal epithelium or its lobules (3). According to the 2018 Global Cancer Observatory Data, breast cancer is a leading cancer case in Indonesia with a prevalence of 16.7% (4). Breast cancer can be treated with chemotherapy treatment, using drugs and natural ingredients that contain antioxidants and have the potential as chemo-preventive agents (5).

Cell damage caused by free radicals is a known factor causing various conditions, from premature aging to diseases such as cancer and coronary heart disease (6). Antioxidants are compounds that can counteract the negative effects of excess oxidants in the body. Naturally, antioxidants are found in food with phenolic structures, particularly flavonoids. Synthetic antioxidants can also be added to foods to prevent damage to the taste, smell, and color. However, previous studies indicated potential toxic effects in synthetic antioxidants; thus, their use has been limited to date (7,8). Therefore, exploring and developing natural food sources with antioxidant properties becomes an alternative solution worth investigating.

Ihau fruit (Dimocarpus longan var. malesianus Leenh.) is an endemic fruit grown in East Kalimantan, Indonesia. Ihau fruit has a morphology similar to the longan fruit, but it is distinguished from the prominent nodules in the skin of the Ihau fruit (9). With a vitamin C content of 66.9 mg/100 g (10), Ihau fruit has the potential as an antioxidant due to its phenolic and flavonoid content. A previous study has demonstrated the antioxidant activity of Ihau fruit extract, with an IC50 value of 698.3 µg/ml for fruit water extract and 681.05 µg/ml for the ethanol extract (11).

The polyphenolic and phenolic compounds in Ihau fruit, such as gallic acid, gallic acid, flavone glycosides, quercetin glycosides, and kaempferol, contribute to its anticancer potential and anti-tyrosinase activity (12). Quercetin acts as an immunomodulator and activates signal transduction pathways to inhibit cancer cell proliferation and induce cancer cell apoptosis (13).

In addition, phytochemicals present in Ihau fruit exhibit antibacterial properties. Flavonoids can inhibit bacterial growth by targeting DNA gyrase, while the hydroxyl groups of flavonoids lead to changes in organic components and nutrient transport in bacteria (14, 15). Quercetin can denature proteins in bacteria, leading to reduced bacterial metabolism and bacterial growth inhibition (16). Therefore, this study aims to investigate the potential cytotoxic activity in aqueous and ethanol extracts of Ihau fruit flesh on the MC7 breast cancer cell line.

METHODS

Study Design

This study utilized an in vitro true experimental laboratory approach using a
post-test-only control group design. The Ihau fruit used was obtained from Ihau fruit suppliers in Melak District, West Kutai Regency, East Kalimantan, Indonesia. Ihau fruit was extracted by maceration method using two different solvents, water and 96% ethanol. The cytotoxicity effect was assessed using an MTT assay.

**Material preparation**

Ihau fruits were selected on specific criteria: not slimy, not rotten, having yellow to brown skin surface color, and no change in aroma and foreign matter. Then, the fruits were separated from the skin and seeds to obtain the whole flesh. The flesh was squeezed and chopped before being dried using a food dehydrator at 75°C for 2 hours (17). The dried material was ground using a blender to reduce the particle size and ensure homogeneity, which increases the surface area and enhances the extraction process, allowing for better penetration of the extraction solvent into the cells to extract more bioactive compounds (18).

**Extraction process**

For the water solvent extraction, a total of 500 ml distilled water was boiled to 100°C and then allowed to stand to a temperature of 70-80°C. As much as 125 grams of powdered material was added to the distilled water in a ratio of 1:4, stirred for 30 minutes, and filtered using filter paper to obtain the filtrate. The filtrate was centrifuged at 10 rpm for 10 minutes. Then, the result was evaporated in an oven at 100°C to remove the water solvent and obtain a thick extract. The characteristics of the thick extract included a very small amount of water content, not in a paste form, and no liquid residue when rubbed against a paper towel. The final extract weighed 54.55 g.

For the ethanol solvent, a total of 113 grams of powdered material was macerated with 791 ml of 96% ethanol solvent in a ratio of 1:7 at room temperature for 7 days and stirred for 5 minutes every day. Then, the mixture was centrifuged at 10 rpm for 10 minutes, and the supernatant was collected and distilled at a temperature range of 70-80°C, as ethanol has a boiling point of 78.6°C (19) to obtain a thick extract. To evaporate the remaining ethanol, the extract was heated until the smell of ethanol was gone, and the desired consistency was achieved. The weight obtained was 49.1 g of ethanol extract.

**Cytotoxicity test**

MCF-7 cell lines were planted in 100 ml of RPMI medium with a cell count of 5x104 cells/well. The solution was resuspended every time it filled 12 wells to ensure homogeneous distribution of cells (20). Then, the cells were incubated in a 5% CO₂ incubator at 37°C for 24 hours to obtain good cell growth (21) and allow for cell recovery after harvesting (20). After incubation, the microplate containing the cells was inverted, and the remaining liquid in the plate wells was drained using a tissue. The cells were then washed with 100 L of PBS once before being treated with four series of concentrations in the range of 125 ppm, 250 ppm, 500 ppm, and 1,000 ppm. After that, the cells were placed in a CO₂ incubator for 24 hours.

MTT staining was performed by removing the culture medium containing the test compound and washing the cells with 100 μl of PBS. A total of 100 μl MTT was added to the culture medium at a concentration of 0.5 mg/ml. The microplate was put in a CO₂ incubator for 4 hours. To stop the MTT reaction, 10% SDS (in 0.01 N HCl) was added to the media containing 100 μl of MTT and incubated at room temperature for 12 hours, with the microplate covered in aluminum foil (20). After 4 hours of incubation, observations were made by observing the purple formazan crystals

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formed under a microscope (21). To dissolve formazan, the microplate was shaken on a shaker at 100 rpm for 10 minutes. The absorbance results were then read using an ELISA reader at a wavelength of 570 nm. The resultant cells that were still alive would produce a purple color in response to MTT (20).

**Statistical analysis**

Cytotoxic activity was assessed by determining the IC50 value, which is a concentration that causes the death of 50% of the cell population (22). The percentage of inhibition of cancer cell proliferation was determined by using the following formula:

\[
\text{Treatment [abs] – Media Control [abs]} \times 100\% \\
\text{Negative Control [abs] – Media Control [abs]}
\]

\*abs = absorption

Regression curves were generated using the sample concentration data (x) and the percentage inhibition of cancer cell proliferation (y) to obtain the regression equation and the \( R^2 \) value (20). The IC50 value was obtained from the regression equation, \( y=ax+b \). A smaller IC50 value indicates a higher cytotoxic activity. Table 1 shows the toxicity characteristic cut-off values for IC50 value.

<table>
<thead>
<tr>
<th>IC50 Value</th>
<th>Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;100 ( \mu )g/ml</td>
<td>Potentially cytotoxic</td>
</tr>
<tr>
<td>100 – 1000 ( \mu )g/mL</td>
<td>Moderately cytotoxic</td>
</tr>
<tr>
<td>&gt;1000 ( \mu )g/mL</td>
<td>No toxic</td>
</tr>
</tbody>
</table>

To detect statistical differences across the treatment groups, a one-way ANOVA test was performed. The significance level used was a p-value of <0.05. All data were analyzed using SPSS version 25.

**RESULTS**

This study aimed to determine the potential toxicity of water and ethanol extract of Ihau fruit against MCF-7 breast cancer cell lines. In vitro cytotoxicity test is one of the research models commonly used to study the effect of anticancer molecules of a medicinal plant (23). Cytotoxicity tests were performed on water and ethanol extracts using concentrations of 125 ppm, 250 ppm, 500 ppm, and 1000 ppm. The blank was used as a control solvent. Based on Table 2, the lowest percentage of alive MCF-7 cells were found in water and ethanol extract at the highest concentration, and vice versa, indicating an inverse correlation between extract concentration and percentage of alive cells. However, no significant difference was found among the four concentrations in both of water and ethanol extract.

A linear regression curve (Figure 1) was created based on the sample concentration and the percentage of alive MCF-7 cells (Table 2). The water extract exhibited an \( R^2 \) value of 0.9969, meaning that 99.69% of the decrease in MCF-7 cell proliferation was influenced by the concentration of the sample, which acts as an antioxidant.
Table 2. Absorbance Value to % Alive MCF-7 Cell Lines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (ppm)</th>
<th>Mean Absorbance*</th>
<th>% Alive Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0</td>
<td>0.386</td>
<td>0.00</td>
</tr>
<tr>
<td>125</td>
<td>0.333</td>
<td>86.90</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>0.327</td>
<td>84.31</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>0.307</td>
<td>74.25</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.271</td>
<td>56.91</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>0.352</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>0.308</td>
<td>71.74</td>
<td></td>
</tr>
<tr>
<td>250</td>
<td>0.290</td>
<td>66.15</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>0.285</td>
<td>63.88</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>0.264</td>
<td>53.66</td>
<td></td>
</tr>
</tbody>
</table>

* Statistical analysis using One-way ANOVA

![Figure 1. Regression Curve of Ihau Fruit Sample Concentration Against % Alive MCF-7 Cell Lines (a) Water Extract (b) 96% Ethanol Extract](image)

**DISCUSSION**

Ihau fruit, belonging to the Dimocarpus longan species, contains various potential nutrients that offer health benefits. According to a previous study, Ihau fruit contains a total of 66.9 mg/100 g of vitamin C (10) and several secondary metabolites, such as flavones, quercetin, kaempferol, alkaloids, corilagin, ellagic acid, and flavonoids (12). Vitamin C and antioxidant contents present in Ihau fruit have the potential to suppress oxidative stress and improve the immune system. Flavonoids are antioxidant compounds that play a major role in inhibiting cancer cell growth by binding to the death receptor (TNF-R) and Fas-Associated Death Domain (FADD), which form the Death Inducing Signaling Complex (DISC). Then, the DISC complex activates caspase 8, which will stimulate the Bid protein, leading to Bax activation in the mitochondrial membrane and the release of cytochrome C as a proapoptotic molecule. Apoptosomes formed through the binding of cytochrome C with Apoptosis Activating Factor 1 (APAF-1) will trigger the process of apoptosis (24,25). In addition, quercetin acts as a metal ion chelating agent, namely Fe2+ and Cu2+ in the formation of free radicals (26).

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However, the IC50 value obtained in this study showed that water and ethanol extract of Ihau fruit has very weak antioxidant activity. It can be due to the high sugar content in Ihau fruit. The antioxidant activity of an extract tends to decrease with higher sugar content, thereby impacting its ability to scavenge free radicals in the body (27). Sugar content in Ihau fruit increases during the ripening process, which consists of sucrose, fructose, and glucose (12). The sugar content in Dimocarpus longan fruits, including Ihau fruit, comprises polysaccharides with 1→6)-α-D-glucan glycosidic bonds and a chemical shift from C6. The molecular weight of polysaccharide content is 108 kDa, with a total glucose content of 661. Earlier study performed cytotoxicity tests on MCF-7 breast cancer cells and HepG2 liver cancer cells showed that the polysaccharide content had a cytotoxic effect on HepG2 cells and did not show cytotoxicity on MCF-7 cells (28). These findings align with the results of this study, which showed a low cytotoxic effect of Ihau fruit on MCF-7 breast cancer cells.

In addition, the choice of the Ihau plant part used can also influence the level of antioxidant and anticancer potential. Ihau fruit and longan fruit belong to the Sapindaceae family and share similar morphological characteristics. Research on longan plant extracts, particularly ellagic acid, demonstrated high antioxidant levels in the leaf, stem, fruit skin, and seed extracts. From the results of the DPPH test, it showed that the highest source of antioxidants in longan plants was in the stem and leaf extracts. The antioxidant effect increased with higher extract concentrations, as evidenced by IC50 values of 0.057 g/ml and 0.058 g/ml in the stem and leaf extracts, respectively. The total content of ellagic acid in the stem extract was 0.091 mg/g and 3.723 mg/g in the longan plant leaf extract (29). Longan seed extract has the ability to scavenge free radicals. Factors that affect the content of phytochemicals and antioxidants in a plant vary, including the soil condition, varieties, genetics, pesticide use, environment, and harvest timing. However, the polysaccharide content in Ihau fruit is able to undergo methylation reactions, which can reduce its effectiveness as an anticancer agent in scavenging free radicals (12).

The extraction method employed can also affect the anticancer ability of Ihau fruit. The maceration method used in this study involved stirring the material powder at room temperature and heating in an oven to obtain a thick extract. Although heating can accelerate the extraction process of phenolic compounds and flavonoids from fruit extracts, flavonoids are phenolic compounds with a conjugated aromatic system that are susceptible to damage at high temperatures. The prolonged extraction process has shown to decrease the total phenol and flavonoid content. The results of previous studies showed that high-pressure-assisted extraction (HPE) and ultrasonic waves were more effective than conventional extraction in extracting bioactive compounds from the longan pericarp. Optimizing the HPE extraction method at temperatures ranging from 30 to 90°C, durations of 2.5 to 30 minutes, and pressures of 200 to 500 mPa yielded a pericarp extract of longan fruit with cytotoxic abilities against HepG2, A549, and SGC7901 cancer cells (12).

The heating process employed after the extraction process to obtain a thick extract from water and ethanol solvents can also affect the antioxidant levels in the sample. Damage resulting from heating when drying material can be controlled by adjusting the

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temperature and duration of the heating process. However, even with the correct temperature, the heating process can still potentially damage the antioxidant compounds contained in the material (29). Therefore, it is also thought to affect the total antioxidants in the sample. This study had some limitations, including the absence of analytical methods, such as proliferation test and Gas Chromatography/Mass Spectrometry (GC/MS) analysis to further investigate the anti-cancer potential of Ihau fruit.

CONCLUSION

Ihau fruit is one of the endemic fruits from Indonesia that need deeper research to find the potential health effects on human. The cytotoxic activity of water and ethanol extracts of Ihau fruit on MCF-7 breast cancer cells using the MTT assay method showed low cytotoxicity. This could be due to the high content of polysaccharides in Ihau fruit, the extraction method used, and the phytochemical compounds present in the extract. Even though no significant difference in the percentage of alive MCF-7 cells was found across different sample concentrations, as the sample concentration increased, the percentage of MCF-7 cell proliferation decreased.

Further study should consider conducting anti-proliferation tests and analyzing the bioactive compounds using techniques such as using GC/MS to investigate the anti-cancer potential of the crude extract of compound contained in Ihau fruit.

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