Anticancer Effect of Red Fruit Fractions Toward Breast Cancer in T47D Cell and Oral Squamous Cancer in KB Cell

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Abstract. Indonesia is one of the mega-biodiversity countries, with many plant species that are useful as medicine. Red Fruit (*Pandanus conoideus* Lam), found in Jayawijaya, Papua, is empirically believed to have numerous health benefits due to having a huge amount of bioactive compounds, especially carotenoids. This study aimed to examine the cytotoxicity effect of red fruit fractions against breast cancer in T47D cells and oral cancer in KB cells compare to doxorubicin. Methanol extracts were fractionated by vacuum liquid chromatography using silica gel H-60 and eluting solvents n-hexane, ethyl-acetate, and methanol in various ratios. In this experimental *in-vitro* study design, T47D cells and KB cells were induced by four red fruit fractions (I, II, IV, VI) with 6 concentrations (0.0625 µl, 0.125 µl, 0.25 µl, 0.50 µl, 1.00 µl, 2.00 µl) and Doxorubicin as positive control used in this cytotoxicity assay. All data were analyzed by One way ANOVA and Tukey HSD with $\alpha = 0.05$. The result showed that all red fruit fractions reduced the viability of T47D cells and KB cells in a concentration-dependent manner. Red fruit fraction IV 1 µl had the best cytotoxic effect, which caused 71.48% cell death in T47D cells and 81.03% in KB cells. It can be concluded that all red fruit fractions showed cytotoxicity effect towards breast cancer in T47D cell and oral squamous cell cancer in KB cell. Fraction IV red fruit showed the best effectiveness as anticancer in breast and oral squamous cancer.

Keywords: Breast cancer · Oral squamous cell cancer · Red fruit fractions · T47D cell · KB cell

1 Introduction

Indonesia is one of the mega-biodiversity countries, a country that has the richest biodiversity in the world. Many types of plants that are useful as medicinal ingredients are also found in Indonesia, such as Buah Merah (Red Fruit). This red fruit can only be found in the mountainous area of Jayawijaya, Papua, Indonesia. Red fruit (*Pandanus conoideus* Lam) is a plant in the pandanus family and has about 30 cultivars. One of the red fruit cultivars, which is empirically believed to have many health benefits, is the long red cultivar [1]. Red fruit contains a large number of bioactive compounds such as phenolic, flavonoids, carotenoids, tocopherols, and unsaturated fatty acids [2, 3]. The

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bioactive compound with very high levels in red fruit is carotenoid, as much as 3027–19959 mg/kg, much higher than carotenoid sources from other natural ingredients. Local people in Papua use red fruit as a natural remedy for various diseases, including cancer [3, 4].

Cancer is still a health problem for the Indonesian population, with an incidence rate of 136.2/100,000 and ranks 8th in Southeast Asia. Based on Riskesdas data, the prevalence of cancer in Indonesia increased from 1.4 per 1000 population in 2013 to 1.79 per 1000 population in 2018. The highest type of cancer in Indonesian women is breast cancer, 42.1 per 100,000 populations with a mortality rate of 17 per 100,000 populations [5]. Oral cavity squamous cell carcinoma is the most common type of malignancy in the oral cavity, and its incidence in Indonesia is 3–5% of all cancers [6].

Our previous study found that red fruit extract has a cytotoxicity effect against cervical cancer cells but not toxic to normal cells such as fibroblasts [7]. To be used as herbal medicine, this plant should be safe. Based on Wismandanu's research (2016), with acute toxicity test, red fruit can be categorized as Category 5 GHS (Globally Harmonized System for Chemical Classification Substances and Mixtures) as practically non-toxic materials [8].

As a comparison, we used doxorubicin, which is a chemotherapeutic agent, part of the anthracycline group, widely used for various types of cancer, including breast cancer and soft tissue sarcoma. The mechanism of action of doxorubicin is to intercalate within DNA base pairs, inhibit the topoisomerase II enzyme, cause DNA damage, inhibit DNA and RNA synthesis, and ultimately induce apoptosis of cancer cells. Although doxorubicin has the good ability as a chemotherapeutic agent, doxorubicin has a dangerous side effect that can cause acute cardiac toxicity in the manifestations of reversible myopericarditis, left ventricular dysfunction, or arrhythmias, even can cause irreversible cardiomyopathy in the long-term use [9].

This study aimed to examine the cytotoxicity effect of red fruit fractions against breast cancer in T47D cells and oral cancer in KB cells compare to doxorubicin.

2 Materials and Methods

The methods used in this study is an experimental *in-vitro* study design. The red fruit used in this study was a fresh long red cultivar of red fruit, originating from Jayawijaya, Papua. The fractionated of red fruit by vacuum liquid chromatography was done at Bandung Institute of Technology. The cytotoxicity assay towards T47D cell line and KB cell line with a trypan blue dye exclusion was done at Laboratory of Biological Sciences Gadjah Mada University.

2.1 Preparation of Red Fruit Fractionation

Red fruit (*Pandanus conoideus* Lam) as much as 200 g was macerated with 400 ml of methanol. The methanol red fruit extract was taken 40 g for fractionation by Vacuum Liquid Chromatography (VLC) using silica gel H-60 as an absorbent and an elution solvent with increasing polarity. Sequential elution of n-hexane: ethyl acetate (95:5), n-hexane: ethyl acetate (1:1), n-hexane: ethyl acetate (1:2), ethyl acetate 100%, ethyl

acetate: methanol (1:1), and methanol 100%, using a vacuum pump to facilitate the withdrawal of the eluent. From VLC obtained 51 fractions, each of which has a volume of 80–100 ml. The fraction was monitored using Thin Layer Chromatography with a dual expansion system. The fractions with the same or similar color profile were combined to obtain nine fractions showing different chromatogram profiles. In this study, we used four fractions that have an enormous volume. A qualitative evaluation was carried out for these four fractions based on the retention factor (Rf). The result is Fraction I with Rf 0.58; fraction II Rf 0.36; Fraction IV Rf 0.12 and Fraction VI Rf 0.56. The results of the analysis using Gas Chromatography-Mass Spectroscopy revealed that the extract and ethanol fractions of red fruit contained flavonoid compounds, phenols (glycosides), anthraquinones, and coumarins. In addition, the n-hexane fraction of red fruit contains a group of fatty acid compounds, steroids, and triterpenoids, as well as carotenoids.

2.2 Preparation of T47D Cell and KB Cell Line

Cell culture T47D was grown using a growth medium (RPMI 1640, Fetal Bovine Serum, Streptomycin/Penicillin, and fungizone), then incubated in the 5% CO_2 incubator, 37 °C. The medium should be changed every two days. When the cells are at about 90% confluence, pour out the medium, apply Trypsin 0.25%, and incubate at 37 °C for 10–15 min. Verify under an inverted microscope to ensure that all the cells are detached from the flask; the suspension was transferred in a 15 ml Falcon tube and centrifuged 2000 rpm for 10 min. Aspirated the supernatant, re-suspended the pellet in 1 ml RPMI, then pipetted 20 μl, mixed with 50 μl trypan blue. Next pipetted 10 μl and put in the Haemocytometer, counted the cell under an optical microscope. Add RPMI into the conical tube, which already has the pellet, until the volume is 12 ml, then pour 100 μl to each 96-microwell plate and incubated the microwell plate in the CO_2 incubator 37 °C, 3 h. In 100 μl, there were 225×10^3 live cells. Do the same procedure with KB cell but used DMEM as the medium [10].

2.3 Cytotoxicity Assay

There were 4 Red Fruit fractions (Fractions I, II, III, IV) with six various concentrations (0.0625 μl, 0.125 μl, 0.5 μl, 0.75 μl, 1 μl, 2 μl), used in this cytotoxicity assay towards breast cancer cell line (T47D) and oral squamous cell cancer cell line (KB cell). The concentrations used in this research based on our previous study towards cervical cancer cell line [7].

Take out the 96-microwell plates from the incubator, which in each well already contains 100 μl cells with medium (T47D cells and KB cells), which contains 225×10^3 live cells in each well. Then, add 100 μl Red Fruit fractions (4 fractions) with six various concentrations (triplicate). The negative control only contains cells with the medium, and the positive control contains cells, medium, and doxorubicin with the same dose with Red Fruit fractions. Then incubated the microwell plates in the incubator with CO_2 5% and temperature 37 °C for 24 h. The last step is to count the live cells using Cell Counting Direct with trypan blue and continue by analyzed the result using the formula: % cell death = {(Σ live cell control - Σ live cell treatment)/ Σ live cell control} *100% [10].

2.4 Data Analysis

In this study, the data were the percentage of cell death after treatment with various concentrations of red fruit fraction and doxorubicin as positive control. First, the data tested with the Levene Test for homogeneity and the Shapiro-Wilk normality test. Due to the data was normally distributed and the data distribution has the same variance, then the data analyzed by One Way ANOVA test and followed by the Post Hoc Tukey HSD test, with the significance level of 5% ($\alpha = 0.05$).

3 Results

3.1 Cytotoxicity of Red Fruit Fractions Towards T47D Cell

The four red fruit fractions (Fractions I, II, IV, VI) with 6 concentrations (0.0625 µl, 0.125 µl, 0.25 µl, 0.50 µl, 1.00 µl, 2.00 µl) were exposed to T47D cells with Doxorubicin as positive control and incubated for 24 h. The results can be seen in Fig. 1.

In Fig. 1 it can be seen that all the Red Fruit fractions give the cytotoxic effect towards T47D breast cancer cells, if compared to negative control. Red fruit fractions I, II and VI at the largest dose of 2 µl, the cytotoxicity effect did not reach 50%, only fraction IV gave the best cytotoxicity effect, which at a dose of 0.250 µl caused 54.10% cell death. The fourth fraction will be further analyzed and compared with the positive control, doxorubicin.

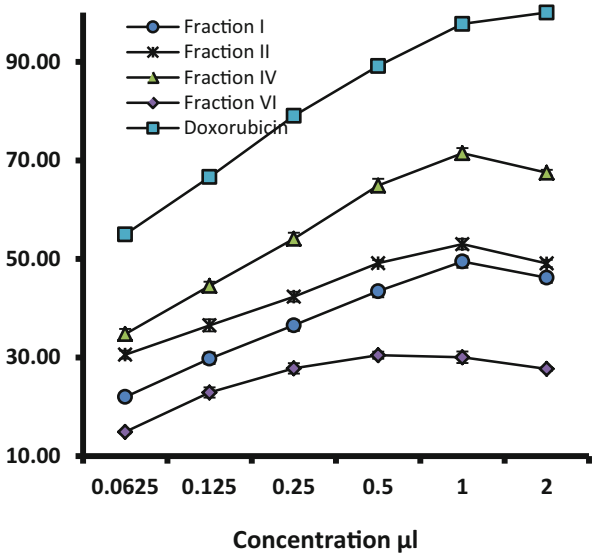

Fig. 1. T47D cell: percentage of cell death (Y axis) after treatment by red fruit fractions I, II, IV, VI and doxorubicin as positive control.

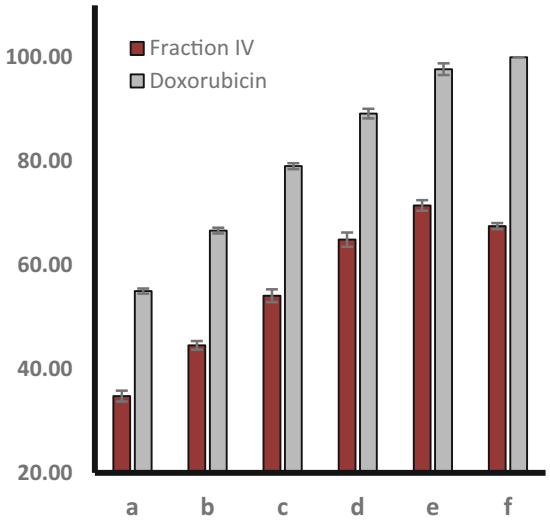

Fig. 2. T47D cell: comparison the percentage of cell death (Y axis) between fraction IV red fruit and doxorubicin in various concentration (X axis).

When the cytotoxicity effects of fractions IV and doxorubicin were analyzed, the percentage of T47D cell death due to treatment with Red Fruit Fraction IV 0.250 ml was not significantly different from that of doxorubicin 0.0625 μl ($p = 0.980$) with a percentage of cell death of 54.99 ± 0.48% (Fig. 2).

Likewise, the treatment by Red Fruit Fraction IV 0.50 μl and 2.00 μl compared to Doxorubicin 0.125 μl, the percentage of cell death was not significantly different with $p = 0.442$ for a concentration of 0.50 μl and $p = 0.980$ for a concentration of 2.00 μl, with the percentage of cell death 66.64 ± 0.55%. This result showed that Red Fruit Fraction IV effective as anti- cancer towards T47D breast cancer cells but requires a larger dosage, 4–8 times greater than Doxorubicin.

3.2 IC_{50} of Red Fruit Fractions on T47D Cell

To find out how much concentration of each red fruit fraction can 50% decreased the viability of T47D cells, IC_{50} is calculated (Fig. 3).

In T47D cells, the red fruit fraction with the smallest IC_{50} was fraction IV (c), which was 1.02 ± 0.01 μl. This means that fraction IV has the best anti-cancer effectiveness against breast cancer in T47D cells. This IC_{50} still distinct compare to IC_{50} of doxorubicin, which was 0.71 μl. The statistical analysis results also showed that all IC_{50} fractions of red fruit were highly significant difference ($p = 0.001$) compared to doxorubicin (e).

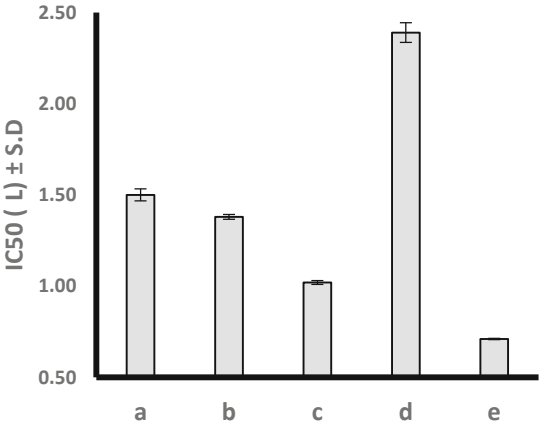

Fig. 3. IC_{50} of red fruit fractions on T47D cell. (a = Fraction I, b = fraction II, c = fraction IV, d = fraction VI, e = Doxorubicin).

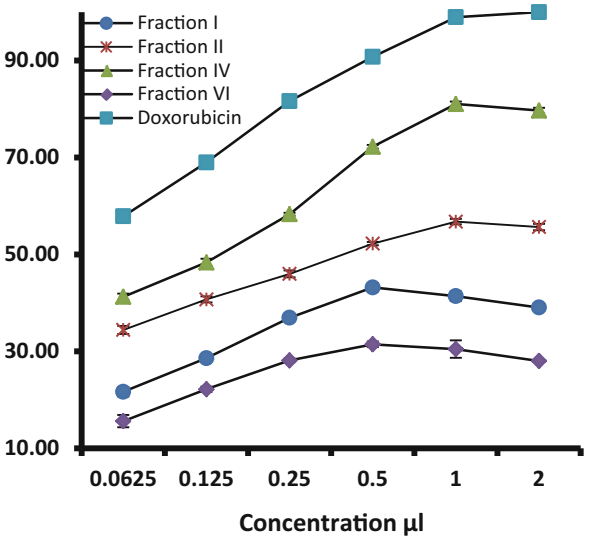

Fig. 4. KB cell: percentage of cell death (Y axis) after treatment by red fruit fractions I, II, IV, VI and doxorubicin as positive control.

3.3 Cytotoxicity of Red Fruit Fractions Towards KB Cell

The results of the cytotoxicity test of the red fruit fractions on oral squamous cell cancer in KB cells can be seen in Fig. 4

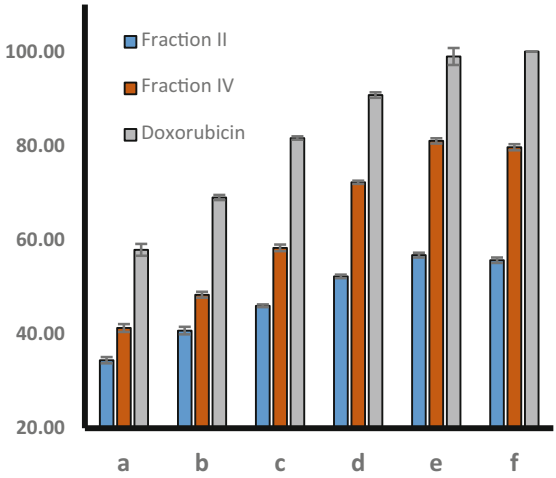

Fig. 5. KB cell: comparison percentage of cell death (Y axis) after treatment with red fruit fraction II, fraction IV and doxorubicin in various concentration (X axis).

It turned out that the cytotoxicity test of the red fruit fraction towards squamous oral squamous cell cancer cells in KB cells was the same as in breast cancer T47D cells, where fraction IV showed the best results and almost the same as doxorubicin. Fractions I and VI only caused cell death of 41.39 ± 0.91% and 31.46 ± 0.85%. Therefore, the fractions II and IV will be analyzed further. Comparison of the percentage of KB cell death after treatment with fraction II, fraction IV, and doxorubicin showed in the next figure.

Figure 5 showed that the highest cell death caused by fraction II was 56.77% at a concentration of 1 μl, but then the cytotoxicity effect decreased again at a concentration of 2 μl. Fraction IV has a better cytotoxicity effect, and even it can reach 81.03% at a concentration of 1 μl.

The statistical analysis results showed that the percentage of cell death after treatment with fraction IV 0.25 μl (c) was not significantly different from doxorubicin 0.0625 μl (a) with $p = 1{,}000$, and concentration 1 μl was not significantly different from doxorubicin 0.25 μl with $p = 0.999$. Nevertheless, fraction II not as effective as fraction IV in reducing the availability of KB cell.

3.4 IC_{50} of Red Fruit Fractions on KB Cell

The IC_{50} calculation results from all red fruit fractions in KB cells can be seen in Fig. 6.

In KB cells, the red fruit fraction with the smallest IC_{50} was fraction IV (c), which was 0.89 ± 0.00 μl, but statistical analysis still showed a highly significant difference ($p < 0.001$) compared to doxorubicin (e) with an IC_{50} of 0.71 μl.

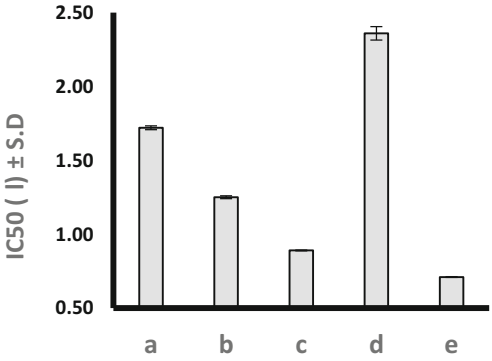

Fig. 6. IC_{50} of red fruit fractions on KB cell (a = Fraction I, b = fraction II, c = fraction IV, d = fraction VI, e = Doxorubicin).

4 Discussion

Red fruit has been known to have health benefits and empirically used as anticancer. Several in vitro studies have been reported on the anti-cancerous activity of the red fruit extract, such as in lung cancer, myeloma cancer, colorectal cancer, and could induce apoptosis in cervical cancer cell [11]. The benefits of red fruit are supported by a large number of bioactive compounds, such as carotenoid, tocopherols, flavonoids, phenolic, and unsaturated fatty acids [2, 3]. Red fruit oil is known to have high carotenoid levels, 3,027–19,959 mg/kg, much higher than other carotenoid sources [12, 13]. The types of carotenoids found in red fruit are α-carotene, β-carotene, α-cryptoxanthin, and β-cryptoxanthin. Carotenoids found in extremely high amounts were β-carotene 1,980 g/100 g and β-cryptoxanthin 1,460 g/100 g. Besides carotenoids, red fruit also contains -tocopherol 21.2 mg [1, 12, 13].

This study showed that the red fruit fraction decreased breast cancer cell viability in T47D cells and oral squamous cell cancer in KB cells in a concentration-dependent manner. Fraction IV has the best effectiveness, and the smallest IC_{50} is 1.02 ± 0.01 μl in T47D cells and 0.89 ± 0.00 μl in KB cells. Carotenoids as bioactive substances in this fraction IV have the potential to cause cytotoxicity to cancer cells. Several studies have shown that some carotenoids have strong anti-cancer effects, both *in vitro* and *in vivo*. Dietary carotenoid intake has been correlated with reduced cancer risk [14]. Carotenoids have multiple targets and several mechanisms that contribute to their efficacy as anti-cancer agents [15].

It has long been known that α-carotene and β-carotene reduced cancer incidence of skin, lung, liver, and colorectal cancer, even α-carotene can protect pre-neoplastic colorectal adenocarcinoma lesions from developing into malignant cancer [16].

α-carotene, β-carotene, β-cryptoxanthin, and α-tocopherol have antioxidant and provitamin A activity, leading to immune-enhancement effects, decreasing the risk of some cancers, and anti-inflammatory effect. In addition, these antioxidants play an essential role in reducing oxidative damage, breaking free radical chains to reduce mutagenesis and thereby reduce carcinogenesis because they can reduce DNA damage [15].

Antioxidants are compounds that can neutralize free radicals by capturing or donating electrons. Therefore, these compounds are essential in maintaining a healthy body. The human body produces antioxidant compounds, but not enough to compete with the free radicals produced every day by the body itself. Free radicals are relatively unstable molecules having one or more unpaired electrons in their outer orbits. These molecules are reactive in looking for electron pairs from other molecules and are very easy to attack healthy body cells. If it is formed in the body, a chain reaction will occur and produce new free radicals, which eventually continue to increase in number. These free radicals can cause DNA damage which eventually leads to carcinogenesis. Cells will try to eliminate free radicals and keep the damage to a minimum. Several enzymatic and non-enzymatic systems play a role in the inactivation of free radical reactions, namely by forming antioxidants that can block the initiation of free radical formation or free radical inactivation and prevent DNA damage. Various enzymes that can act as free radical scavenging systems and reduce hydrogen peroxide and superoxide anions are catalase enzymes in peroxisomes, superoxide dismutase, and glutathione peroxidase [17].

Several studies have shown that antioxidants can work as anti-cancer agents by activating the transcription factor Nrf2, which is bound to ARE (Antioxidant response element). Then the gene that forms enzymes that play a role in free radical scavenging will be expressed. In studies with Nrf2-deficient mice, it was proven that there would be a decrease in free-radical scavenging enzymes [15].

In this study, fraction IV of red fruit contains carotenoids, especially β-carotene and β-cryptoxanthin, an antioxidant, to kill cancer cells in breast and oral squamous cell cancer. However, this anti-cancer activity is not equivalent to doxorubicin but requires four times higher concentration.

Red fruit fractions I, II, IV, VI reduced the viability of T47D cells and KB cells in a dose-dependent manner. Red fruit fraction IV showed the best effectiveness as anti cancer in breast cancer T47D cell as well as oral squamous cancer in KB cell. The IC_{50} of red fruit fraction IV is 0.102 ± 0.01 μl in T47D cell and 0.089 ± 0.00 μl in KB cell, but statistical analysis showed significant different compare to doxorubicin with IC_{50} 0.07 ± 0.00 μl in both cell lines. Red fruit fraction IV, as a typical Indonesian medicinal plant, can be develop as an anticancer for breast cancer and oral squamous cancer.

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Authors' Contributions. HR conceived the original idea, YC and E screened and summarized all obtained literatures. HR, YC and E analysed the bias of the study. The manuscript was initially written by YC and E, and the improved and revised by HR All authors read and approved the final manuscript.

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