

## Cytotoxicity Assay of Dètente Instantanée Côntrôlée Pre-dried *Pandanus conoideus* Lam. Extracts

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### Artikel Penelitian

**Abstract:** Red fruit (*Pandanus conoideus* Lam.) contains antioxidant compounds in high levels, including beta-carotene and tocopherol, which neutralize free radical compounds which can cause cancer. Red fruit contains 46% water so red fruit can only last 5 days in this condition. To maintain its stability, it is necessary to dry it with various methods, for example dètente instantanée contrôlée (DIC) is a revolutionary pressure drop drying procedure. Every procedure for drying raw material can affect its quality and activity, for example the anticancer activity of red fruit. In-vitro cytotoxicity of DIC pre-dried red fruit extracts on HeLa cells has never been tested, so it is important to study. Red fruit which was obtained from Klamono, Sorong, a highland in Papua was extracted with ethanol or hexane which previously dried by DIC pre-drying or conventional drying methods were weighed then dissolved in DMSO. Cytotoxicity assays was conducted using method the MTT with five levels of extract concentration. Result of this research showed that cytotoxicity assay of red fruit extracts on HeLa cells showed that red fruit extract can inhibit cell viability. The conventionally dried red fruit extract shows stronger cytotoxicity against HeLa cells than the DIC pre-dried extract. Red fruit extract cytotoxicity against HeLa cells: ethanolic extract of red fruit previously dried by conventional drying technique > ethanolic extract of red fruit previously dried by DIC pre-drying process > hexane extract of red fruit previously dried by conventional drying method > hexane extract of red fruit previously dried by DIC pre-drying process.

**Keywords:** cytotoxicity assay, Dètente Instantanée Côntrôlée, HeLa cells, *Pandanus conoideus*, pre-drying, red Fruit

**Abstrak:** Buah merah (*Pandanus conoideus* Lam.) mengandung senyawa antioksidan yang tinggi, antara lain betakaroten dan tokoferol, yang dapat menetralkan senyawa radikal bebas penyebab kanker. Buah merah mengandung 46% air sehingga buah merah hanya bisa bertahan 5 hari. Untuk menjaga stabilitasnya, perlu dilakukan pengeringan dengan berbagai metode, misalnya dètente instantanée control (DIC) yang merupakan prosedur pengeringan dengan penurunan tekanan yang revolusioner. Metode pengeringan bahan baku dapat mempengaruhi kualitas dan aktivitasnya, misalnya aktivitas antikanker buah merah. Uji sitotoksitas in-vitro ekstrak buah merah pra-kering DIC pada sel HeLa belum pernah diuji, sehingga penting untuk dipelajari. Buah merah yang diperoleh dari Klamono, Sorong, dataran tinggi di Papua diekstraksi dengan etanol atau heksana yang sebelumnya dikeringkan dengan metode DIC pra-pengeringan atau pengeringan konvensional ditimbang kemudian dilarutkan dalam DMSO. Uji sitotoksitas dilakukan dengan metode MTT dengan lima jenis konsentrasi ekstrak. Hasil penelitian menunjukkan uji sitotoksitas ekstrak buah merah terhadap sel HeLa menunjukkan bahwa ekstrak buah merah dapat menghambat viabilitas sel. Ekstrak buah merah yang dikeringkan secara konvensional menunjukkan sitotoksitas yang lebih kuat terhadap sel HeLa daripada ekstrak pra-kering DIC. Sitotoksik ekstrak buah merah terhadap sel HeLa: ekstrak etanol buah merah yang sebelumnya dikeringkan dengan teknik pengeringan konvensional > ekstrak etanol buah merah yang sebelumnya dikeringkan dengan proses DIC pre-drying > ekstrak heksana buah merah yang sebelumnya dikeringkan dengan metode pengeringan konvensional > ekstrak heksana buah merah yang sebelumnya dikeringkan dengan proses pra-pengeringan DIC.

**Kata kunci:** buah merah, Dètente Instantanée Côntrôlée, *Pandanus conoideus*, pra-pengeringan, sel HeLa, uji sitotoksitas

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## Introduction

Red fruit (*Pandanus conoideus*) contains nutritional substances and active compounds in high levels, including beta-carotene and tocopherol. The content of tocopherol in the red fruit is high which has a biological activity as antioxidant (1). The compounds such as tocopherol and flavonoid that is found in plants can act as an antioxidant primer (2,3).

In the human body, antioxidant can neutralize and break free radical compounds which can cause cancer. Moreover, Red fruit also has an activity as immunomodulator. Immunomodulator is a compound that capable of influencing the positive reaction in the body against cancer. These compounds can stimulate various cells that play a role in the immune response, such as lymphocytes T, NK cells, and macrophages (4,5).

The previous study (6) showed that the red fruit extract had cytotoxicity against cancer cells (HeLa cells). The sample which was used was methanol extract of red fruit and partitioned with n-hexane and ethyl acetate. According to the result, methanol extract of red fruit which was partitioned with n-hexane and 50% ethyl acetate, and partitioned with n-hexane and 100 % ethyl acetate have anticancer activity. Both of the samples could inhibit HeLa cells viability as many as 100%.

Red fruit has moisture content approximately 46%. This condition makes the red fruit can not be preserved more than 5 days. In room temperature (25-30°C), the red fruit become easily damaged. However, in cold temperature (10°C), the red fruit can be preserved for 7-10 days. The damage can cause the degradation of the beneficial content which is needed as medicine/supplement. Because of this condition there are only two ways to prevent the damage, directly processes into the red fruit oil or preserved by drying method. Drying method of each crude drug must be considered because a different drying method can affect the quality of the crude drug (7).

The conventional drying method, such as drying under sunlight or exposing the simplicia in the air, is the preferred method in Indonesia. Since the method needs long exposure in an open area, the risk of contamination, both particulate

and microbiological contaminants, is higher than any other drying methods. Besides, the temperature of the conventional one is uncontrolled. Some compounds are volatile and some are easily degraded when directly exposed under sunlight, hence the active compound will be lower than expected.

For that reason, a new process of drying by instantaneous control pressure drop, called *détente instantanée contrôlée* (DIC) is developed as a pre-drying treatment. This treatment is also categorized as a High Temperature Short Time (HTST) process (8). Advantages of DIC processing are enhancing the desired outcome and the product has a better quality in terms of flavor, appearance, and other intrinsic factors. Besides, this process can significantly shorten production time and cut the production cost.

The in-vitro cytotoxicity of DIC pre-dried red fruit extracts have never been studied before. Thus, the cytotoxicity of DIC pre-dried red fruit extracts on HeLa cells is important to studied. The cancer cell that is used is HeLa cells. The HeLa cells are easy to be cultured and can be 90-100% confluence rapidly.

One of assay to study the cytotoxicity is MTT assay. MTT assay based on the conversion of the tetrazolium salt (yellow color) by the enzyme succinate dehydrogenase with the helped of NADH, turn into dark blue product called formazan. Formazan crystal is proportional for the number of living cells in various cell types (9). These methods are rapid, sensitive, accurate and a large number of samples can be tested automatically using a microplate reader.

In this study, the effect of pre-drying treatment by DIC on the cytotoxicity of the red fruit extract against HeLa cells will be explored using MTT assay.

## Materials and Methods

### Materials

#### *Extract powder of red fruit*

Red fruit sample is the red fruit (*Pandanus conoideus* Lam.) which was obtained from Klamono, Sorong, a highland in Papua on 5<sup>th</sup> October 2012. The powder of red fruit extract was

obtained from several treatments, modified from the previous research (10,11).

### Chemicals

Roswell Park Memorial Institute Medium (RPMI) 1640, Fetal Bovine Serum (FBS) (gibco® by lifes technology), Sterile *aquabidestilata*, antibiotics (Penicillin-streptomycin) (Sigma), Alcohol 70%, Phosphate Buffered Saline (PBS) (gibco® by lifes technology) 0.25% Trypsin-EDTA (gibco® by lifes technology), DMSO (Jacob Lab), Ethanol p.a., HeLa cells (American Type Culture Collection® Catalog No. CCL-2TM), and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Vybrant® by lifes technology).

### Methods

#### Cell Culture Condition

Human cervical cancer cell lines, HeLa cells, were maintained in RPMI supplemented with 40 ml penicillin-Streptomycin, 40 ml fungizone, 10% heat-inactivated fetal bovine serum (FBS) and 0,2% sodium bicarbonate. The suspension of HeLa cells was added to each well for cultured in a 96-wells plate, then incubated in the CO<sub>2</sub> incubator overnight until 90%-100% confluence (5x10<sup>4</sup> cells /well).

#### Drying condition

There are four samples that are tested in this research. The first sample is red fruit of optimum parameter of DIC pre-drying (2.5 bar, 15 seconds, 4 cycles) followed by optimum extraction condition (60% ethanol, 30°C, 1 hour. The second sample is red fruit dried by conventional drying followed by optimum extraction condition (60% ethanol, 30°C, 1 hour) (11). The third sample is red fruit of optimum parameter of DIC pre-drying (1.5 bar, 15 seconds, 2 cycles) followed by optimum extraction condition (hexane, 45°C, 1.5 hours). The fourth sample is red fruit dried by conventional drying followed by optimum extraction condition (hexane, 45°C, 1.5 hours) (11).

#### Preparation and Conditions for Assay

Ethanolic extract of red fruit, which previously dried by DIC pre-drying and conventional drying methods, were weighed 180 mg, which was then dissolved in 2%DMSO. The

mixture was homogenized by vortex and centrifuged at 1600 rpm for 5 minutes. The concentration of extract was diluted with RPMI culture medium to yield five assay concentrations: 900 ppm, 2,700 ppm, 5,400 ppm, 7,200 ppm and 9,000 ppm (8, 12, 13, 14).

Hexane extracts of red fruit, which previously dried by DIC pre-drying and conventional drying methods, were weighed 500 mg, which was then dissolved in solvent mixture which contained 1.25% DMSO and 0.8% ethanol. The concentration of extract was diluted with RPMI culture medium to yield five assay concentrations: 10,000 ppm, 20,000 ppm, 40,000 ppm, 60,000 ppm and 80,000 ppm (12-14).

For this assay, a positive control, 0.003 % Doxorubicin, for indicating the toxicity observed in the direct contact assay was due to toxic components in the extract, was included in the assay. As a negative control material is a hydrophilic fumed silica that can aid improvements in drying efficiency, Cab-O-Sil, was used. For ethanolic extract, Cab-O-Sil was weighed 45 mg, which was then dissolved in 1% DMSO, while for hexane extract, it was weighed 50 mg, and then dissolved in 1% DMSO.

#### Cytotoxicity assay

HeLa cells were seeded in a 96 wells tissue culture plate at a concentration of 5x10<sup>4</sup> cells /well. After overnight cell culture, tissue culture medium was discarded and the extraction solutions, positive control and negative control were added. The incubation was performed for 24-68 hours at 37°C. The cell viability was determined with MTT-assay.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed using the cell proliferation kit I MTT according to the manufacturer's protocol. After 24 h of incubation with test compounds, 100 µl of medium were removed from each well, then 25 µl of MTT solution was added into each well and plates incubated 4 hours at 28°C in a CO<sub>2</sub> incubator. After incubation process was complete and formazan crystals has been formed, the solution tests are discarded and 2% DMSO was added. Then the absorbance of the formazan was measured at 595 nm with 680 nm as reference

wavelength using an ELISA microplate reader (15).

For data evaluation, the viability percentage of HeLa cells are calculated and the  $IC_{50}$  value is determined using GraphPad Prism 6 software for windows, version 6.03, For data evaluation, background values from wells without cells were subtracted and average values for the triplicates calculated and expressed as % cytotoxicity referring to the untreated control containing only the solvent DMSO. Cytotoxicity was then calculated according to the following equation:

$$\text{Cytotoxicity (\%)} = \frac{(\text{experimental value} - \text{DMSO control})}{(\text{positive control} - \text{DMSO control})} \times 100$$

## Result and Discussion

### Samples Preparation

Red fruits which are used in this study were obtained from high land in Klamono, Papua. The red fruit powder from previous study was used for this study. The powder of 40/50 mesh after DIC pre-drying and after conventional drying from previous research are used for extraction. For the red fruit with optimum parameter of DIC pre-drying based on flavonoid and total phenol content as response (2.5 bar, 15 seconds, and 4 cycle), the optimum extraction condition is 60% ethanol (1:10), 30°C, for 1 hour (11), and for red fruit dried by conventional drying was extracted with the same condition as well. The powder of 40/50 mesh is used because if the particle size is reduced to increases the surface area of the red fruit particles for the extraction process later, thereby increasing the rate of extraction. Increasing the surface area of the particles is to increase the contact area of the particles with the specific solvent called *menstrum*.

The advantage of using the kinetic maceration is to achieve the equilibrium concentration faster in the help of the stirring motor and also this method is the best suitable for use in case of the thermolabile drugs (16). The Cab-O-Sil (1/10 powder) is added to thicken the extract, and to adsorb the remaining water in thick extract. The bulk (extract of red fruit and Cab-O-Sil) was dried in an oven at 50°C until constant weight. Drying is

aimed to prevent damage caused by microorganism, especially fungus. The chosen temperature was 50°C, it is intended to prevent damage of the heat sensitive components.

For the red fruit with optimum parameter of DIC pre-drying based on  $\alpha$ -tocopherol content as response (1.5 bar, 15 seconds, and 2 cycle), the optimum extraction condition is n-hexane (1:25), 45°C, for 1.5 hours (11). And for red fruit dried by conventional drying was extracted with the same condition as well.

### Harvesting And Culturing Hela Cells

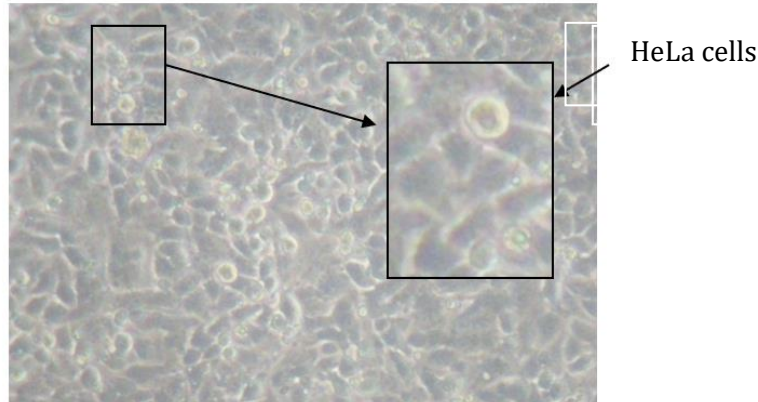
The trypsin is added in HeLa cells after thawing. This is done to break the bonds between the cells of HeLa cells to become single cell.

The result of harvesting is a suspension form of single HeLa cells. The purpose of breaking the bonds of HeLa cells is to make it possible to be cultured in the 96-wells plate, and the purpose of overnight incubation process is to make the HeLa cells 90-100 % confluence.

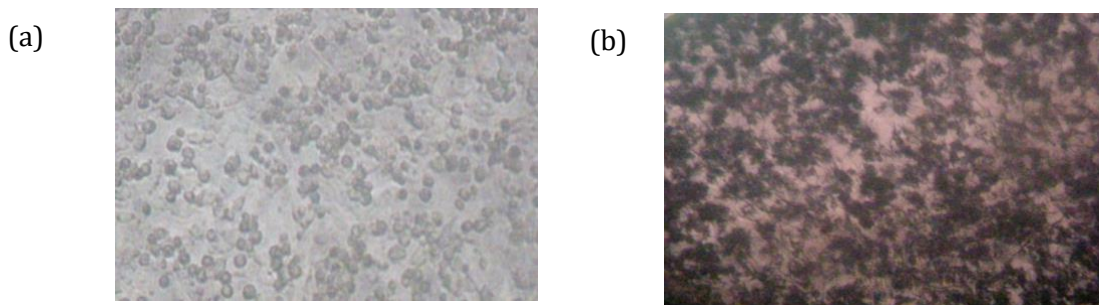
HeLa cells must be 90-100 % confluence for the purpose of a strong bond between the cells and indicates that the cells which is used is in healthy condition and ready for the next test. If the growth of HeLa cells is not 90-100% confluence, the binding of cells is not strong and do not have enough nutrition and the HeLa cells will die. **Figure 1** showed the HeLa cells after 90% confluence, the bonds between cells are dense, the cells attached to the base plate and the cells shape is monolayer. It indicates that cells which is cultured is in healthy condition and ready for testing.

### Treatment Of Red Fruit Extract on Hela Cells and MTT Assay

Cytotoxicity assay of the red fruit extracts on HeLa cells culture using MTT assay is started with the determination of concentrations of all extracts which can inhibit 50% HeLa cells proliferation in the population or  $IC_{50}$ . In this experiment, there are two negative controls which are used. The control solution for ethanolic extract is cab-o-sil diluted in DMSO 1%, and for hexane extract, a combination solvent of DMSO and ethanol (60:40) is used.



**Figure 1.** HeLa cells (ATCC@catalog No. CCL-2TM) (magnification 40x10)



**Figure 2.** The HeLa cells after (a) the addition hexane extract of red fruit (by conventional drying) at the concentration of 4,275 ppm, (b) after the addition of the red fruit extract and MTT (3 - (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent and incubated for 4 hours (microscopic, magnification 40x10)

As the positive control, *doxorubicin* (30  $\mu\text{g}/\text{mL}$ ) is used. For the hexane extract, the mixture of DMSO 60 % and 40 % Ethanol is used because the solvent can dissolve hexane extract and proven to be safe for cells. For the ethanolic extract, DMSO 1% is used because DMSO can dissolve the extract and proven to be safe for cells (17).

HeLa cells culture with the addition of red fruit extract is incubated for 24 hours. Then, MTT is added to the cells culture and incubated for 4 hours. Next, DMSO is introduced. After DMSO was added, formazan crystals which were formed was dissolved, so the color of formazan crystal was changed and became purple. The higher the concentration of the red fruit extract, the lower formazan crystals is formed. It indicates that the extracts have cytotoxicity to inhibit the HeLa cells proliferation. The ability of the cells to reduce tetrazolium (MTT (3 - (4,5-dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide)) showed the integrity of mitochondria and its activities can be interpreted as a measurement of the number of cells, cells proliferation, cells viability, and cell

ability to survive (15). The HeLa cells after the addition of the red fruit extract and incubated for 24 hours was observed by microscope, and was shown in **Figure 2a**.

**Figure 2a** showed the characteristics of HeLa cells which are different from the normal HeLa cells (ATCC@Catalog No. CCL-2TM) culture, i.e. the cells shape is circle, the cells is not attached to the surface of a 96-wells plate base, and the bond between cells is estranged. It showed the decrease of cell viability after the addition of red fruit extract, the cells like changing into the final phase of the stationary phase and did not have the ability to adhere on the base in 96-well plate.

The HeLa cells after the addition of the red fruit extract and MTT (3 - (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent and incubated for 4 hour was observed by microscope as shown in **Figure 2b**. The shape of formazan crystals look like needles and fine fibers. The crystals will dissolve in the addition of DMSO, so the color change into purple, the absorbance of formazan can be detected by ELISA

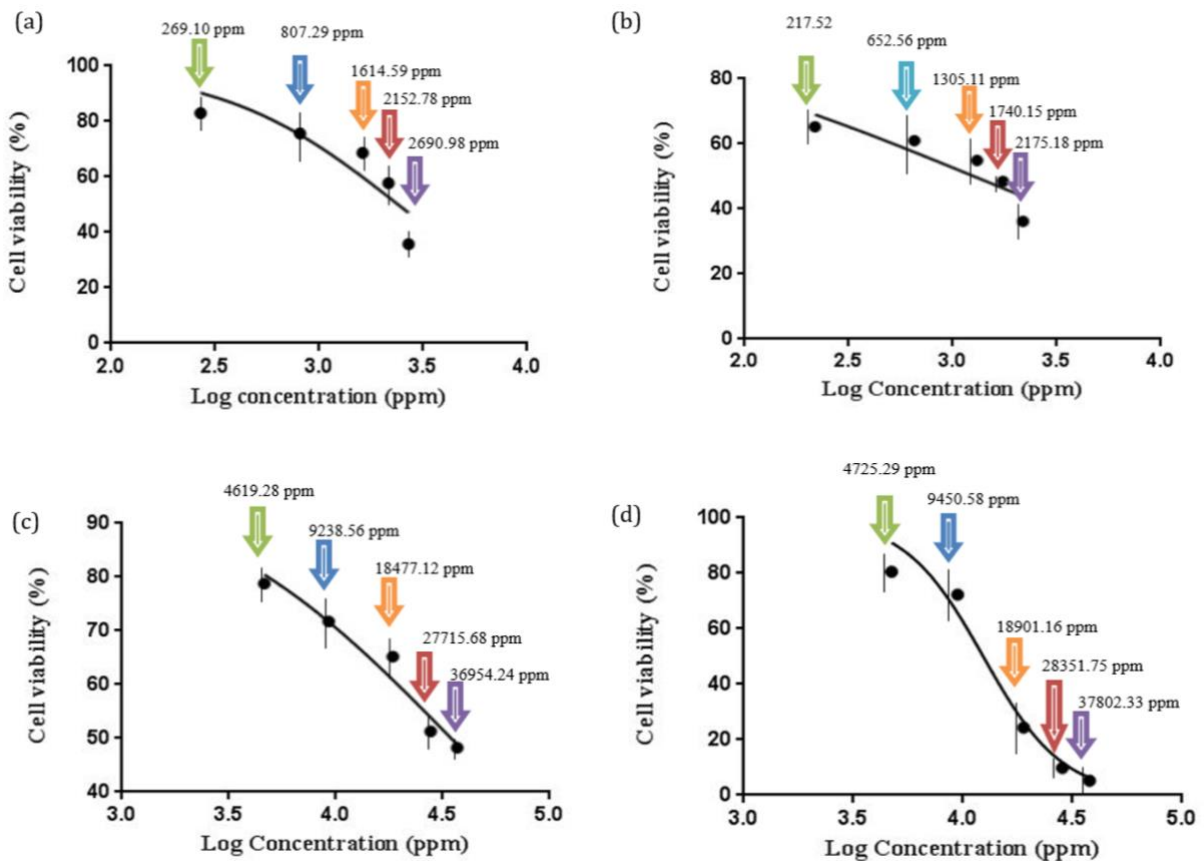
microplate reader. The maximum wavelength of formazan crystal is 595 nm. The result of absorbance which was obtained was compared to the absorbance of the negative control. The result obtained showed the percentage of cell viability.

**Figure 3** shows that percentage of inhibitory activity of HeLa cells increased with the increasing of the concentrations of ethanolic extracts and hexane extracts from red fruit (by DIC pre-drying and by conventional drying). At the higher concentrations, it is possible that the cytotoxicity will increase, decrease or remain stable.

The observation of the inhibitory cells proliferation with the ELISA microplate reader is resulting an optical density (OD) at  $\lambda$  595 nm, then the data is converted to cells viability percentage and the value of the data is further analyzed in a

logarithmic curve with the software GraphPad Prism 6 for windows, version 6:03.

The inhibitory activity percentage of HeLa cells of ethanolic extract of red fruit, by DIC pre-drying and by conventional drying can be seen **Figure 3a** and **Figure 3b**. The result of the  $IC_{50}$  value of the ethanolic extract of red fruit (by DIC pre-drying) is 2,397.5 ppm, while  $IC_{50}$  value of the ethanolic extract of red fruit (by conventional drying) is 1,270 ppm. **Figure 3c** and **Figure 3d** show inhibitory activity percentage of HeLa cells of hexane extract of red fruit, by DIC pre-drying and by conventional drying. The result of the  $IC_{50}$  value of the hexane extract of red fruit (by DIC pre-drying) is 34,567.25 ppm, while  $IC_{50}$  value of the hexane extract of red fruit (by conventional drying) is 12,366.75 ppm.



**Figure 3.** The percentage of viability of HeLa cells in various concentrations of (a) ethanolic extract of red fruit (by DIC pre-drying), (b) ethanolic extract of red fruit (by conventional drying), (c) hexane extract of red fruit (by DIC pre-drying), (d) hexane extract of red fruit (by conventional drying)

IC<sub>50</sub> value is a parameter which is used to determine cytotoxicity test. IC<sub>50</sub> value indicates that the concentration is capable to inhibit cell proliferation as many as 50% and it indicates the potential toxicity to the cells. The higher IC<sub>50</sub> value indicates that the compound is less toxic (18).

The cell viability percentage after the addition of positive control (*doxorubicin* 30 ppm) is 28.7449 %. It indicates that the concentration is lower than the concentration of the red fruit extract, so that *doxorubicin* is able to provide a higher cytotoxic effect against HeLa cells.

The difference of this study with another research (6) is the extract and treatment of the extract that are used. It used the methanol extract of red fruit and partitioned with hexane and ethyl acetate, but used the same cancer cell (HeLa cells) as a model for toxicity assay of red fruit.

In the previous research the cancer cells used as a model for toxicity assay is myeloma cells (2020), and comparing the difference of incubation time (20).

All of the extracts of red fruit can not be considered as a potent cytotoxicity against HeLa compared to *doxorubicin*, because all of the IC<sub>50</sub> value of extracts have lower cytotoxicity than *doxorubicin* against HeLa cells. This condition may be due to the high percentage of Cab-O-Sil in the bulk, which is causing the active substances that act as cytotoxicity in the extracts can not dissolved completely because the active substances in the extracts are bound to a high percentage of Cab-O-Sil. So, for the further study, the percentage of cab-o-sil in bulk is needed to be reduced.

Besides, this may be due to the kinetic maceration extraction method which can not extract all the active substances optimally. A further study is needed to search for another method of extraction, for example kinetic re-maceration.

The solvents which were used to pull the active substance out of the red fruit extract are 60% ethanol and hexane. Ethanol is a protic polar solvent because its dielectric constant of 30. While hexane is a non-polar solvent due to its dielectric constant of 2.0. It shows that the higher constant dielectric is, the higher the polarity of

solvent. Because this research only used ethanol as a protic polar solvent and hexane as a non-polar solvent, so a further study is needed to search for solvents which are capable to extract more of the active substances of red fruit. Probably an aprotic polar solvent can be used to dissolve not only polar, but also non polar active substances.

The example of active substances that contained in the ethanolic extract of red fruit extract is flavonoid. The examples of flavonoid which was having cytotoxicity on cancer cells are quercetin and rutin (21). The example of active substances which was contained in the hexane extract of red fruit extract are  $\alpha$ -tocopherol, and  $\beta$ -carotene (1). The concentrations of active compounds in the extracts are not high, and it also influence its biological activities.

## Conclusion

Based on the results, the red fruit extract, which previously dried by conventional drying method has better cytotoxicity against HeLa cells than red fruit extract which previously dried by DIC pre-drying method). The cytotoxicity of extract of red fruit against HeLa cells, are as follows: ethanolic extract of red fruit which previously dried by conventional drying method) > ethanolic extract of red fruit which previously dried by DIC pre-drying method > hexane extract of red fruit which previously dried by conventional drying method > hexane extract of red fruit which previously dried by DIC pre-drying method.

Some suggestions are offered for further research i.e. using other solvents such as aprotic polar solvent to get the optimal active substances in extraction process, optimizing the extraction process to get optimal cytotoxic substances in the extract, and reducing the percentage of the cab-o-sil in the bulk to concentrate the extracts.

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