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**Research Paper**

# IN-VITRO Activity of Roselle Petal Ethanol Extract (*Hibiscus sabdariffa* L.) On Viability Of 264,7 RAW CELL

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***ABSTRACT:*** *Rosella (Hibiscus sabdariffa L.) is one of the herbal plants that contains many natural phytochemical compounds that has various benefits to the immune system. Rosella is widely used by the public as an herbal drink to maintain the immune system. A viable cell count is essential to evaluate the kinetics of cell growth and the activity of the test sample. This study aims to determine whether rosella petal extract has any effect on viability in RAW 264.7 cells by in-vitro. In this study, ethanol extract of rosella petals was tested for its activities on cell viability at concentrations of 0.1; 1; 10; 25; 50; 75; and 100 µg/mL using the microtetrazolium (MTT) method. The results showed that rosella petal extract at concentrations of 0.1; 1; 10; 25; and 50 µg/mL had a viability value of >90%. These results indicate that rosella petal extract did not affect RAW 264.7 cell viability and safe to consume.*

***Keywords:*** *Hibiscus sabdariffa L, immune system, viability, RAW 264.7 cells*

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## I. INTRODUCTION

Current lifestyle trends in Indonesia show a shift towards "back to nature," especially when it comes to maintaining a healthy body. Many people today tend to prefer traditional medicine over modern medicine because it is considered safer. This is due to the perception that traditional medicine has fewer side effects than modern medicine. For many years, Indonesian people have had knowledge about medicinal plants that have been passed down from generation to generation, and they utilize them as a way to overcome health problems [1].

Traditional medicines are natural mixtures or components that can be parts of plants, animals, mineral substances, tested formulations (galenics), or a combination of these elements. The use of these traditional medicines has been going on for generations for medicinal purposes, and one example is Jamu [2].

Rosella flowers (Hibiscus sabdariffa L.) have traditional medicinal uses and can be applied in food and beverage products. Traditionally, people have consumed rosella petals by brewing them in the form of tea or beverages. Rosella flowers are widely recognized for their benefits as antioxidants that can fight free radicals [3]. In Indonesia, people generally utilize rosella petals as a health drink that is believed to boost the immune system. Research conducted by Mahadevan et al. in 2009 showed that rosella petals contain various compounds such as terpenoids, phenolics, and flavonoids, including anthocyanins [4].

One of the cells that play a role in the body's immune system is macrophage cells. Macrophages are white blood cells that perform the function of ingesting and digesting pathogens such as microbes, foreign substances, and cancer cells. The number of macrophage cells can be used as an indication of the activity of certain compounds, this is seen from the number of living cells. If there are few living cells, it means that the compound is toxic. However, if the administration of a compound does not affect the living cells, then the compound is not toxic.

Cell-based assays are often used to test a number of compounds with the aim of assessing whether these compounds affect cell development or produce direct cytotoxic effects and ultimately lead to cell death. There are several methods that can be used to detect the number of viable cells, including tetrazolium reduction (MTT), resazurin reduction and ATP (Adenosine triphosphate) detection [5]. The MTT assay is used to measure cell metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This viability assay is based on the reduction of the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT to purple formazan crystals by metabolically active cells [6].

Cell viability calculation is essential for studying cells for different purposes such as cell culture management in biological research, cell population titration in diagnostics and control in cell culture processes [7]. Among the well-known and developed cell counting methods so far, manual counting with a hemocytometer has been the most commonly used method due to its low cost and high flexibility [8].

Cultured cells, also known as cell lines, refer to cells that are capable of proliferating in culture media outside the body (in vitro). Cultured cells can be derived from native tissue or can be propagated from existing cells. In in vitro level research, cultured cells are often used, including in studies involving the testing of new drug compounds or extracts. In addition, cellular-level research also relies on the use of cultured cells, and these cultured cells are also sometimes referred to as continuous cell lines [9].

## II. MATERIAL AND METHODS

3.2.1 Tools

The tools used included measuring cups (Herma), funnel (Duran), Erlenmeyer flask (Pyrex), rotary flask (Pyrex), analytical balance (Shimadzu AUX 220, Japan), rotary evaporator (Buchi), 100 and 500 ml infusion bottles, filter paper, spatel, chromatography vessel, Ultraviolet Analyzer, spray bottle, micro pipette (Ecopipette), refrigerator (Samsung), water bath (Memert), conical tube (Falcon), inverted microscope (Zeiss), eppendorf tubes, hemasitometer, 96 and 24 well plates, centrifuge (Thermo Scientific), T-25 flask (Iwaki), 37oC 5% CO2 incubator (Thermo Scientific), microbiological safety cabinet air flow class II (Thermo Scientific), microplate reader (Bio-Rad), tissues, plastic bags, and stationery.

3.2.2 Materials

The materials used for this research include rosella flower petals (Hibiscus sabdariffa L.), ethanol (70% alcohol), ethanol (70% alcohol), and ethanol (70% alcohol). ), ethanol (70% alcohol), distilled water, RAW 264.7 cells (ECACC), Dulbecco' Modified Eagle Medium (DMEM) (Gibco), Fetal Bovine Serum (FBS) (Gibco), Dimethyl sulfoxide (DMSO), penicillin-streptomycin 2% (v/v) (Gibco), trypsin-EDTA 0.25% (Gibco), trypan blue (Gibco), 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetra zolium bromide (MTT) (Sigma), Phosphate Buffer Saline (PBS), Lipopolysaccharide (LPS) (Sigma)

***Sampling***

*Rosella flower petals used in this study were taken in Bari Village, Padang Pariaman District, West Sumatra. Samples were taken when rosella had grown for 3 months and was ready for harvest.*

***Sample Processing***

*Rosella flower petals that have been harvested, then selected and cleaned from dirt and insects that stick. After that the sample is washed using running water and drained. Samples that have been clean are then separated from the seeds one by one. Samples are dried in the sun in a place protected from direct sun exposure. After obtaining dry simplisia, sorting the dried petals of good flowers is carried out. After that, knit with a size of approximately 1-2 cm and store the sample in a closed container.*

**Sample Identification**

*The identification of rosella flower petals was carried out at the ANDA Herbarium, Department of Biology, Faculty of FMIPA, Andalas University, Padang, West Sumatra.*

**Sample Extraction**

*Rosella flower petals that have been knitted then weighed and obtained the results of 265.7 g. Then the extraction is carried out using the maceration method. Then the extraction was carried out using the maceration method with 70% ethanol solvent as much as 1500 mL for 3 days. After the maceration process is complete, the extract is filtered using filter paper and evaporated using a rotary evaporator until a thick extract is obtained.Kultur Sel*

***Cell Culture***

**Preparation of DMEM Complete Medium (MK DMEM)**

DMEM medium + 1% penicillin-streptomycin + 10% fetal bovine serum (FBS), then filtered using a 0.2 µm filter.

**Cell Preparation**

The cells used are RAW 264.7 cells available at the Faculty of Pharmacy, Andalas University. Cells are removed from the nitrogen tank, then thawed using a water bath with a temperature of 37 oC for 1 minute while shaking. After the cells melt, then transfer the cells into a conical tube containing 5 mL MK DMEM and centrifuge for 10

minutes at 2000 rpm. The results of the centrifuge will form a pellet at the bottom of the tube, discard the supernatant carefully by pipetting so that the pellet is not wasted. Put 2 mL of MK DMEM into the conical tube by passing it through the tube wall. Transfer to a T-25 flask, add 2 mL of MK DMEM and incubate in a 5% CO2 incubator at 37 oC. The growth medium can be changed once every two days, and when the number of cells in the T-25 flask has reached 70-90%, cell sub-culture can be done [10].

**Cell counting**

Pipette 10 µL of cell suspension and add 10 µL of trypan blue, place on the edge of the counting box of the haemacytometer, and perform counting under a microscope. Hemasitometer consists of 4 chambers, each chamber consists of 16 boxes. Perform cell counting in all 4 chambers of the haemacytometer, cells that are blue (dead) and cells that are at the outer boundary are not counted. Calculate the total number of cells/mL using the formula: Cells/mL = average number per chamber x dilution factor x 104 [11].

**Cell Viability Test**

a. Plating of Test Solution

Put 20 µL of the prepared test solution into each test well on a 96-well plate that has been filled with cell suspension. The control well contains 180 µL of cell suspension, and the blank contains 200 µL of medium. After that, incubate the plate for 48 hours in a 5% CO2 incubator at 37 oC [10].

b. MTT Solution Plating

The medium that has been used is discarded and wash each well using 100 µL of PBS solution. Pipette 100 µL of 0.5 mg/mL MTT solution for each well, incubate for 4-6 hours in a 5% CO2 incubator at 37 oC. After 4-6 hours a purple precipitate of formazan crystals will form, discard the supernatant. After that, the purple crystal formazan precipitate is dissolved with 100 µL of DMSO in each well, and measure the absorbance using a microplate spectrophotometer with a wavelength of 550 nm [10].

Data Analysis

From the data obtained from the calculation of percent viability, the relationship between the concentration of the test solution and cell viability is displayed in a graph using Microsoft excel.

## III. RESULTS AND DISCUSSION

Cell culture work is carried out in an aseptic work area which aims to prevent external contamination. Before working the tools and materials to be used are sterilized using an oven and also an autoclave. For the process of research work carried out in the biological safety cabinet air flow class II. Tools and materials to be used are placed in a biosafety cabinet in an aseptic state by spraying 70% ethanol. Biological safety cabinet class II provides the aseptic environment required for the cell culture process, and for testing potentially hazardous materials [12]

The cells used were RAW 264.7 cells from the United Kingdom (UK) under the brand name European Collection of Authenticated Cell Cultures (ECACC). RAW 264.7 cells are monocyte or macrophage-like cells derived from the transformation of ableson leukemia cells from BALB/c mice. The reason for using these cells is because RAW 264.7 cells are commonly used in in vitro testing as a model of macrophage cells, and can present the results of the immune response [13]

The first step in the process is to implant the cells in the medium. It takes a medium that is similar to the biological fluid of the cells when the cells are in the body of the original organism so that the cells can grow. In this study, the medium used for RAW 264.7 cells is Dubleco's Modified Eagle Medium (DMEM) which is considered ideal for RAW 264.7 cell growth. DMEM medium was added with 1% penicillin-streptomycin antibiotics and 10% fetal bovine serum (FBS), so this medium is called DMEM complete medium (MK DMEM). This addition is done because DMEM alone is considered insufficient to meet the nutritional needs of cells during the culture period. FBS functions as a provider of essential nutrients, hormones and growth factors, protein binding, and protection, while penicillin-streptomycin antibiotics function as antimicrobials [14]. DMEM medium contains four times more vitamins and amino acids, and two to four times more glucose than the original formula [15].

After the cells were put into the medium, a centrifugation process was carried out to separate the cells from the medium. This centrifugation is done at 2000 rpm for 10 minutes. The basic principle of centrifugation is that the object will rotate horizontally at a certain distance. When an object rotates inside a tube or cylinder containing a mixture, it will tend to move towards the center of rotation. However, this is not the case due to the presence of a force in the opposite direction towards the outer wall of the tube, known as centrifugal force. This force causes the particles in the mixture to move towards the wall of the tube and eventually settle, forming a precipitate [16]. After that, the supernatant was discarded and MK DMEM was added and transferred into a T-25 flask.

Furthermore, cells are incubated in a 5% CO2 incubator with a temperature of 37 oC because this temperature is the optimal temperature for cells and CO2 functions so that cells are able to convert digest food

from the medium into energy so that cells can grow properly and also as a buffer for the pH of the medium. pH has an important role in the cell culture process, the pH of the medium must be kept stable, because during incubation the pH can change, this change is due to the results of cell metabolism in the form of lactic acid which will cause a decrease in the amount of cell growth. The normal pH of RAW cells cultured from primary cells is 7.4. Incubator temperatures that are too hot will cause cell damage, and usually the temperature in the incubator is set slightly lower than the optimal temperature to prevent cell damage [12]

After incubation for 24 hours, observation of cells that have grown using an inverted microscope is carried out, if the cells are 70-85% confluent in the flask, the cells are ready to be harvested. The optimal number of cells used for testing is 85%. This can be seen from the color change in the cell medium from red to yellow. After cell growth is optimal, the cell harvesting process can be done using a scraper that serves to release cells from the flask. After that, centrifugation is carried out again to get pure cells which will later be used for this research process.

Before the cells were used, cell counting was done using a haemacytometer observed under an inverted microscope with 4x magnification. Hemasitometer has 2 parts, namely top and bottom, where in each part there are 4 rooms, and each room consists of 16 boxes. Cell counting is done so that the amount of cell suspension that is put into each well is as needed. The calculation is done by mixing the cell suspension with trypan blue and then calculated using a hemasitometer box. Trypan blue is a diazo dye that is widely used to color dead tissue or cells. Dead cells will appear blue when viewed using a microscope, this is because there is damage to the cell membrane in dead cells, so that trypan blue is able to enter the cell [17]. While cells that are still alive will look more luminous and round in shape. Cell calculation is done by calculating the average living cells multiplied by a correction factor of 104 [14].

From the calculation results, the number of cells in replication 1 is 605,000 cells/mL, replication 2 is 1,550,000 cells/mL, and replication 3 is 4,500,000 cells/mL. Cell calculations can be seen in appendix 3. The cell suspension needed for the viability test is 10,000 cells for each well. The number of cells needed is adjusted to the well plate used and for MTT testing a 96 well plate is used.

The cell viability test was carried out with the microtetrazolium (MTT) method. The purpose of the MTT test is to measure living cells and to determine the cytotoxicity of several samples at different concentrations, which in this study is to see the effect of giving rosella petal extract on cell viability. The principle of the MTT test is that living cells have functioning mitochondria, mitochondrial activity is reflected in the conversion of tertrazolium salts into formazan crystals [18]. The % viability value of samples that are safe and non-toxic to cells has if the value is >90% [11].

The concentration variations of rosella petal extract used in the MTT test are 0.1; 1; 10; 25; 50; 75; and 100 µg/mL. The concentration variation was made to see at what concentration the cells had a viability value of >90%. Concentration was made by dissolving rosella petal extract with dimethylsulfoxide (DMSO). The reason for using DMSO is because DMSO is an organic solvent that can dissolve polar and non-polar compounds [19].

Cell viability observations were made quantitatively and qualitatively. In quantitative observations, the % cell viability value at each concentration was calculated with a total of 3 repetitions. The average value of percent cell viability obtained from each concentration. Based on table 4.5, it can be seen that at concentrations of 0.1; 1; 10; 25; and 50 µg/mL the % cell viability value is >90%, while at concentrations of 100 µg/mL and 75 µg/mL the average % cell viability value of RAW 264.7 is <90%. This can be caused by the presence of chemical compounds in the extract that are toxic to cells, causing cell death and resulting in a low percent viability.

Table 1. Data on Percent Viability of RAW 264.7 Cells treated with ethanol extract of rosella flower petals

| Concentration | Replication | | | Average | SD |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | | |
| 0,1 | 107,346 | 84,502 | 89,902 | 93,949 | 11,923 |
| 1 | 104,841 | 82,288 | 80,147 | 89,092 | 13,681 |
| 10 | 91,486 | 109,779 | 95,147 | 98,804 | 9,679 |
| 25 | 89,816 | 107,749 | 89,118 | 95,561 | 10,561 |
| 50 | 72,955 | 114,022 | 84,951 | 90,659 | 21,112 |
| 75 | 67,817 | 106,642 | 85,294 | 86,572 | 19,462 |
| 100 | 69,282 | 106,642 | 87,206 | 87,680 | 18,685 |

In qualitative observation, color change is induced by the formation of purple formazan crystals that occur as a result of the reaction between MTT salts and the succinate tetrazolium reductase system present in the mitochondria of living cells [17]. The purple color that appears is an indication of the number of active living cells. A lower sample concentration will produce a more intense purple color, and conversely, the higher the sample concentration, the more intense the purple color will be. In other words, the intensity level of the purple color reflects the number of living cells [19].

From the data obtained from the cell viability test results, it can be concluded that the ethanol extract of rosella flower petals (Hibiscus sabdariffa L.) at concentrations of 0.1; 1; 10; 25; and 50 µg/mL has the highest

percentage of cell viability where the extract does not show toxicity to RAW 264.7 cells, namely cell viability is at> 90%.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

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