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VIABILITY STUDY OF TAPAK LIMAN (*Elephantopus scaber* Linn) LEAVES TOWARD RAW 264.7 CELLS

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ABSTRACT

Tapak liman (*Elephantopus scaber* Linn) is a plant that is easily found in Indonesia which is traditionally used for various treatments. The chemical constituents of the *E. scaber* plant are sesquiterpenes, triterpenoids, steroids, flavonoids, tannins, and saponins. The aim of the stydy is to determine the safety of using this plant, by looking the effect of this plant's leaf extract on cell growth by carrying out a viability study. A viability study was carried out on raw 264.7 cells. This study was design as laboratory experiment. *E.scaber* was extracted using cold maceration method with ethanol, then ethanol was evaporated with rotary evaporator. Before cell viability study, cells were cultivated in 96 well plates and providing ethanol extract from *E. scaber* leaves at concentrations of 500, 100, 50, 25, 12.5, 6.25, and 1 µg/ml. Then cells were cultured at 37ºC with 5% CO_2 for 48 hours. After 48 hours the cells added with 100 µl of MTT solution with a concentration of 0.5 mg/ml in PBS for about 4 hours. The resulting crystals of formazan then dissolved in 100 µl DMSO, and their absorbance was determined using a microplate reader. Cell viability was observed in the ethanol extract of *E. scaber* leaves, each of them at concentrations of 500, 100, 50, 25, 12.5, 6.25, and 1 µg/ml were 15.35%, 94.29%, 115.30%, 121.36%, 120.23%, 111.74%, 102.29%. From the study results it can be seen that the cell viability of *E.scaber* ethanol extract for concentrations 1-100 µg/ml gives a cell viability percentage above 80%. From this study, it can be concluded that *E. scaber* leaf extract was not harmful to raw cells and and shows potential for further study into plant's medicinal properties because the extract able to maintain cell viability above 80% at concentrations ranging from 1 to 100 µg/ml.

Keywords: *elephantopus scaber, MTT assay, raw 264.7 cells, tapak liman.*

INTRODUCTION

COVID-19 or Coronavirus disease 2019 is a new kind of pneumonia that caused by the SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2). This illness has spread rapidly throughout the world begin in December 2019 from Wuhan, China (1). It was recorded that in May 2023 more than 765,903,278 people were verified to be diagnosed with the SARS-CoV-2 virus and this virus has also caused 6,927,378 deaths worldwide (2). After entering the body, the SARS-CoV-2 virus will infect the lower respiratory tract, which then causes symptoms in patients. Naturally, the body has the ability to protect itself from virus attacks, namely the immune system. Immunostimulants can improve immune system of the body (3). Recently,

herbs or traditional medicine tend to be preferred use to treat various ailments and problems such as to boost the immune system because it does not produce any side effect (4). In clinical trial, synthetic immunostimulant produced several side effect such as vomiting or nausea, headache, transient liver enzyme rises, arthralgia, skin rash, tachycardia, bone marrow damage, agranulocytosis, thrombocytopenic purpura (5), (6).

Based on research, some plants having immunomostimulant activity. One of them are often used as a traditional medicine is *Elephantopus scaber* Linn or called tapak liman in Indonesian, plant from the Asteraceae family (7). *E. scaber* widely available in Indonesia (8). It contains chemical substances as an example isodeoxyelephantopin, deoxyelephantopin, isoscabertopin and scabertopin have been found to be potent anticancer activity. There are numerous other biological activities have been reported in various research papers such as antioxidant, antimicrobial, antidiabetic, hepatoprotective, analgesic, antiasthamatic, anti-inflammatory, wound healing and antiplatelet (9). The latest research show that the *E. scaber* acts as an immunostimulant, boosting the the immune ability to protect the body against pathogenic microbes such as viruses, fungi, bacteria and protozoa. Accordingly, *E. scaber* leaves may serve as an alternate source of medication because they are readily available, easy to process and cheap (7). The use of *E. scaber* leaves as an immunostimulant needs to be proven up by scientific evidence. The cell viability is an initially necessary stage to determine the safety using plant by looking the effect of this plant leaves extract on cell growth by carrying out a viability study. A viability study was carried out on raw 264.7 cells. After this viability study, further testing regarding immunostimulant activity can be conducted (10). These data provide a perspective for further research, which will contribute to further understanding of the non-toxic dose or maximum dose that can be administered for advanced activity assays while maintaining cell viability of at least 80% (11).

MATERIALS AND METHODS

Raw 264.7 cells was obtained from The European Collection of Authenticated Cell Cultures (ECACC) United Kingdom, Penicillin-Streptomycin, DMEM (Dulbeccos's Modified Eagle Medium) and FBS (Fetal Bovine Serum) were obtained from Gibco (United State), Dimethyl sulfoxide (DMSO) was obtained from Vivantis (United State) and 3-(4,5-dimethylhiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (United State). Fresh *E. scaber* leaves were collected from a single location at Balai Kurai Taji in Pariaman, West Sumatra, Indonesia. Which had been identified in Herbarium of University Andalas (ANDA). The leaves were washed, cut into little pieces that were between 3-5 mm thick, and dried by air at room temperature. The leaves that were dried were crushed into a powder using a blender. *E. scaber* leaves extracted by cold maceration in distilled ethanol for three days with intermittent shaking. The macerate was evaporated using a rotary evaporator until formed a thick extract.

Raw 264.7 cells in DMEM culture media supplemented with 10% FBS and 1% pen/strep (penicillin-streptomycin) were cultured for 24 hours on 96 well plates at 37°C, 5% CO_2. Ethanol extract of *E. scaber* at the concentrations 500, 100, 50, 25, 12.5, 6.25 and 1 µg/ml add into raw 264.7 cells and incubated it approximately 48 hours at 37ºC with 5% CO_2. After a 48 hour incubation, the medium was taken out and washed with PBS. As much as 100 µl solution MTT reagent at a concentration 0.5 mg/ml was added, and then incubated it for 4 hours at 37°C with 5% CO_2. After the MTT solution was thrown away, the formazan crystals that had formed were dissolved in 100 µl DMSO. The absorbance of purple formazan crystals was measure using a microplate reader with a 550 nm wavelength. Utilizing the formula below, one may determine the viability test (12),(13):

$$cell\ viability = \frac{cell\ absorbance\ with\ treatment - cell\ absorbance\ controls}{cell\ absorbance\ DMSO\ (solvent) - cell\ absorbance\ controls} x\ 100\%$$

The data was given as the mean ± standard deviation. SPSS 25.0 software (SPSS Inc., USA) was used for statistical analysis. The acquired data were analyzed using a one-way ANOVA test, subsequently analyzed using Duncan post hoc with a $p<0.01$ significance level.

RESULT AND DISCUSSION

The percentage of cell viability was determined from *E. scaber* leaves extract of ethanol, respectively at concentrations of 500, 100, 50, 25, 12.5, 6.25, and 1 µg/ml were 15.35%, 94.29%, 115.30%, 121.36%, 120.23%, 111.74%, 102.29%.

Table 1 : Mean persentage cell viability of *E. scaber* ethanol extract toward raw 264.7 cells

Concentration (µg/ml)	Mean ± standard deviation (%)
500	15.35% ± 1.73%
100	94.29% ± 1.73%
50	115.30% ± 3.66%
25	121.36 ± 2.22%
12.5	120.23% ± 0.20%
6.25	111.74% ± 0.52%
1	102.29% ± 2.25%

The outbreak of the new COVID-19 has become one of the huge challenges in the world. Medicinal plants have become one of the main focuses of researchers to obtain new compounds that can be used as an alternative to bring down the COVID-19 outbreak. Considering that there are still few immunostimulant compounds derived from natural ingredients and is frequently thought to have less side effects than synthetic medications (14), (15), (16). Before carrying out further tests on the effectiveness of a plant extract, it is necessary to test the activity of the extract on living cells, the aim of which is to determine an effective dose to produce a non-toxic effect on the cells. Cell viability refers to the ability of cells to remain alive and function properly. It is a crucial parameter in cell biology and biomedical research, as it indicates whether cells are healthy and capable of carrying out their normal physiological functions. Understanding cell viability is crucial in various research and medical contexts to ensure accurate experimental results and evaluate the impact of different treatments or conditions on cell health. Several studies on viability have been conducted. *Citrus* extract at ranging concentrations from 1 to 100 µg/mL provides cell viability above 100% (17). *Antirrhinum majus* extract at concentrations 50, 100, 150, 300, and 500 µg/ml showed cell viability above 90% up to the concentration of 300 µg/ml, revealing non cytotoxicity, whereas 500 µg/ml *Antirrhinum majus* extract showed cell viability below 90%. On the basis of these results, the 50, 100, 150, and 300 µg/ml *Antirrhinum majus* samples were selected for further experiments (18). *Curcuma longa* L extract and *Carthamus tinctorius* L extract at concentrations 10, 25, 50, 100 and 200 µg/ml showed cell viability above 100% (19). Other research on viability study, namely from water extracts of *Cistanche deserticola*, using concentrations of 15.625 to 1000 µg /ml obtained data that at all concentrations provide cell viability above 100%, so extract is safe to continue testing (20).

No previous studies have evaluated the effect of *E. scaber* ethanol extract on cell viability. In this research, we evaluated the viability effect of various concentrations of *E. scaber* ethanol extract using the MTT assay (21). This assay is a common procedure for determining the cytotoxicity of a substance, according to ISO10993: 2009. It determines cell viability (14). The percentage of living cells in a population is called cell viability. The MTT assay is one of the most widely used methods for assessing proliferation and cell viability, which uses a colorimeter as its basis. This is because it is inexpensive, has a high level of accuracy, and can be completed quickly (21). The basis of the MTT assay mechanism are depend on ability of nicotinamide adenine dinucleotide phosphate (NADPH) to convert the yellow, water-soluble tetrazolium dye (MTT) into purple, insoluble form of formazan. Thus, the enzymatic conversion of tetrazolium substances towards insoluble in water formazan crystals by dehydrogenases take place in the mitochondria of living cells is utilized for assessing cell viability, though reducing agents and enzymes found in other organelles, such as the endoplasmic reticulum, also play a role. The formazan product that is insoluble in the MTT test is dissolved by adding DMSO solution. At a certain wavelength, usually 500-600 nm, the absorption of this purple solution can be measured using a spectrophotometer (22).

In the MTT assay there is a linear connection between the color produced and cell metabolic activity so that accurate measurements can be made and cell viability values can be obtained. Cells that are healthy can easily produce formazan crystals, while inactive or dead cells cannot produce them. Inactive or dead cells lose the capability to convert tetrazolium crystals into purple colored formazan crystals (23). The intensity of the purple color obtained is directly proportional to the number of formazan crystals formed. The number of formazan crystals formed is directly proportional to the number of living cells. The higher intensity of the purple color formed, resulting the higher absorbance of cell. The higher absorbance of cell, correlation with the greater number of living cells in the culture (24). This study is the first in a series aiming at discovering active chemicals in *E. scaber* that can be used as immunostimulants. It is possible to determine the safety of medicinal plants to raw cells by assesing cell viability using the MTT assay. In line with ISO 10993-5 : 2009 for biological evaluation of medical devices part 5: tests for in vitro cytotoxicity, the percentage of cell survival is classified into above 80% as non cytotoxicity, 80-60% as weak cytotoxicity, 60-40% as moderate cytotoxicity and below than 40% as strong cytotoxicity (25). Cell viability percentange more than 80% may be used for further biological activity testing. Figure 1 presents the results of the cell viability test.

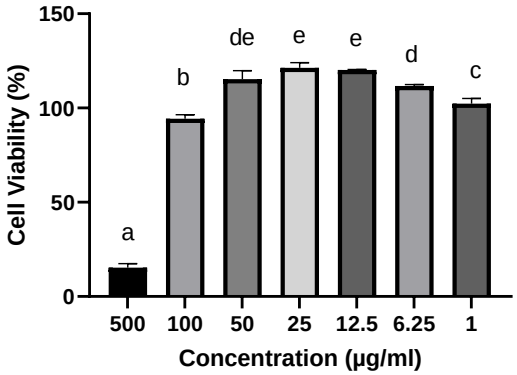

Figure 1 : Persentage cell viability of *E. scaber* ethanol extract

Evaluation of *E. scaber* ethanol extract on raw 264.7 cell viability at 500, 100, 50, 25, 12.5, 6.25, and 1 µg/ml. To determine the cytotoxicity of each concentration, three duplicates were employed. The data were presented in the form of mean ± standard deviation. Using ANOVA Duncan post hoc, different letters demonstrate significant differences in concentrations with $P<0.01$. In this study, cell viability tests were carried out using the MTT method. There were seven concentrations used at 500, 100, 50, 25, 12.5, 6.25 and 1 µg/ml. The *E. scaber* ethanol extract cell viability increases as concentration decreases. The cell viability of *E. scaber* ethanol extract at concentrations 500, 100, 50, 25, 12.5, 6.25, and 1 µg/ml were 15.35%, 94.29%, 115.30%, 121.36%, 120.23%, 111.74%, 102.29%. Based on research results and correlated with ISO 10993-5 : 2009, it was determined that *E. scaber* ethanol extract at a concentration of 500 µg/ml the viability value was obtained 15.35% and it is included in the strong cytotoxicity. Whereas at concentrations from 100 to 1 µg/ml the viability value was obtained above 80% and included in the non cytotoxicity.

CONCLUSION

From this study, it can be concluded that *E. scaber* leaf extract was not harmful to raw cells and can be used for further study because the extract able to maintain cell viability above 80% at concentrations ranging from 1 to 100 µg/ml.

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