Plumeriastuti *et al*. MKH (2022). 233-239
DOI: 10.20473/mkh.v33i3.2022.233-239

Cytotoxicity of Kembang Bulan (*Tithonia diversifolia*) Ethanolic Leaf Extract on Rabbit Limbal Mesenchymal Stem Cells

Hani Plumeriastuti[1*], Vida Seanita Zahro[2], Nusdianto Triakoso[3], Fedik Abdul Rantam[4], Iwan Sahrial Hamid[5], Mustofa Helmi Effendi[6], Annise Proboningrat[1]

[1]Division of Veterinary Pathology, Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga
[2]Bachelor of Veterinary Medicine Study Program, Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga
[3]Division of Veterinary Clinic, Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga
[4]Division of Veterinary Microbiology, Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga
[5]Division of Veterinary Basic Medicine, Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga
[6]Division of Veterinary Public Health, Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universitas Airlangga

*E-mail: hani-p@fkh.unair.ac.id

ABSTRACT

Kembang Bulan (*Tithonia diversifolia*) is known as an herbal plant that has many useful properties to cure disease, such as diabetes, malaria, and other infections. Instead of much usefulness, *T. diversifolia* has main biological properties that may induce toxicity in this plant known as sesquiterpene lactones. The aim of this study was to identify the cytotoxicity effect of *T. diversifolia* leaf extract on limbal mesenchymal stem cells of rabbits using the MTT assay. *T. diversifolia* leaf extract was divided into 4 concentrations (0.5%, 0.25%, 0.125%, and 0.0625%) and given to limbal mesenchymal stem cells of rabbits in 96-well microplates, which would be incubated for 24 hours. The cytotoxicity result was obtained using the MTT assay method. Every well would be observed to see the cell life level of each concentration. The optical density absorbance was calculated using a microplate reader. *T. diversifolia* leaf extract with a concentration of 0.0625% has the biggest viability with a level of existence of limbal mesenchymal stem cells of 50%, and the other *T. diversifolia* leaf extract concentrations of 0.5%, 0.25%, and 0.125% have less than 50% level of existence of limbal mesenchymal stem cells.

Keyword: LC50, live cell, Mexican sunflower, MTT assay

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Received: 29-06-2022, Accepted: 14-08-2022, Published online: 08-09-2022
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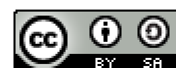

INTRODUCTION

The usage of traditional medicine is safer compared to synthetic medicine (Yuan et al., 2016). One of the most common traditional medicines was made from *Tithonia diversifolia* leaves, which belong to the family Asteraceae and are commonly used by people from South Asia to cure many kinds of illnesses (Ajao and Moteetee, 2017). In Indonesia, this plant is usually called "Daun Insulin" (Ammar et al., 2021). *T. diversifolia* can be used as medicine for diabetic, malaria, and many other infectious diseases (Di Giacomo et al., 2015).

T. diversifolia has the potential to be developed as a standardized herbal medicine that is safe for health purposes. The first step in drug development is the pre-clinical test, which is a toxicity test conducted both in vitro and in vivo (Fischer et al., 2020). A cytotoxicity test was conducted for evaluation and screening using culture in vitro. The advantages of this test include its simplicity, speed, sensitivity, and lack of the need for an animal experiment (Li et al., 2015).

The MTT (methyl thiazolyl diphenyl-tetrazolium) assay is an easy and low-cost technique commonly used to evaluate the cytotoxicity of many drug candidates. The idea behind this assay was that the amount of formazan crystal formation was proportional to the number of living cells, including their metabolic activities (Li et al., 2012).

The MTT assay employs 3 - (4,5-dime thylthiazol-2-yl) - 2,5 - diphenyltetrazoli um bromide via a redox reaction inside the cell. It is determined by the cell's ability to convert MTT salt into formazan, as indicated by the color changing from yellow to a blue-purple like form. The MTT assay works on the premise that only living cells have the ability to change the tetrazolium ring via mitochondrial activity. The result of this process can be examined spectrophotometrically using a microplate reader (Buranaamnuay, 2021; Proboningrat et al., 2021). The intensity of the purple color correlates with the number of living cells. The percentage of death cells is then calculated using the number of living cells (Proboningrat et al., 2021).

The standard for measuring toxicity is LC50 (lethal concentration at 50%). It depends on the incubation period (Zhang et al., 2007). A toxicity test on primary cells used lethal concentration (LC50) as the standard, where the sensitivity of the drug was measured according to the rising number of death cells after drug treatment compared to the decreasing number of life cells in the untreated control. While the cell line inhibitory concentration (IC50) is suitable to measure the toxicity of the drug (He et al., 2016).

Limbal mesenchymal stem cells have recently been investigated as a cell-based alternative therapy for treating degenerative diseases. The ability of stem cells to self-renew and

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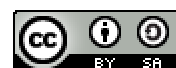

differentiate is intriguing for cytotoxicity assay applications. Stem cells are an excellent model for predicting toxicity because they can be used to assess cytotoxic effects on cell viability and the cell differentiation process. Besides, they may be more relevant for predicting human or animal toxicity. Furthermore, they are a well-established in vitro model for human or animal multipotent cell populations, they do not require as many supplements as NHK, and they are commercially available or can be self-isolated in our laboratory (Abud et al., 2015).

The aim of this study was to observe the effect of the serial concentration of *Tithonia diversifolia* leaf extract on rabbit limbal mesenchymal stem cells.

MATERIALS AND METHODS

Materials

Materials used in this study were *Tithonia diversifolia* leaves, ethanol 70%, aerosol carboxyl, sterilized aquadest, limbal mesenchymal stem cell isolated from rabbit, phosphate buffer saline (PBS) 10%, fetal bovine serum (FBS), 3-(4.5-dimethylthiazol-2-yl) 2.5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), modified minimum essential medium eagle-alpha (MEM-α), and trypsin EDTA.

Experimental Design

This study consists of two control groups and four treatment groups. The control groups were cell control (limbal mesenchymal stem cells cultured in MEM-α medium) and media control (MEM-α medium only). Treatment groups consisted of limbal mesenchymal stem cells treated with *T. diversifolia* leaf extract at concentrations of 0.5%, 0.25%, 0.125%, and 0.0625%.

Preparation of *Tithonia diversifolia* Leaf Extract

2.5 kg of *T. diversifolia* leaf powder was macerated in 25 L of 70% ethanol (60 °C, 3 hours). The liquid extract was then evaporated at 60 °C to leave 4.5 liters of liquid. Then, it was mixed with 58 grams of carboxyl aerosol for 5 minutes until it became homogeneous. The resulting thick extract was dried in an oven at 45 °C. Prior to the in vitro assay, the dried powder of *T. diversifolia* leaf extract was dissolved in MEM-α medium with concentrations of 0.5% (5,000 µg/mL), 0.25% (2,500 µg/mL), 0.125% (1,250 µg/mL), and 0.0625% (625 µg/mL).

MTT Assay

The cytotoxicity of *T. diversifolia* leaf extract was evaluated using 3-(4.5-dimethylthiazol-2-yl) 2.5-diphenyl tetrazolium bromide (MTT) assay. Rabbit limbal mesenchymal stem cells were cultured in 96 well microplate with density of 3-5×103 cells/well for 24 hours (CO2 5%, 37 °C). First, the medium from all wells was removed. Cells were then treated with four concentrations of *T. diversifolia* leaf extract (0.5%, 0.25%, 0.125%, and 0.0625%) for 24 h. The medium was removed, and the cells were incubated with 25 µL MTT (5 mg/mL) for 4 hours (37 °C). The cells were observed using an inverted microscope to see the presence of formazan crystals. Next, 50-

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100 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance of dissolved formazan was measured spectrophotometrically using a microplate reader with a wavelength of 595 nm. The darker the color, the higher the optical density (OD), which indicates the number of living cells. The percentage of live cells is calculated using the formula:

Live cells (%) = ((OD of treatment cells) + (OD of media control)) : ((OD of cell control) + (OD of media control)) × 100%

Data Analysis

The regression relationship between the concentration of *T. diversifolia* leaf extract and the percentage of viable limbal mesenchymal stem cells was analyzed using probit analysis to obtain a lethal concentration (LC50) value.

RESULTS AND DISCUSSION

The result showed that the rise in *Tithonia diversifolia* leaf extract concentration caused the death of limbal mesenchymal stem cells as well, due to the cell's inability to reduce MTT into formazon crystal. It is observed that the blue-purple form cannot be observed while the color of the medium containing phenol red is dominant (Figures 1b, 1c, and 1d). The positive control produced a different result. It can be observed that there was a transformation from MTT into formazon crystal marked by a blue-purple color with high density (Figure 1a). Most living cells were found at the lowest concentration of *T. diversifolia* leaf extract (Figure 1e).

A material that will be developed into a new drug candidate must pay attention to its toxic properties. The drug substance used must be non-toxic, non-irritating, have biocompatibility properties, and not have adverse effects on the biological environment, both locally and systemically (Meizarini, 2005).

According to Passoni et al. (2013), the toxic compounds in *T. diversifolia* leaf extract that are thought to be responsible for causing toxic effects are sesquiterpene lactones and chlorogenic acid derivatives, which cause damage to the liver and kidneys. Leaf extracts containing higher concentrations of chlorogenic acid derivatives were given to rats for 90 days and caused liver damage. Meanwhile, extracts rich in sesquiterpene lactones cause kidney damage. The reported safe dose of *T. diversifolia* leaf extract is less than 100 mg/kg body weight.

The mechanism of toxicity of *T. diversifolia* leaves related to sesquiterpene lactones includes alkylating and non-alkylating mechanisms. Alkylation toxicity by sesquiterpene lactones is known to induce ROS accumulation. Meanwhile, the non-alkylating mechanism of sesquiterpene lactones is known to inhibit a class of CA2+ transport enzymes, which causes cytosolic calcium levels to increase, thus leading to oxidative DNA damage (Amorim et al., 2013).

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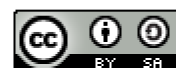

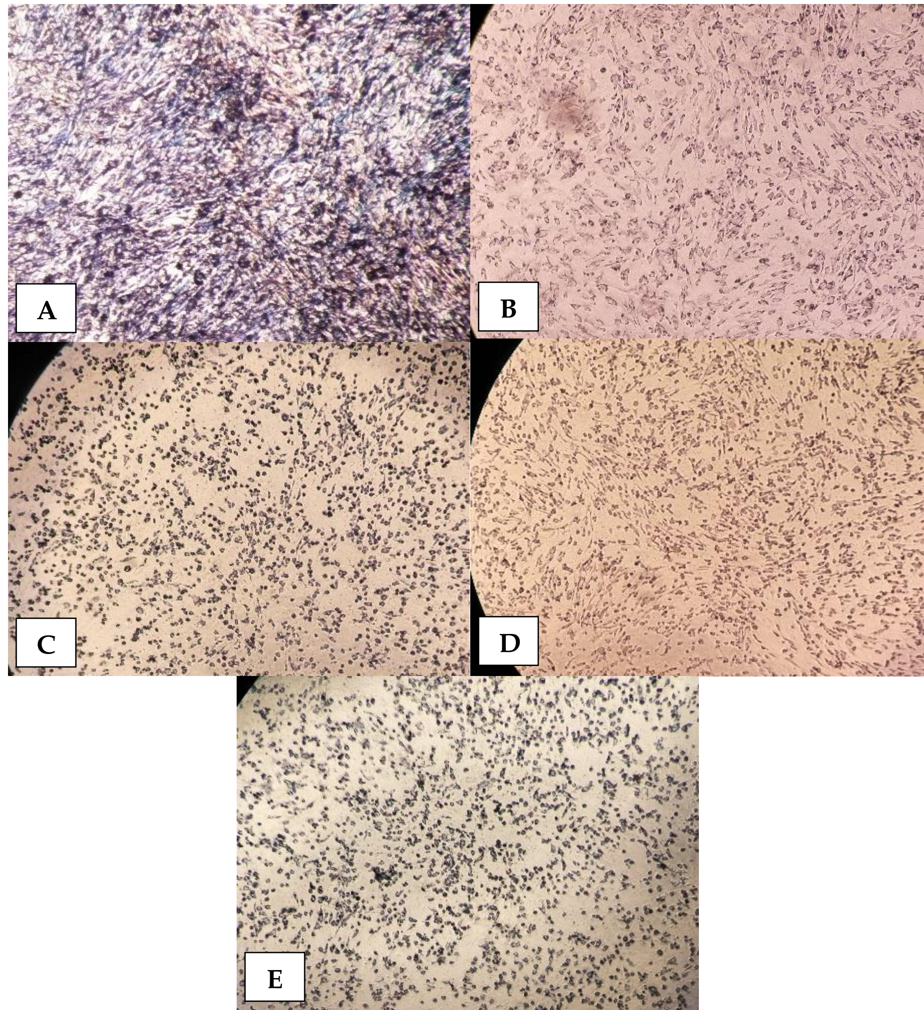

Figure 1. Microscopy of limbal mesenchymal stem cells at magnifications of 40-100x. (a) Cell control; (b) extract treatment with a concentration 0,5%; (c) extract treatment with a concentration 0,25%; (d) extract treatment with a concentration 0,125%; and (e) extract treatment with a concentration 0,0625%.

The parameter commonly used in the cytotoxicity test of an herbal substance is the LC50 value. LC50 indicates a concentration value that results in 50% inhibition of cell proliferation and indicates the potential toxicity of a compound to cells. This value is a benchmark for conducting cell kinetics observation tests. The LC50 value can indicate the potential of a compound as a cytotoxic agent; the greater the LC50 value, the less toxic the compound will be (Djajanegara et al., 2009).

Observations on the cytotoxicity test of *T. diversifolia* leaf extract with concentrations of 0.5%, 0.25%, 0.125%, and 0.0625% in rabbit limbal mesenchymal stem cells for 24 hours showed a different percentage of living

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cells in each treatment. The percentage of limbal mesenchymal stem cells that survived the treatment with *T. diversifolia* leaf extract at 0.0625% was 50%. *T. diversifolia* leaf extract with concentrations of 0.5%, 0.25%, and 0.125% yielded a value of less than 50%. It was noted that at concentrations of 0.5%, 0.25%, and 0.125%, the number of living cells that could be observed was 14%, 29%, and 33%, respectively.

The result of this study revealed that the higher the concentration of *T. diversifolia* leaf extract, the lower the percentage of living cells from limbal mesenchymal stem cells, indicating a higher toxic effect of *T. diversifolia* leaf extract.

CONCLUSIONS

It can be concluded that the greater the concentration of *T. diversifolia* leaf extract, the lower the number of viable rabbit limbal mesenchymal stem cells. The concentration of *T. diversifolia* leaf extract required to kill 50% of rabbit limbal mesenchymal stem cells (LC50) within 24 hours is 0.059%.

ACKNOWLEDGEMENTS

The authors express their gratitude to the Stem Cell Research and Development Center, Universitas Airlangga, Surabaya, for providing the rabbit limbal mesenchymal stem cells for this study.

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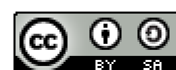

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