

Cytotoxicity Test of *Tithonia diversifolia* Leaf Extract on Bone Marrow Mesenchymal Stem Cell (BMSC) of Rats Using MTT Assay Method

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ABSTRACT

The aim of this study was to identify the cytotoxicity effect of *Tithonia diversifolia* leaf extract on bone marrow mesenchymal stem cell using MTT assay. The sample of this research using bone marrow mesenchymal stem cell of rats. The *Tithonia diversifolia* leaf extract divide into 4 concentration (0.5%, 0.25%, 0.125%, and 0.0625%) and given to bone marrow mesenchymal stem cell of rats in each well and incubated for 24 hours. The toxicity result was obtained using the MTT assay method and incubate for 4 hours. The optical density absorbency which indicates cell viability, calculated by ELISA reader with a wavelength of 595nm. Cytotoxicity parameters using LC₅₀ analyzed by probit analysis. The extract of *Tithonia diversifolia* leaf with the concentration of 0.5%, 0.25%, and 0.125% found to kill cell up to 50% and only the concentration 0.0625 of *Tithonia diversifolia* leaf extract with the highest cell viability. The LC₅₀ value of *Tithonia diversifolia* leaf extract on bone marrow mesenchymal of rat is 0.167%.

Keywords: *Tithonia diversifolia* leaf extract, mesenchymal bone marrow stem cell of rats, cytotoxicity, LC₅₀, MTT assay

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INTRODUCTION

Consumption of herbal or traditional medicines in Indonesia continues until now when referring to basic health research data by the Indonesian Ministry of Health which shows that 30.4% of households in Indonesia use traditional health services, including 49.0% of households using herbs (1). Medicinal plants that are widely known by Indonesians as "insulin leaves" are the leaves of *Kembang Bulan* (*Tithonia diversifolia*) which are famous for their antidiabetic properties (2), besides that there are also plants that produce essential oils that can function as antidiabetic ingredients (3-6). The proven efficacy of *Tithonia diversifolia* leaf extract is also followed by other findings, where the toxicity test of *Tithonia diversifolia* leaf extract by administering *Tithonia diversifolia* leaf extract to rats at a concentration of 400 mg / kgBW and 800 mg / kgBW shows thickening of the tubular wall in the presence of many damage to the tubular cell nucleus and blood vessels began to appear, while at a dose of 1600 mg / kgBW shows widespread tubular necrosis of the kidney, the liver concentration of 400 mg / kgBW has shown significant periportal hepatocyte damage with cellular vacuole with a higher severity of 1600mg / kgBW (7). Toxicity tests were continued to determine the toxic compounds in leaf extracts known to be the compounds responsible for causing cytotoxicity effects are

sesquiterpene lactone (STL) compounds (8). Sesquiterpene lactones (STLs) present a variety of biological activities, based largely on their alkylation abilities. This mechanism is known to be very nonspecific with low selectivity and hence non-specific toxicity is very high (9). Medicinal plants to be consumed and their safety guaranteed must be developed into standardized herbal medicines and phytopharmaca. One of the stages in the development of herbal medicines into phytopharmaca is the preclinical testing stage which consists of toxicity tests that can be carried out in vitro and in vivo (10). Cytotoxicity test is a toxicity test using tissue cells in vitro (11). bone marrow mesenchymal stem cells (BMSC) for screening in vitro toxicology tests related to the realization that mesenchymal stem cells can be easily isolated, ready to be expanded in culture, and well differentiated under suitable stimulation. Bone marrow mesenchymal stem cells (BMSC) are known to be very sensitive in cytotoxicity testing of cancer drugs and some plant extracts are known to have a cytotoxic effect and some cytotoxic drugs are known to cause stromal damage in bone marrow such as cytosine arabinoside (12). This is the reason to support the usefulness of *Tithonia diversifolia* leaf extract which has been used as an antidiabetic treatment, it is necessary to test the leaf extract cytotoxicity. With the hope that the results can be used as reference material.

MATERIAL AND METHODS

In this study used bone marrow mesenchymal stem cells which were implanted into a 96 well microplate. 36 wells were used for 6 treatment groups and 6 repetitions. The 6 treatments in this study are, K (-) or control media that only contain cell growth media (α MEM), k (+) or control media that contain bone marrow mesenchymal stem cells and the growth media (α MEM), 0.5% extract administration, 0.25% extract administration group, 0.125% extract administration group and 0.0625% extract administration group. The treatment of bone marrow mesenchymal stem cells was carried out for 24 hours in a 5% CO₂ incubator at 37 °C. Cells that were incubated 24 hours were then given MTT salt and incubated for 24 hours at 37 °C. Formazan crystals formed by the reductase of living cell mitochondrial dehydrogenase enzymes are added DMSO to dissolve formazan crystals. Absorbance of formazan crystals can be calculated with a spectrophotometer called ELISA reader with a wavelength of 599 nm. The research data obtained in the form of OD (Optical Density) values were

analyzed using the ANOVA test and LC50 values were calculated.

RESULTS AND DISCUSSION

The results of observations of MTT salt administration on Bone marrow mesenchymal stem cells (BMSC) rats given moon flower extract with a concentration of 0.5%, 0.25%, 0.125%, 0.0625% were carried out by six replications for each group, then incubated for 24 hour. Cell viability is calculated using the MTT assay method, dehydrogenase enzymes from mitochondria. Bone marrow mesenchymal stem cells (BMSC) that are still alive will reduce yellow MTT salts to formazan salts that are blue-purple that can be observed under a light microscope at an enlargement of 40-100 x . The results obtained showed that the higher the concentration the more mesenchymal cells die, which is indicated by the higher concentration of the extract, the more mesenchymal cells which cannot reduce MTT salt to formazan crystals that are blue-purple in color (figure 1.).

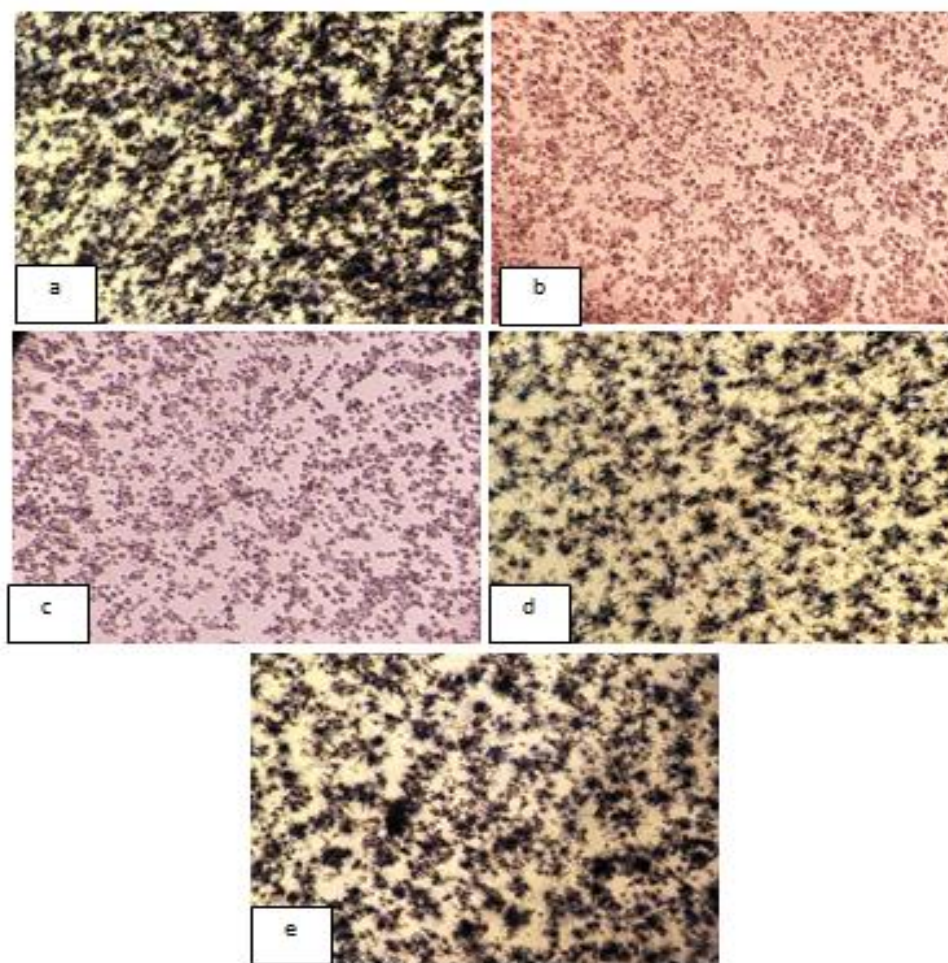


Figure 1. Observations of mesenchymal cells under a light microscope: 40-100x (a) control cells (b) extract concentration of 0.5%, (c) extract concentration of 0.25%, (d) extract concentration of 0.125%, and (e) extract concentration of 0.0625%.

Absorbance Blue-purple formazan crystals by the mitochondria of living mesenchymal cells are quantified by a spectrophotometer called an ELISA reader, to obtain an Optical Density (OD) result. Data in the form of OD (Optical Density) were obtained in order to know the normality of the data and the presence or absence of significant differences in all groups of research results, the data in all treatment groups were analyzed using the ANOVA test and followed by the Duncan test. ANOVA test showed that

the treatment given to each group was significantly different, so that the Duncan test was continued which showed the results of research between cell control, media control, administration of *Tithonia diversifolia* leaf extract concentrations of 0.125% and 0.0625% significantly different from all treatment groups ($P < 0,05$), but did not show a real difference ($p > 0,05$) between treatments giving extracts of the hibiscus leaf extract concentration of 0.5% and 0.25%. Duncan's test results are listed in table 1.

Table 1. Mean and standard deviations of OD value of mesenchymal stem cells (BMSC) that live after being given leaf extract (*Tithonia diversifolia*) for 24 hours.

Group	Mean (Living cell) \pm SD
Media Control	1,78 ^e \pm 0,05
Cell Control	4,30 ^e \pm 0,10
Concentration of 0,5%	2,83 ^d \pm 0,11
Concentration of 0,25%	2,98 ^d \pm 0,26
Concentration of 0,125%	3,28 ^c \pm 0,07
Concentration of 0,0625%	3,76 ^b \pm 0,16

Information: superscript differences in the same column indicate significant differences ($p < 0,05$)

OD (Optical Density) in each treatment group is entered into a formula to determine the percentage of living cells by comparing OD treatment - OD media divided by OD values of cell control - OD media and multiplied by 100%. The calculation results found the number of live mesenchymal cell percentage after administration of *Tithonia diversifolia* leaf extract to bone marrow mesenchymal stem cells (BMSC) mice at a concentration of 0.0625% showed a percentage of

live cells 72%. Extract concentration of 0.125% shows the percentage of living cells is 49%. Extract concentration of 0.25% shows the percentage of living cells is 41%. The 0.5% extract concentration shows the percentage of living cells 33%. This shows that the higher the concentration of the extract given, the higher the dead mesenchymal cells as shown in Figure 2.

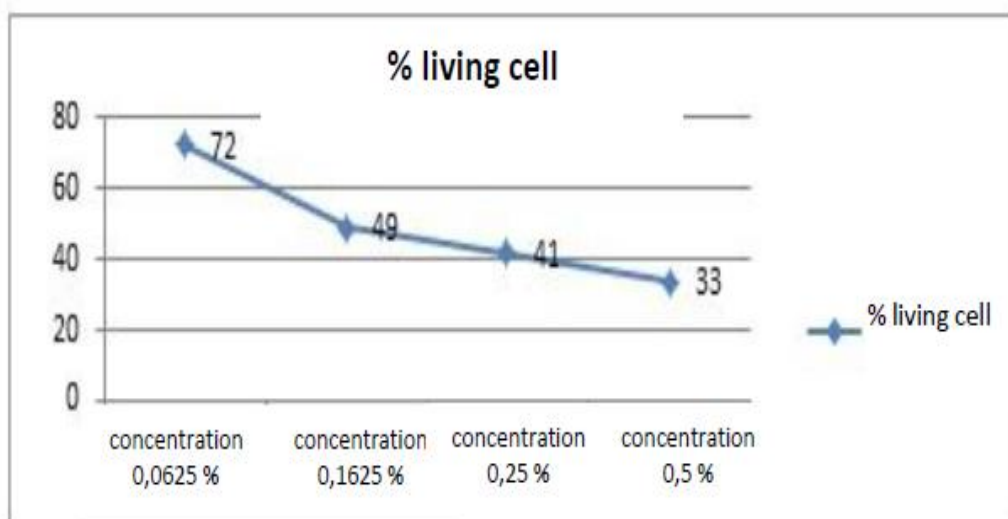


Figure 2. Graph of the number of living mesenchymal bone marrow cells

Data in the form of the percentage of mesenchymal cells that died from 100% of the percentage of living mesenchymal cells, then analyzed probit to determine the value of Lethal Concentration (LC50) of bone marrow mesenchymal stem cell rats that were given *Tithonia diversifolia* leaf extract for 24 hours. The

concentration of *Tithonia diversifolia* leaf extract which can kill 50% of the population of living mesenchymal cells lies in the concentration of 0.167%. Graph of number of mesenchymal cell bone marrow cell death rats (figure 3).

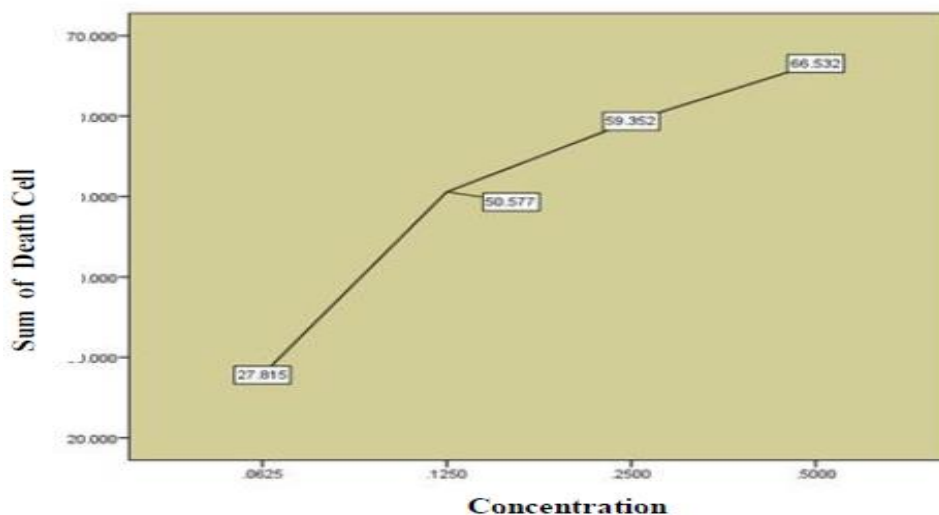


Figure 3. Graph of death cell percentage of mice mesenchymal bone marrow

The observations obtained showed that the higher the concentration of *Tithonia diversifolia* leaf extract, the lower the percentage of mesenchymal cells of living mice, which means the higher the concentration of *Tithonia diversifolia* leaf extract, the higher the cytotoxicity effect of rat mesenchymal cells, so that the lowest concentration 0.0625% produces the highest cell viability, while extracts of the moon flowers with a concentration of 0.5% produce the lowest cell viability. The results obtained are in accordance with the theory which states that the toxicity of an ingredient is directly proportional to the exposure. The determinants of exposure are in the form of material concentration (13). Another factor that causes the higher concentration of extract given, the more mesenchymal cells die due to phenolic compound activity will decrease in administration of extracts with higher doses, this is caused by increased concentrations of extracts at high doses which results in phenolic compounds being difficult to be absorbed, so Antioxidant activity decreases (14). Biological activity exhibited by *Tithonia diversifolia* leaf has been linked to the presence of sesquiterpenen lactones, more than 2% of the dry weight of *Tithonia diversifolia* leaves containing sesquiter-lactone (STL) compounds (15).

Sesquiterpene lactones (STLs) present a variety of biological activities, based largely on their alkylation abilities. Alkylation is the main mechanism of Sesquiterpene lactones, with the formation of covalent bonds with biological macromolecules. The alkylation mechanism of Sesquiterpene lactones (STLs) in addition to presenting many benefits, this mechanism is known to be very nonspecific with low selectivity and hence non-specific toxicity is very high (9). The parameter to show cytotoxicity is LC50 where the concentration of a given substance is lethal to 50% of cells. This amount depends on the time of incubation with the agent (16). In this study the administration of *Tithonia diversifolia* leaf extract to bone marrow mesenchymal stem cells for 24 hours at a concentration of 0.5%, 0.25%, 0.125% killed up to more than 50% mesenchymal cells and only a concentration of 0.0625% killed less than 50% cell.

Probit analysis results from the administration of *Tithonia diversifolia* leaf extract to mesenchymal stem cell bone marrow rats for 24 hours obtained LC50 located at a concentration of 0.167%.

The studies stated that the young stems and leaves of *T. diversifolia* contain lots of glandular trichomes which function as storing sesquiterpene lactones as the main constituent and act as an antidiabetic agent and antimicrobial defense (17, 18). The mechanism of toxicity of *Tithonia diversifolia* leaves is related to the mechanism of sesquiterpene lactone (STL) which includes the mechanism of alkylation and non-alkylation. The mechanism of toxicity of sesquiterpene lactone (STL) alkylation is known to induce accumulation of ROS due to its peroxidic binding (19), and interfere with the function of many cysteine-containing proteins that cause various lesions in different organ systems. ROS accumulation caused by Sesquiterpene lactone (STL) alkylants also causes GSH depletion (20). The depletion of GSH as an endogenous antioxidant causes an imbalance between the amount of free radicals and the amount of antioxidants in the body which leads to oxidative stress and continues to cause cell / DNA damage (20).

The non-alkylating mechanism of sesquiterpene lactone is known to inhibit the class of Ca²⁺ + transport enzymes (serca pumps). Calcium is then released from the endoplasmic reticulum (ER) which increases cytosolic calcium levels. Increased Ca²⁺ + levels (serca pumps) cause changes in many cellular functions, which can lead to mitochondrial stress and increased ROS release from mitochondria which also leads to oxidative DNA damage (21). Increased cytosolic calcium levels result in the release of several enzymes (ATPase, phospholipase, protease, and endonuclease that can damage cell membranes (phospholipase, and proteases). Endonuclease is known to cause the death of cell nucleus chromatin. Damage to cell membranes and cell nuclei causes cell death (22, 23). The parameter to show cytotoxicity is LC50 in which the concentration of a given substance is lethal to 50% of cells This number depends on the incubation time with the agent (16). In this study the administration of *Tithonia diversifolia* leaf extract to bone marrow

mesenchymal stem cells for 24 hours at a concentration of 0.5%, 0.25%, 0.125% kill up to more than 50% mesenchymal cells and only a concentration of 0.0625% kill less than 50% of cells. Probit analysis of the results of the administration of *Tithonia diversifolia* leaf extract mesenchymal stem cell bone marrow rats for 24 hours obtained LC50 located at a concentration of 0.167%.

CONCLUSION

The number of living mesenchymal cells shows a significant value. Most living cells in the administration of *Tithonia diversifolia* leaf extract 0.0625%, so that the extract of the *Tithonia diversifolia* leaf shows the highest percentage of cell viability, which means a concentration of 0.0625% is safe to use. The value of Lethal Concentration (LC50) in the administration of *Tithonia diversifolia* leaf extract is at a concentration of 0.167%.

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