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**TOXICITY TEST OF BAY LEAF EXTRACT ON BHK-21
 FIBROBLAST CELLS IN VITRO**

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ABSTRACT

Background: Denture stomatitis is an inflammatory reaction in the oral mucosa that support the denture. This lesion is caused by the fungus *Candida albicans* and can be avoided by always maintain the cleanliness of denture. Bay leaf (*Syzygium polyanthum*) has flavonoid as the highest content which has antifungal and antioxidant properties so that bay leaf can be used as alternative ingredient for denture cleanser. Toxicity test needs to be done to determine the safety of this material. **Objective:** to analyze the toxicity of bay leaf extract (*Syzygium polyanthum*) to BHK-21 fibroblast cells using the MTT assay method. **Method:** This study is a true experimental study, which using posttest-only with control group design. The group, treated with bay leaf extract, were 25%, 20%, 15%, 10% and 5% concentrations including 2 control groups which were media control and cell control. Absorbance was read using ELISA reader and cell viability was calculated. **Results:** The percentage of living cells in all groups which treated with bay leaf extract was 100%. The parametric analysis of One Way Annova showed that there was no significant difference. **Conclusion:** Bay leaf extract (*Syzygium polyanthum*) does not have any toxic effect to BHK-21 fibroblast cell using the MTT assay method because cell viability in all treatment groups was $\geq 60\%$.

Keywords: Bay leaf extract, flavonoid, toxicity test.

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INTRODUCTION

Denture stomatitis is an inflammatory reaction in the oral mucosa which support the denture. The clinical appearance of soft tissue is more red-colored than the surrounding tissue, almost painless and associated with the presence of yeast from *Candida albicans*.^{1,2} In Indonesia, the incidence of denture stomatitis is quite high. Although, there is no official data from the government. According to the Lahama et al. (2015), a study in Batu Kota, the incidence of denture stomatitis is reached 83.95%.³

Denture stomatitis can be prevented by always maintaining the cleanliness of the denture.⁴ On the other hand, denture cleanser is difficult to find because it should be imported and relatively expensive as well as the influence of denture cleanser on decreasing the hardness of the acrylic resin denture base. So the research has been done to develop natural ingredient from medicinal plant that

efficient to reduce costs and minimize the side effect of chemical denture cleanser.⁵ One of the plants that can be used for denture cleanser is bay leaf.⁶

Bay leaf (*Syzygium polyanthum*) is one of the original Indonesian medicinal plants, which has been used as a spice and traditional medicine for various diseases.⁷ The extraction of bay leaf using methanol solvent is known to contain flavonoid group of 14.87 mg, flavonoid act as antifungal and antioxidant.⁸ Saputera et al. (2017) showed that 25% bay leaf extract was able to inhibit the growth of *Candida albicans* on the surface of the acrylic resin plate. The use of bay leaf as an alternative material for denture cleanser needs to be carried out for toxicity test.⁹

Toxicity test is a test that aims to determine the harm of an ingredient, can be done in vitro using BHK-21 fibroblast cell culture by the method of 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) assay.¹⁰ The MTT assay method is

used because it is valid and relatively faster.¹¹ Based on the description above, it is necessary to investigate the administration of bay leaf extract (*Syzygium polyanthum*) with toxicity to BHK-21 fibroblast cells using the MTT assay method. This study aim to show the toxicity effect of bay leaf extract to BHK-21 fibroblast cells.

METHOD AND MATERIAL

This research was initiated by managing the research license and ethical conduct by the Health Research Ethics Commission of the Faculty of Dentistry, Lambung Mangkurat University No.130/KEPKG-FKGULM/EC/I/2009. This research was true experimental with a posttest-only with control group design.

The population of this study was BHK-21 fibroblast cells which were divided into seven groups; five groups which treated with bay leaf extract in various concentrations (25%, 20%, 15%, 10% and 5%) and two control groups (media control and cell control). The number of repetitions for each treatment was four times using the Federer formula.

Extracting bay leaf required 70% methanol, maceration vessel, filter paper, measuring cup, beaker glass, measuring flask, stirring rod, blender, water bath, and vacuum rotary evaporator. Toxicity test required BHK-21 fibroblast cells, Eagle's culture media, FBS 10%, PBS, trypsin versene, MTT assay, DMSO, flask/roux culture bottle, 96 well microplates, ELISA reader, automatic plate shaker, 37°C 5% CO₂ incubator, multichannel micropipette, inverted microscope, and Laminary flow.

Production Of Bay Leaf Extract Using Maceration Method

1 kg of bay leaf was washed under running water, then dried in a 45°C drying cabinet for 48 hours. Then, the bay leaf was cut into small pieces and mashed using a blender until became powder. The powder was soaked with 70% methanol in a maceration vessel for 3x24 hours at room temperature. After three days, filtering was carried out using filter paper, and the filtrate was taken to be concentrated using a rotary evaporator and dried up again in the water bath until thick bay leaf extract was obtained.⁶ The extract was calculated using the formula below:

$$\% \text{Yield} = \frac{\text{The weight of the extraction obtained (g)}}{\text{The weight of the original simplicia (g)}} \times 100\%$$

Production Of BHK-21 Fibroblast Cells

BHK-21 fibroblast cells were cultured in flask/roux bottles with eagle's media and FBS 10%, incubated using a 37°C incubator for 48 hours. After the cells filled the wall of the flask/roux bottle, a media eagle's solution and 10% FBS were removed and washed with PBS three times. ½ ml of Trypsin versene was added to release cells from the bottle wall.¹⁰

Toxicity Test Of Bay Leaf Extract

BHK-21 fibroblasts were transferred into a 96-well microplate and treated with bay leaf extract, incubated using a CO₂ incubator for 24 hours. Samples were placed in the container then added media eagle's and FBS 10%. The microplate was washed with PBS three times to remove the remaining serum. The MTT reagent was added 10 µL for each well, incubated using a CO₂ incubator for 4 hours.

Furthermore, the MTT solution was removed and given a DMSO stopper to stop the reaction between MTT and the cell. The microplate was shaken for 5-10 minutes and inserted into ELISA reader with a wavelength of 620 nm to read the cell viability. The calculation of cell viability was done with the formula below:¹²

$$\% \text{Viability} = \frac{(\text{OD treatment} - \text{OD media control})}{(\text{OD cell control} - \text{OD media control})} \times 100\%$$

Based on calculation with the formula above, a material was categorized as non-toxic if the percentage of living cells was ≥ 60%.¹ Data were analyzed by probit analysis to determine IC₅₀ values with SPSS software.

RESULT

1 kg of bay leaf produced 500 mg of simplicial powder. Extraction of bay leaf was carried out by maceration method using 70% methanol solvent so that thick and concentrated greenish brown extracts were obtained as much as 29.1gr and the yield was 5.82%.

The toxicity test of bay leaf extract to BHK-21 fibroblast cells on a microplate 96 well had a color change into dark purple. This showed that the darker color was produced, the more BHK-21 fibroblast cells (Baby Hamster Kidney 21) live (**Figure 1**). The calculation results of the BHK-21 fibroblast cells viability in all treatment groups were ≥ 60% (**Graph 1**), indicated that bay leaf extract was not toxic to BHK-21 fibroblast cells at concentrations of 25%, 20%, 15%, and 5%.

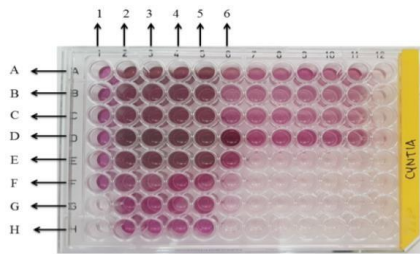


Figure 1. Microplate 96 Well Was Given Bay Leaf Extract And MTT Assay.

Information :

A1, B1, C1, D1, E1, F1 : Cell control

G1, H1 : Meda control

A2-5: Bay leaf extract 25%

B2-5: Bay leaf extract 20%

C2-5: Bay leaf extract 15%

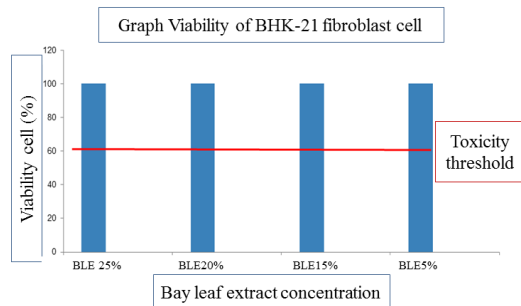
E2-5: Bay leaf extract 5%

A6 : Bay leaf extract 25% control

B6 : Bay leaf extract 20% control

C6 : Bay leaf extract 15% cotrol

E6: Bay leaf extract 5% control



Graph 1. Viability of BHK-21 fibroblast cell

Toxicity test produces an IC₅₀ (inhibitory concentration) value if there is a concentration that causes cell growth inhibition as much as 50% of the population. However, the calculation in this study showed that the cell viability for all concentrations was 100%, so that the IC₅₀ value could not be determined.¹³ The result of the study was tested for normality and homogeneity tests. The normality test was carried out by the Shapiro-Wilk test because the number of sample was less than 50; the result of the normality test can be seen in **Table 1**:

Table 1. Average Result, Standard Deviation and Normality Test, Toxicity Test of Bay Leaf on BHK-21 Fibroblast Cells In Vitro.

Viability	Average ±Standar Deviation	Normality Test (Sig.)
EDS 25%	129,25±9,21	p=0,381
EDS 20%	151±12,98	p=0,629
EDS 15%	137,5±12,76	p=0,226
EDS 5%	150,75±11,29	p=0,694

Data in the table shows that the data distribution is normally distributed because the significance value was $p > 0,05$. Data analysis was continued with the homogeneity test to find out the variance between the sample groups. Homogeneity test of the data, using Levene's Test, got the Sig value = 0.929 ($p > 0.05$), so the data was declared homogeneous. The normally distributed and homogeneous data was then performed parametric analysis of One Way ANOVA. The One Way ANOVA test resulted the Sig. 0.056 ($p > 0.05$), which meant there was no significant difference between groups of BHK-21 fibroblast cell viability.

DISCUSSION

BHK-21 fibroblast cells that were treated with bay leaf extract at concentrations of 25%, 20%, 15% and 5% showed cell viability $\geq 60\%$ as in **table 1**. It means that the bay leaf extract has no toxic effect over the fibroblast cells. But, in this study, IC₅₀ value cannot be determined.¹ The toxicity test with the MTT assay method has principles the tetrazolium salt metabolism by the dehydrogenase enzyme that contains in the cell mitochondria. When the tetrazolium ring is broken, it turns into purple formazan crystal.¹⁴ Purple intensity shows the viability of BHK-21 fibroblast cells that was affected by bay leaf extract and is directly proportional to the number of cells that live or metabolize. The morphology of BHK-21 fibroblast cells, treated with bay leaf extract at a concentration of 25%, using an inverted microscope with 100x magnification can be seen in **Figure 2**.

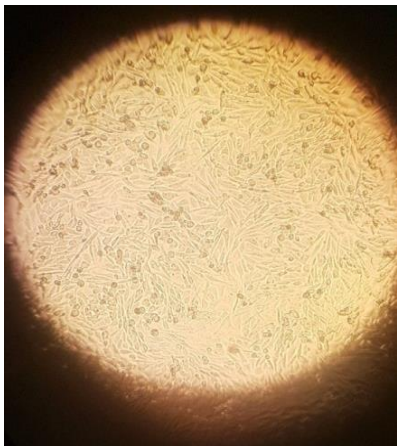


Figure 2. Morphology cell of BHK-21 Fibroblast cell with enlargement 100x

Flavonoid is the highest content of secondary metabolite in bay leaf methanol extract.⁸ Flavonoid is a group of phenol which the highest concentration is in the leaf compared to other plant tissues.¹⁵ One of the active compounds of flavonoid was quercetin. Methanolic extract of bay leaf contains quercetin as much as 100gr.⁸ Quercetin in flavonoid has the strongest antiradical or antioxidant properties of hydroxyl radical and superoxide anion.¹⁵

Antioxidant causes cells to maintaining the permeability, so they can prevent damage due to Reactive Oxygen Species (ROS).⁹ The role of flavonoid as an antioxidant is by activate antioxidant enzyme, bind (chelate) Fe_2^+ and Cu^+ ions and reduce free radical (free radical scavenging), so that it inhibits superoxide (O_2^-) anion formation enzyme.¹⁵ Superoxide anion is a reactive molecule that is formed when a cell metabolizes; superoxide anion will be convert to hydrogen peroxide (H_2O_2) by the Superoxide Dismutase enzyme (SOD). Hydrogen peroxide is a relatively weak oxidant, capable of initiating oxidative reactions and forming free radical species. Catalase enzymes (CAT) and Glutathione Peroxidase (GSHP-x) will convert H_2O_2 to H_2O or water.¹⁶ H_2O_2 , which is not converted to H_2O , will change the shape to hydroxyl (OH) radical which occurs through a catalyzing reaction of Fe_2^+ or Cu .¹⁷ OH group gets hydrogen ion donation from flavonoid, so that the radical can be reduced to be more stable. The presence of the flavonoid causes the enzyme system in cells to control metabolic processes and protect cells from oxidative damage resulting in cell viability.¹⁸

The SOD, CAT, and GSHP-x enzymes are a group of enzymatic antioxidant, that catalyze oxidant and reactive free radical to be less reactive molecule and maintain oxidative balance to avoid an increasing

in excessive amount or level of oxidant, while flavonoid derived from bay leaf is a class of fat-soluble non-enzymatic antioxidant.^{15,19} It is the collaboration between enzymatic antioxidant and non-enzymatic antioxidant that will protect cells from damage caused by free radical.¹⁹ The flavonoid content of bay leaf will trigger the expression of antioxidant encoding enzymes through the activity of Nuclear factor erythroid 2 relates factor (Nrf2), which translocates from the cytoplasm to the nucleus that releases its bond with Kelch-like ECH-associated protein-1 (Keap-1) in the nucleus Nrf2, will bind to the Antioxidant Respond Element (ARE) to produce enzymatic antioxidant.¹⁵

Bay leaf extracted using methanol 70%, solvent to attract efficacious substances in simplicia. Nature material extract that use methanol have some various contents, one of the those is flavonoid.⁸ It is supported by the research of Apriasari et al (2014), which proved that methanol were able to attract flavonoid and the result of toxicity test showed that Maui banana stem extract with methanol was not toxic at 25% concentration. Based on the result of the study, it can be concluded that bay leaf extract (*Syzygium polyanthum*) has no toxic effect to BHK-21 fibroblast cells using the MTT assay method, because cell viability in all treatment groups was $\geq 60\%$.¹¹

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