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TOXICITY TEST OF THE MIXED MOUTHWASH OF MAULI BANANA STEM AND BASIL LEAF AGAINST FIBROBLAST CELL STUDY IN VITRO

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INTRODUCTION

Mauli banana stem and basil leaf are herbal ingredients that have the potential to be used as a mouthwash. Mauli banana stem proves as wound healing booster among people in South Kalimantan, because of its antibacterial property. Basil leaf inhibits the growth of pathogenic bacteria in the mouth.[1,2] Mauli banana stem contains tannin which has been proven to have antiseptic, antibacterial, antioxidant and antifungal effects.[3,4] In the previous study, it stated that mauli banana stem can be used as an alternative herbal mouthwash, but it has bitter taste.[3] The unpleasant taste can be minimized with basil leaf as mixture. Basil leaf expect to give a fresh effect and fragrant aroma.[2,6] Mauli banana stem contains 67.59% tannin, while basil leaf contains 96.7% essential oil.[7,8]

One of the requirements for a nature material to be used on daily basis is to be proven that it is safe and do not have any detrimental effect in the human body.[9,10] In vitro toxicity test can be carried out to test the safety of the material against cell, which oftenly used for the test in dentistry is BHK-21 fibroblast cell culture.[11] The method which commonly used for toxicity test is the methylthiazol tetrazolium (MTT) assay method. The MTT assay method are valid and relatively faster. The parameter for the toxicity test is the IC_{50} or Inhibitory Concentration.[50,12] IC_{50} value can indicate the potential of a toxic compound. The greater IC_{50} value, the more non-toxic it is.[13] Balantyne (1999) made toxicity categories from nature materials based on the number of its IC_{50}, as stated in the following table.[14]

Tabel 1. Toxicity classification for nature material

IC50	Category
$\frac{\text{weight of exract (g)}}{\text{initial weight of simplicia (g) x 100\%}}$	
5 μg/mL (10^6 Sel/mL) < IC_{50}	very toxic
10 μg/mL < IC_{50} < 100 μg/mL	toxic
100 μg/mL < IC_{50} < 1000 μg/mL	moderate
IC_{50} > 1000 μg/mL	non toxic

The toxicity test of mixed mouthwash of Mauli banana stem and basil leaf on BHK-21 fibroblast cell had not been done yet. Based on this reason, it might be necessary to do a research to determine the safety of mixed mouthwash of Mauli banana stem and basil leaf is not toxic to BHK-21 fibroblast cells.

MATERIALS AND METHOD

This study was started by managing research permit and ethical clearance issued in the Ethics Committee of Faculty of Dentistry, Lambung Mangkurat University No. 146 / KEPKG-FKGULM / EC / I / 2019. This study was a true laboratory experimental study with *posttest-only with control group design,* with a true random design using 10 treatment groups. The 10 treatment groups contained mixed mouthwash of Mauli banana stem and basil leaf with 8 concentrations. Those were 31,25 µg/mL, 62,5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL, 2000 µg/mL, 4000 µg/mL and 2 control groups, which was cell control and media control. The minimum number of repetitions for each treatment group was 5 times based on Federer formula. The population of this study was Baby Hamster Kidney-21 fibroblast cells (BHK-21) which were bred on eagle's media at the Farma Veterinary Center Laboratory (PUSVETMA) Surabaya.

Extraction of Mauli Banana Stem

Mauli banana stem was taken from Banjarbaru Vocational High School, South Kalimantan. The one-year-old mauli banana stem was cut 10 cm from the root stump. Mauli banana stem was then weighed 14 kg, washed with running water, cut into small pieces, and dried using an oven with a temperature of 40–50°C for 5 days. After that, the mauli banana stem was mashed with a blender until they were in the form of simplicia powder with 750 g of weigh. The simplicia powder was then immersed in 70% ethanol in a closed container. The soaking process was done for 5x24 hours and stirred several times during the immersion period. Then, filtering process was done using filter paper and stored in a glass bottle. The solution was left for 4 days to precipitate it. The extraction was evaporated with a rotary evaporator with a heating temperature of 40°C and evaporated again in the water until became thick extract of Mauli banana stem. After dried up in the waterbath, thick blackish brown extract of Mauli banana stem as much as 169,22 g were obtained. Then the yield was calculated using the following formula:

$$\%\text{yield} = \frac{\text{weight of exract (g)}}{\text{initial weight of simplicia (g) x 100\%}}$$

Through these calculations obtained a yield of 22,67%

Extraction of Basil Leaf

Basil leaf was collected at Jl. Kurnia Banjarbaru, South Kalimantan. The 2-month-old fully-bloomed basil leaf which was already formed yellow seeds and lower leaf. Basil leaf was taken fresh and then weighed as much as 12 kg and washed with running water. After, they were dried using an oven with a temperature of 40-50°C for 5 days. The leaves then were blended until smooth and became 900g simplicia powder. The powder was then soaked with 70% ethanol for 3x24 hours at room temperature, then it was filtered using filter paper and stored in a glass bottle. The solution was left for 3 days to precipitate it. The extraction result was evaporated with a rotary evaporator with a heating temperature of 40°C and dried up again in the waterbath until the thick basil leaf extract was obtained. There was thick blackish brown dark basil leaf extract weighing 146,44 g. Then, the yield was calculated using the following formula:

$$\%\text{yield} = \frac{\text{weight of exract (g)}}{\text{initial weight of simplicia (g) x 100\%}}$$

Through these calculations obtained a yield of 16,27%

Mixing Mauli Banana Stem and Basil Leaf Extract

After the thick extract was obtained, the extract of mauli banana stem was mixed with basil leaf extract weighing 1 g each, so that the main weight was 2 g. Then, it was followed by making the main concentration of 4000 µg/mL, by mixing 0,04 g parent extracts, which was then added by 10 ml of DMSO to obtain a parent concentration of 4000 µg/mL. It was diluted using DMSO to obtain various concentration of 31,25 µg/mL, 62,5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL, 2000 µg/mL. Those concentrations were diluted according to the following formula:

$$\mathbf{V_1 \times N_1 = V_2 \times N_2}$$

Explaination:
V_1 = Initial volume
N_1 = Initial concentration
V_2 = Final volume
N_2 = Final concentration

Preparation of Fibroblast Cells

BHK-21 fibroblast cells, in cell-line form with eagle's media and FBS 10%, were planted in roux bottles and incubated using an incubator (*Cooperation 1989/90*) for 48 hours. Cell proliferation was carried out until the BHK-21 fibroblast cell attached and filled the wall of the flask / roux bottle. After the cell was full, the eagle's media solution and FBS were removed to the container bottle. The flask / roux bottle was washed with PBS 3 times, to remove the remaining serum. It was added by tripsine versene ½ ml to release the cell from the bottle wall and separated the bond between cells, so that they would not be clustered. This was done by tapping the bottle until the flask / roux bottle wall was clean and the liquid in the flask / roux bottle became cloudy. Afterward, the eagle's media and FBS 10% were given and BHK-21 fibroblast cell were transferred to microplate-96 well according to the number of samples and control using micropipette-multichannel (*Eppendorf*).

Toxicity Test

Each concentration of mixed mouthwash of Mauli banana stem and basil leaf was put into a well containing BHK-21 cells. Then, it was incubated using a CO^2 incubator (*Cooperation 1989/90*) for 24 hours. After that, the sample was removed, the microplate was washed with PBS 3 times, then added with eagle's media and FBS 10% on each well. Yellow MTT reagent was added as much as 10 μl, then incubated using a CO^2 incubator (*Cooperation 1989/90*) for 4 hours. The reaction between MTT and the cell was stopped by giving DMSO stopper solution. Microplate-96 well was shaken for 5 minutes, so that the reaction stop could occur evenly and released formazan. Then, the absorbance readings of each well was done by using ELISA reader (Thermo scientific) with a wavelength of 620 nm. After the toxicity test, the removal of BHK-21 fibroblast cell culture was carried out. The absorbance data were used to calculate the viability percentage of BHK-21 fibroblast cells using the Optical Density formula.

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

Examination:

% viability	: Percentage of living cell after treatment
OD. treatment	: *Optical Density* fibroblast cell each sample
OD. media	: *Optical Density* fibroblast cell on control media
OD. Sel	: *Optical Density* fibroblast cell on cell control

After obtaining the percentage value of cell viability, the IC_{50} value was calculated. Cells were expressed as viable if > 60% of cells were alive.

RESULT

Based on the results of MTT staining purple discoloration was found lighter at 1000 µg/mL, 2000 µg/mL and 4000 µg/mL concentrations than in other concentrations. This suggested that small percentage of BHK-21 fibroblasts exists in this concentration, while darker purple color appeared in MTT staining at the 500 µg/mL, 250 µg/mL, 125 µg/mL, 62,5 µg/mL and 31,25 µg/mL concentrations. Those results indicated that lower concentrations produce darker color, showed higher percentage of BHK-21 fibroblast cells that alive.

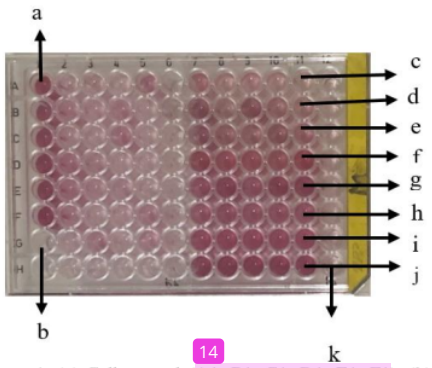

Figure 1. (a) Cell control (A1, B1, C1, D1, E1, F1); (b) control media (G1, H1); (c) concentration of 4000 µg/mL (A7, A8, A9, A10, a11); (d) concentration of 2000 µg/mL (B7, B8, B9, B10, B11); (e) concentration of 1000 µg/mL (C7, C8, C9, C10, C11); (f) concentration of 500 µg/mL (D7, D8, D9, D10, D11); (g) concentration of 250 µg/mL (E7, E8, E9, E10, E11); (h) concentration of 125 µg/mL (F7, F8, F9, F10, F11); (i) concentration of 62,5 µg/mL (G7, G8, G9, G10, G11); (j) concentration of 31,25 µg/mL (H7, H8, H9, H10, H11) dan (k) sample control without cell (A12, B12, C12, D12, E12, F12, G12, H12).

Tabel 2. Viability percentage of fibroblast cell

Concentration	Viability
4000 µg/mL	42,98%
2000 µg/mL	52,39%
1000 µg/mL	48,94%
500 µg/mL	62,41%
250 µg/mL	72,96%
125 µg/mL	74,39%
62,5 µg/mL	80,69%
31,25 µg/mL	80,49%

The measurement of cell viability states that a material is non-toxic if the percentage of living cells is >60%. Based on the result of the viability data, the mouthwash from mixed banana mauli and

basil leaf was non-toxic to BHK-21 fibroblast cells at the concentrations of 31,25 µg/mL, 62,5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL and it became toxic at the concentrations of 1000 µg/mL, 2000 µg/mL and 4000 µg/mL.

The IC_{50} value could be calculated from the result of the toxicity test based on various concentration data and percentage of cell viability in the table that shows data that obtained from 5 replications. The IC_{50} value was analyzed using probit analysis, showed value of 2183,43 µg/mL with a lower bound of 1306,67 μg/mL and upper bound of 4826,48 µg/mL.

DISCUSSION

The toxicity test was done to the mouthwash of mixed mauli banana stem and basil leaf based on various concentrations of 500 µg/mL, 250 µg/mL, 125 µg/mL, 62,5 µg/mL and 31,25 µg/mL resulted in viability percentage of BHK-21 fibroblast cells > 60%, while in other concentrations, 1000 µg / mL, 2000 µg / mL and 4000 µg/mL, the percentage of BHK-21 fibroblast cell viability was < 60%. The ability of BHK-21 fibroblast cells to maintain their permeability comes from the active chemical compound that found in Mauli banana stem and basil leaf.[9] Mauli banana stem contains high amount of tannin, while basil leaf contains a lot of essential oil.[7,8]

Tannin and essential oil are phenolic complex compounds.[3,15] Phenol compound can activate Nrf2 which is a part of antioxidant defense mechanism. When cells are exposed to phenol compound, it reacts with cysteine in Keap1, releases the Nrf2 from Keap1. Nrf2 is translocated to the nucleus and bounded to the Antioxidant Respone Element (ARE). Nrf2 and ARE work simultaneously with the small musculoaponeurotic fibrosarcoma protein in the nucleus to activate the transcription of antioxidant enzyme.[16,18] Antioxidant enzyme, such as SOD, will convert superoxide radicals (O^2-) to hydrogen peroxide (H^2O^2), but they remain reactive. Thus, H^2O^2 will be changed again to H^2O (dihydrogen oxide) and O^2 (oxygen) by CAT and GPx. This enzyme can neutralize ROS that allows the cell to survive.[17,19] The result of this research are similar to the one of Apriasari et al (2015) who also found that mauli banana stem extract was non-toxic to BHK-21 fibroblast cells at a concentration of 25%. It also mentioned that flavonoid content, which are polyphenolic compound are mostly related to the antioxidant and chemoprotective effects[9] Another research also confirmed a very strong correlation between antioxidant activity and total phenol content in fruit extract, in which phenol compound acts as an antioxidant that activates the Nrf2.[16]

In this study, the mouthwash of mixed mauli banana stem and basil leaf at concentrations of 4000 μg/mL, 2000 μg/mL and 1000 μg/mL had higher concentrations. Hence, it was estimated to have a very high antioxidant content. Antioxidant from phenol compound at high concentration can turn into pro-oxidant or free radical.[21] Under normal condition, cells produce physiologically free radical or Reactive Oxygen Species (ROS) as a logical consequence of biochemical reaction in cell metabolism. However, excessive concentration of ROS can cause oxidative damage, mitochondrial dysfunction and cell apoptosis.[22,23,24]

High ROS concentration can be apoptotic stimulus for intramitochondrial intrinsic pathway, which then activate the Permeability Pore Complex (PTPC) within high conductance condition, induce change in mitochondrial permeability transition (MPT) and increase Mitochondrial Outer Membrane Permeability (MOMPS), result in potential reduction in mitochondria. The potential decrease in mitochondria will facilitate apoptotic protein, named cytochrome c, out of the intermembrane space of the mitochondria to penetrate into the cytoplasm and activate the caspase activation pathway. Cytochrome c, comes out into the cytoplasm, binds to a cytoplasmic protein called Apaf-1 and forms the Caspase Recruitment Domain (CARD). CARD forms an apoptosome complex that binds the pro-caspase-9 and activates it into caspase-9 (caspase initiator). The caspase-9 activates procaspase-3 into caspase-3 (caspase effector) and activates the Caspase 6 and 7 to induce cell apoptosis.[25]

This was similar with the finding in Carabelly et al (2017), showed that Mauli banana stem extract is toxic to mesenchymal stem cell at doses of 2,5 mg/ml, 5 mg/ml, 7,5 mg/ml, and 10 mg/ml due to tanin content. Tanin in high concentration become toxic to the cell. As found in this research, mauli banana stem contains proanthocyanidin tannin content which can induce tyrosine phosphorylation in tyrosine kinase receptors on the surface of MSC to activate the MAPK/Erk pathway and PI3K/Akt/mTOR pathway, which regulates apoptosis.[23]

In this research, the IC_{50} value was used as toxicity parameter. IC_{50} value indicates the toxicity of a compound to cells.[12] The nature material is considered non-toxic if IC_{50} value > 1000 µg/mL, and toxic if the IC_{50} value is < 100 μg/mL.[14] Based on the analysis in this research, the IC_{50} value was found at 2183,43 µg/mL with a lower bound of 1306,67 µg/mL and upper bound of 4826,48 µg/mL. Therefore, it could be concluded that the mouthwash of mixed banana mauli and basil leaf is not toxic to BHK-21 fibroblast cells, because the IC_{50} value is 2183,43 µg/mL.

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