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EFFECT OF KARAMUNTING LEAF EXTRACT (*Melastoma malabathricum*) ON THE PROLIFERATION OF BABY HAMSTER KIDNEY-21 (BHK-21) FIBROBLAST CELLS

Aanisah Ramadamayati[1)], Muhammad Yanuar Ichrom Nahzi[2)], Agung Satria Wardhana[3)]
[1)]Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin
[2)]Department of Conservative Dentistry, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin
[3)]Department of Dental Material, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin

ABSTRACT
Background: *Pulpitis is an inflammation of dental pulp due to infection of microorganisms from dental caries. Pulpitis is clinically divided into reversible and irreversible pulpitis. Treatment of reversible pulpitis can be done by applying a pulp capping material. The use of calcium hydroxide as a pulp capping material is still not optimal, so an alternative material is needed, namely karamunting leaf (Melastoma malabathricum). The secondary metabolites in karamunting leaf extract have antibacterial activity. Antibacterial activity is needed in the regeneration of pulp tissue.* ***Purpose:*** *To analyze the effect of karamunting leaf extract (Melastoma malabathricum) on viability and proliferation of Baby Hamster Kidney-21 (BHK-21) fibroblast cells using the MTT Assay method.* ***Methods:*** *This study is a purely experimental study with post test only with control group design which were divided into 8 groups. The group was treated with karamunting leaf extract consisted of 5 groups, were 5%, 10%, 15%, 20%, and 25% concentrations also 3 control groups, were positive control (calcium hydroxide), cell control, and media control. Absorbance value was read using ELISA Reader and the percentage of cell viability was calculated.* ***Results:*** *All treatment groups had cell viability values higher than cell control and cause proliferation of BHK-21 fibroblast cells. The One Way Anova showed a value of sig=0,000 (p<0,05), which means that there was a minimum difference in one treatment group.* ***Conclusion:*** *Karamunting leaf extract and calcium hydroxide can maintain viability and cause proliferation of BHK-21 fibroblast cells. Karamunting leaf extract 25% concentration more effective in causing proliferation of BHK-21 fibroblast cells.*

Keywords**:** *BHK-21 fibroblast cells, karamunting leaf extract, MTT assay test, proliferation, viability.*
Correspondence: Aanisah Ramadamayati, Faculty of Dentistry, Lambung Mangkurat University, Veteran St. No. 128B, Banjarmasin 70232, South Kalimantan, E-mail: aanisahr0117@gmail.com

INTRODUCTION

One of the dental and oral health problems is pulp inflammation (pulpitis). The main cause of pulpitis is infection of microorganisms from dental caries.[1] The main causative microorganism of dental caries is *Streptococcus mutans* bacteria. The products of bacterial metabolism destroy the enamel and dentin so that exposing the pulp tissue. The exposed pulp tissue becomes an entrance for bacteria and their products to infect the teeth and surrounding tissue so that triggering an inflammatory reaction.[1,2]

Inflammation of the pulp tissue (pulpitis) is characterized by pain in response to bacterial toxins. Pulpitis is clinically divided into reversible and irreversible pulpitis.[2] Reversible pulpitis is a mild to moderate inflammatory condition of the dental pulp.[1] Mild or moderate inflammation is required to stimulate the regeneration and repair process. The regeneration and repair process occurs through the activity of growth factors such as Transforming Growth Factor Beta (TGF-β) and Fibroblast Growth Factor Beta (FGF-β).[3] TGF-β and FGF-β play a role in the formation of dentin bridges and tertiary dentin.[4,5]

The formation of tertiary dentin by fibroblast cells by synthesizing type I collagen. This collagen is the main extracellular matrix in dentin which is produced early in the process of tertiary dentin formation, then will combine with mineralized tissue to form dentin bridges and tertiary dentin.[5,7] Pulp capping plays a role in inducing the formation of tertiary dentin so as to maintain pulp vitality.[6,7]

One of the pulp capping materials is calcium hydroxide. Calcium hydroxide has a weakness that causes inflammation occur longer so that the regeneration process of pulp tissue is inhibited.[6] Based on the weakness of calcium hydroxide, an alternative material from nature is needed that has antibacterial activity, high biocompatibility, minimal side effects, and the ability to heal pulp tissue. The alternative material is the karamunting leaf.[8]

Karamunting leaf (*Melastoma malabathricum*) has been used by people in Indonesia as a traditional medicine. This plant is native to South Kalimantan. Secondary metabolites in karamunting leaf extract are flavonoid, phenol, tannin, alkaloid, saponin, terpenoid, and steroid.[8] Flavonoid (102.92 QE mg/g), phenol (171.19 ± 3.19 mg GAE/g), and tannin were the highest secondary metabolites in karamunting leaf.[9,10] Flavonoid, tannin, alkaloid, and saponin have antibacterial activity.[8,11] Research conducted by Marsepriani et al (2017) stated that karamunting leaf extract can inhibit the growth of *Streptococcus mutans* bacteria with 5%, 10%, 15%, 20%, and 25% concentrations.[11]

Antibacterial activity is needed because controlling infection in the pulp chamber is the first stage in the the regeneration process of pulp tissue.[12] Regeneration of pulp tissue occurs through the activity of growth factors, namely TGF-β and FGF-β which play a role in stimulating proliferation of fibroblast cells so that accelerating the formation of collagen components to promote the regeneration of pulp tissue.[4,5] The proliferation of fibroblast cells can be measured using the MTT Assay method.[14]

The MTT Assay method is a commonly used method to measure cell viability and proliferation.[14] This method uses cell culture to test a material or extract in dentistry. The cell culture that is often used is Baby Hamster Kidney-21 (BHK-21) fibroblast cells obtained from baby hamster kidney. The advantages of this cell are that it has the shape, characteristics, and ability to resemble human fibroblast cells, more stable, easy to grow, more sensitive, and easy to re-subculture.[13,14] Based on that, it is necessary to conduct research on the effect of karamunting leaf extract (*Melastoma malabathricum*) on the proliferation of Baby Hamster Kidney-21 (BHK-21) fibroblast cells.

METHOD AND MATERIAL

This research was conducted at the Laboratory of Pusat Veteriner Farma (Pusvetma), Surabaya. This research has obtained a research permit and an ethical permit by the Research Ethics Commission of the Faculty of Dentistry, Lambung Mangkurat University No. 009/KEPKG-FKGULM/EC/II/2021. The method of this research is a pure experimental method with post test only with control group design which were divided into 8 groups. The minimum number of repetitions for each treatment group was 3 times which is obtained from the results of calculation using the Federer formula. The number of samples in this study were 48 samples. The sample used was karamunting leaf extract with 5%, 10%, 15%, 20%, and 25% concentrations also calcium hydroxide as positive control.

Extraction of karamunting leaf required 96% ethanol, aquadest, maceration vessel, analytical balance, measuring cup, beaker glass, volumetric flask, blender, stirring rod, waterbath, and vacuum rotary evaporator. MTT Assay test required BHK-21 fibroblast cells, MTT, Eagle's culture media, PBS, 10% FBS, DMSO, Trypsine Versene, flask/roux culture bottle, laminary flow, multichannel micropipette, automatic plate shaker, microplate 96-well, inverted microscope, 37^{O}C 5% CO_2 incubator, and ELISA Reader.

Production of Karamunting Leaf Extract Using Maceration Method

Karamunting leaf in this study were taken from Kiram Village, Banjarbaru, South Kalimantan. Extraction of karamunting leaf was carried out by selecting and picking karamunting leaf which were green, fresh, and clean free of pests and then weighed as much as 1 kg. Next, karamunting leaf was washed under running water and it was dried in a 45^{O}C drying cabinet for 48 hours, then the dried karamunting leaf was cut into small pieces. Karamunting leaf was mashed using a blender until simplicia powder was formed. After that, the maceration process was carried out by inserting karamunting leaf powder into the maceration vessel and soaked with 96% ethanol for 3 x 24 hours at room temperature. Then, the solution was filtered to get a clear brownish liquid. The solvent was evaporated using a vacuum rotary evaporator and it was heated over a waterbath until all of the solvent has evaporated to get a thick and dark-greenish-brown karamunting leaf extract as much as 17 gr. Ethanol free test was carried out by adding Kalium Dichromate ($K_2Cr_2O_7$). Karamunting leaf ethanol extract 100% was then diluted using aquadest become 5%, 10%, 15%, 20%, and 25% concentrations. Calculation of concentration dilution was done with the following formula:

$$C1 \cdot V1 = C2 \cdot V2$$

Information:
C1 : Initial Concentration (%)
V1 : Initial Volume (ml)
C2 : Final Concentration (%)
V2 : Final Volume (ml)

Production of BHK-21 Fibroblast Cells

Fibroblast cells were taken from BHK-21 cell culture in cell-line form. Cell culture was carried out in a flask/roux bottle with Eagle's media and 10% FBS. Incubation using a $37^{O}C$ incubator for 48 hours. After the BHK-21 fibroblast cells adhered and filled the flask/roux bottle wall, the Eagle's media solution and FBS were removed. Next, the flask/roux bottle was washed with PBS 3 times to remove the remaining serum. ½ ml Trypsin versene was subsequently added to release cells from the bottle wall and to separate the bonds between cells. Then, cells were transferred to a microplate 96-well according to the number of samples using a multichannel micropipette.

MTT Assay Test of Karamunting Leaf Extract

BHK-21 fibroblast cells were prepared in a microplate 96-well. Each concentration of karamunting leaf extract and calcium hydroxide were added into the well. Incubate for 24 hours in a CO_2 incubator. After that, the sample was removed and Eagle's media solution and FBS were added. Then, the microplate was washed with PBS 3 times. Next, add 10 µL of MTT reagent for each well. Incubate again for 4 hours in a CO_2 incubator. After incubation, the MTT solution was removed and the reaction between MTT and cells was stopped using a DMSO stopper solution. The microplate was shaken for 5-10 minutes so that the reaction stopped evenly and purple formazan was released. Microplate 96-well was inserted into ELISA Reader with a wavelength of 620 nm. Absorbance value data was recorded. Calculation of the percentage of cell viability was done with the following formula:

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

The percentage of cell viability obtained from each sample, then cell proliferation value was calculated by entering the cell viability value into the following formula:

$$\% \text{ proliferation} = \% \text{ treatment viability} - \% \text{ cell control}$$

RESULTS

The results of the visual observation of MTT Assay after being treated with karamunting leaf extract and calcium hydroxide on BHK-21 fibroblast cells which were replicated 6 times can be seen in **Figure 1**.

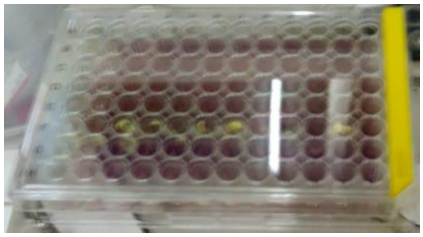

Figure 1. Microplate 96-well was Given Treatment and MTT Assay

The average of absorbance value (Optical Density) in all treatment groups on BHK-21 fibroblast cells that have been measured using the ELISA Reader can be seen in **Table 1**.

Table 1. Average Absorbance Value of All Treatment Groups

Treatment Group	Average Absorbance Value
EDK 5%	0,258
EDK 10%	0,261
EDK 15%	0,272
EDK 20%	0,300
EDK 25%	0,359
Positive Control	0,309
Cell Control	0,248

The percentage of BHK-21 fibroblast cells viability was obtained by entering the absorbance value into the formula. These results can be seen in **Table 2**.

Table 2. Percentage of BHK-21 Fibroblast Cells Viability

Treatment Group	Mean (%) ± Standard Deviation
EDK 5%	112,96 ± 10,51
EDK 10%	114,48 ± 7,63
EDK 15%	121,78 ± 12,04
EDK 20%	140,45 ± 16,52
EDK 25%	178,53 ± 15,82
Positive Control	146,21 ± 14,32
Cell Control	105,99 ± 16,67

The calculation of BHK-21 fibroblast cells proliferation was obtained by entering the cell viability value into the formula. These results can be seen in **Table 3**.

Table 3. Percentage of BHK-21 Fibroblast Cells Proliferation

Treatment Group	Mean (%) ± Standard Deviation
EDK 5%	6,97 ± 11,84
EDK 10%	8,50 ± 14,80
EDK 15%	15,80 ± 17,64
EDK 20%	34,46 ± 23,31
EDK 25%	72,55 ± 12,98
Positive Control	38,85 ± 24,24

The data obtained from each treatment group was tested for normality using Shapiro-Wilk. The results of the Shapiro-Wilk normality test in all treatment groups showed a significance value (p>0,05), so it can be concluded that the data were normally distributed. Furthermore, the data was tested for homogeneity using Levene's Test. The results of the Levene's Test homogeneity test showed a significance value (p>0,05), so it can be concluded that the data were homogeneous.

The research data were normally distributed and homogeneous, so it can be continued with the One Way Anova parametric statistical test. The results of the One Way Anova test showed a significance value of 0,000 (p<0,05), so it was stated that there was a minimum difference in one treatment group. Then Post Hoc Bonferroni test was continued to find out which groups gave a significant difference.

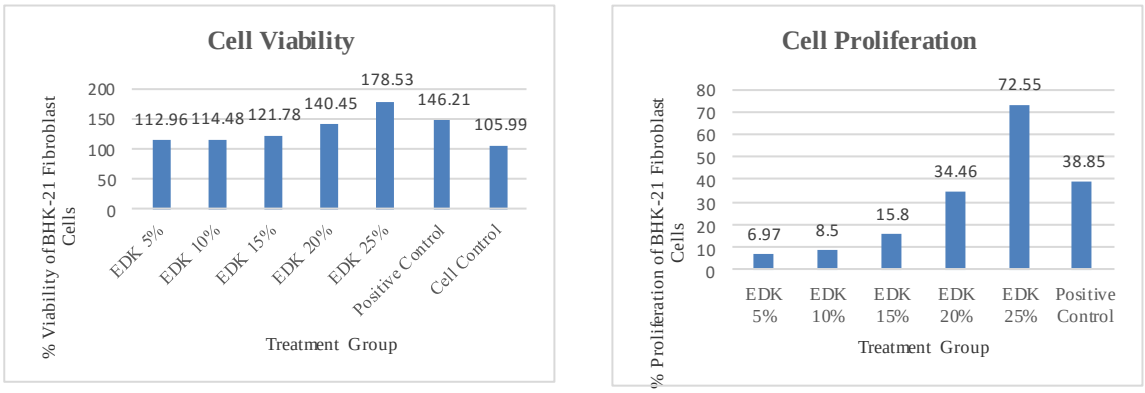

Figure 2. Graph of Viability and Proliferation of BHK-21 Fibroblast Cells in All Treatment Groups

Table 4. Results of Post Hoc Bonferroni Test for Viability of BHK-21 Fibroblast Cells

Treatment Group	(1)	(2)	(3)	(4)	(5)	(6)	(7)
(1) EDK 25%		0,001*	0,000*	0,000*	0,000*	0,005*	0,000*
(2) EDK 20%	0,001*		0,511	0,050	0,030*	1,000	0,002*
(3) EDK 15%	0,000*	0,511		1,000	1,000	0,084	1,000
(4) EDK 10%	0,000*	0,050	1,000		1,000	0,007*	1,000
(5) EDK 5%	0,000*	0,030*	1,000	1,000		0,004*	1,000
(6) Positive Control	0,005*	1,000	0,084	0,007*	0,004*		0,000*
(7) Cell Control	0,000*	0,002*	1,000	1,000	1,000	0,000*	

Information:
* : There is a significant difference (p<0,05)

Table 5. Results of Post Hoc Bonferroni Test for Proliferation of BHK-21 Fibroblast Cells

Treatment Group	(1)	(2)	(3)	(4)	(5)	(6)
(1) EDK 25%		0,015*	0,000*	0,000*	0,000*	0,046*
(2) EDK 20%	0,015*		1,000	0,284	0,202	1,000
(3) EDK 15%	0,000*	1,000		1,000	1,000	0,531
(4) EDK 10%	0,000*	0,284	1,000		1,000	0,104
(5) EDK 5%	0,000*	0,202	1,000	1,000		0,072
(6) Positive Control	0,046*	1,000	0,531	0,104	0,072	

Information:
* : There is a significant difference (p<0,05)

The results of the Post Hoc Bonferroni test in table 5 showed that there was a significant difference (p<0,05) in the proliferation of BHK-21 fibroblast cells of karamunting leaf extract 25% concentration compared to karamunting leaf extract 20%, 15%, 10%, and 5% concentrations also positive control. There was no significant difference in the proliferation of BHK-21 fibroblast cells of karamunting leaf extract 20%, 15%, 10%, and 5% concentrations also positive control.

DISCUSSION

MTT Assay showed that all treatment groups were produced purple color. The purple color produced is proportional to the number of living cells. The color change occurs because the active metabolic substance of BHK-21 fibroblast

cells reacted to tetrazolium salt (initially yellow) to produce a purple formazan product. Observation of the purple color intensity (colorimetry) in a microplate 96-well was seen by a spectrophotometer with ELISA Reader.[14] The results were then calculated using the formula.

The calculation results showed that all treatment groups had cell viability values higher than cell control or more than 100% and cause the proliferation of BHK-21 fibroblast cells. Based on statistical results showed that there was a significant difference between karamunting leaf extract 25% concentration compared to positive control (calcium hydroxide), which means that karamunting leaf extract 25% concentration was more effective in causing the proliferation of BHK-21 fibroblast cells.

Karamunting leaf extract play a role in maintaining the viability of BHK-21 fibroblast cells through the content of active compounds such as flavonoid, tannin, and saponin. Flavonoid and tannin are the most abundant compounds in karamunting leaf.[9,10] The ability of flavonoid to maintain the viability of BHK-21 fibroblast cells is by activating Ca^{2+} in mitochondria so that cells are able to produce ATP and survive. Mitochondria are organelles that act as centers of cellular energy metabolism and storage of Ca^{2+}. Calcium (Ca^{2+}) plays a role in regulating the function of mitochondria and stimulates ATP synthesis.[15]

The ability of tannin to maintain the viability of BHK-21 fibroblast cells is through their antioxidant activity. Antioxidant in karamunting leaf function as inducer for the expression of antioxidant enzymes through the activity of Nuclear Factor Erythroid 2-related factor 2 (Nrf2) which is located in the cytoplasm, when exposed to compounds that act as inducer, Nrf2 will translocate to the nucleus. In the nucleus, Nrf2 will bind to the Antioxidant Response Element (ARE) and will produce antioxidant enzymes, namely Superoxide Dismutase (SOD), Catalase (CAT), and Glutathione Peroxidase (GPx). Antioxidant enzymes play a role in neutralizing free radicals, so that maintaining cell viability or the ability of cells to survive.[16,17]

Karamunting leaf extract play a role in stimulating the proliferation of BHK-21 fibroblast cells through the content of active compounds such as flavonoid, tannin, and saponin that can increase the production of growth factors. Growth factors play a role in stimulating the growth and proliferation of fibroblast cells. Some of the growth factors include Transforming Growth Factor Beta (TGF-β), Fibroblast Growth Factor Beta (FGF-β), Epidermal Growth Factor (EGF), Vascular Endothelial Growth Factor (VEGF), and Platelet-Derived Growth Factor (PDGF).[3] TGF-β and FGF-β were involved in cell proliferation.[18,19] TGF-β is an enabling signal molecule that induces cell proliferation and chemotaxis. TGF-β is produced in an inactive form and will activated when it binds to its receptor. Activation of TGF-β will produce signaling pathways that induce cell differentiation and proliferation.[19] The active compounds in karamunting leaf will activate the TGF-β signaling pathway and increase the ability of its receptors to bind to TGF-β, so that more TGF-β is activated to stimulate cell proliferation.[20]

Cell proliferation by FGF-β occurs through the interaction between FGF-β ligand and FGF Receptor (FGFR). The interaction between the ligand and the receptor will induce receptor transphosphorylation. Receptor transphosphorylation is the cascade of molecules activation to form a transduction signal (intracellular signal communication cascade) to the nucleus and elicited a cellular response. This process will stimulate the activation of Mitogen Activated Protein Kinase/Extracellular Signal-Regulated Kinase (MAPK/ESRK) and Phosphoinositide-3 Kinase/Protein Kinase B (PI3K/Akt). MAPK/ESRK and PI3K/Akt are signaling pathways that play a role in cellular processes such as cell differentiation and proliferation.[18,19]

The results of this study can be used as a reference for further research to develop alternative choices of direct pulp capping materials to accelerate the healing process of pulp tissue made from karamunting leaf extract. The conclusion of this study is that karamunting leaf extract 5%, 10%, 15%, 20%, and 25% concentrations also calcium hydroxide can maintain viability and cause proliferation of BHK-21 fibroblast cells. Karamunting leaf extract 25% concentration was more effective in causing the proliferation of BHK-21 fibroblast cells.

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