THE TOXICITY TEST OF CHANNA STRIATA SCALE CHITOSAN ON BHK-21 FIBROBLAST CELLS IN VITRO

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INTRODUCTION

Oral and dental health is referred to as a port ofentry in supporting holistic health. Diseases that may infect the teeth and oral cavity are commonly found in people around the world, including in Indonesia. Basic Health Research data indicated that the percentage of the population with dental and oralproblems in 2013 was amounted to 25.9% and showed an increase of 57.6% in 2018.[1,2]

Dental and oral problems may be prevented by routinely using mouthwash, toothpaste, and others[3]. Until the present time, a number of research on various uses of chitosan have been widely conducted. Chitosan has been proven capable of being used as a bone graft scaffold for bone cell regeneration and cosmetics for skin cell regeneration. Chitosan in haruan fish scales (*ChannaStriata*) is considered as an ingredient that naturally provides antibacterial and antioxidant properties. Therefore, haruan fish scale chitosan is highly potential to be used as a safe biomaterial for the human body.[4,5,6]

Haruan fish scale chitosan is generated from chitin from haruan fish scales which is processed through deproteination, demineralization, and deacetylation. Haruan fish (*Channa striata*) is commonly recognized as a type of fish that may be found throughout Indonesia, particularly in South Kalimantan, because the fish has been referred to as a popular food ingredient.[7,8] The body part of haruan fish that may be consumed is only amounted to 40-50%, and the rest should be disposed of as waste. Chitosan has a very strong positively charged amine (-NH2) functional group which may attract

negatively charged amino acid molecules that form proteins in bacteria which causes protein denaturation on the bacterial cell wall and results in bacterial cell death.[4,6,9,10]

Pigure 1. Haruan Fish.

Materials that enter and come into contact with the oral cavity should not contribute to any side effects that are detrimental to the local and systemic biological environment, which subsequently requires toxicity tests to be carried out on certain compounds.[11] Prior to use in humans, a material must be initially evaluated by means of a toxicity test. The recommended test medium to be used is Baby Hamster Kidney 21 (BHK-21) fibroblast cells.[12] The method that has been widely used for toxicity testing is the MTT assay method. The MTT assay is highly sensitive to evaluate cell viability. MTT assay method tends to be considered valid and relatively faster than other methods. Live cells with active metabolism tested with MTT will result in changes in MTT to purple formazan products, while dead cells may not be able to convert MTT into purple formazan.[12,13] The toxicity test parameter is referred to as the Inhibitory Concentration 50 (IC_{50}) value, which is defined as the concentration value that inhibits growth in cells by 50% of the cell population and proves the potential toxicity of a compound to cells..[14] Toxicity categories for natural materials proposed by Balantyne (1999) in Mardja etal (2016) can be seen in the following table.[15]

Table 1. Toxicity Classification For Natural Ingredients.

IC_{50}	**Category**
10μg/mL (10^6 Sel/mL) < IC50	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	Not Toxic

Research on the toxic effects of chitosan has been commonly carried out. Referring to a previous research that tested the toxicity of shrimp shell chitosan (*Litopenaeus vannamei*) on BHK-21 fibroblast cells with concentrations of 25%, 50%, 75%, 100%, it was found that shell chitosan (*Litopenaeus vannamei*) was not toxic to BHK-21 fibroblast cells.[16] However, there has been no research focused on the toxicity test of haruan fish scale chitosan (*Channa striata*) until recently. Based on the description above, this research was conducted to determine whether the chitosan of haruan fish scales (*Channa striata*) was toxic to BHK-21 fibroblast cells at concentrations of 25%, 50%, 75%, and 100%.

RESEARCH METHODS

This research is true experimental with a post test only research design with control group design which has been declared ethically feasible by the Health Research Ethics Commission, Faculty of Dentistry, Lambung Mangkurat University with No. 050/KEPKG-FKGULM/EC/III/2021. The population in this study were BHK-21 fibroblast cells consisting of six groups; Four groups were given treatment with haruan fish scale chitosanwith concentrations of 25%, 50%, 75%, and 100% with a minimum of five repetitions per treatment group and two control groups consisting of control media and control cells.

Production of Kitosan Solution

Haruan fish scale chitosan obtained at the Biomedical Laboratory, Faculty of Dentistry, Lambung Mangkurat University was dissolved using 1% acetic acid by utilizing the formula % = B/V for each concentration required in this research.

Production of BHK-21 Fibroblast Cells

BHK-21 fibroblast cell culture was carried out in a flask/roux bottle by inserting eagle's media and 10% FBS which was incubated for a period of 24 hours using a 37° incubator. After the cells had filled the walls of the flask/roux bottle, the eagle's media and 10% FBS were removed and the flask/roux bottle was cleaned using ½ ml trypsin versene to remove BHK-21 fibroblast cells adhering to the bottle wall. The fibroblast cells were then transferred to a 96-well microplate for toxicity testing.

Toxicity Test of Haruan Fish Scale Chitosan

BHK-21 fibroblast cells on a 96-well microplate were treated with haruan fish scale chitosan, cells were incubated with a CO^2 incubator for 24 hours, the sample was subsequently poured into a container and was added with eagle's media and 10% FBS. The microplate was washed three times by using PBS to purge the remaining serum from the microplate. 10 μl of MTT reagent was added to each well and incubated again in a CO^2 incubator for 4 hours. The MTT reagent was then discarded and a DMSO stopper was added to each well to stop the reaction between MTT and cells. The microplate was shaken for 5-10 minutes and then inserted into an ELISA reader with a wavelength of 620 nm to read the viability of BHK-21 fibroblast cells after treatment. The reading

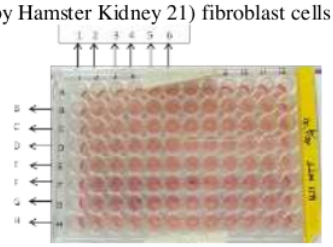
was successfully carriedout by calculating the cell viability value using the following formula:

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

Based on the above formula, the percentage of cell viability from each treatment was successfully obtained. The data were then analyzed by means of probit analysis by using SPSS software.

RESULTS

The results of MTT staining on 96 Well Microplates that had been treated with chitosan of haruan fish scales with concentrations of 25%, 50%, 75%, and 100%, indicated a color change to dark purple. This may be stated that the dark purple color that is mostly produced will lead to many live BHK-21 (Baby Hamster Kidney 21) fibroblast cells.



Pigure 2. *96-well Microplate After Giving Haruan Fish Scales Chitosan and MTT Assay*

Information :

B_1, C_1, D_1, E_1 : Control Sample Concentration 25%, 50%, 75%, 100%

H_2-H_6 : Medium ControlF_1-F_6, G_1-G_6 : Cell Control cells.

Toxicity test research is declared to show theIC_{50} (Inhibitory Concentration) value, if a concentration that causes cell growth inhibition is detected by 50% of the population. Data obtained from this research were then analyzed by utilizing probit analysis which determines IC_{50} byusing SPSS software.

Table 3. IC_{50}Probit Analysis

	95% *Confidenc*	*e Limit*for Co	ncentratio n	
	Probability	***Estimate***	***Upper Bound***	***Lower Bound***
Probit	0.5	684871.064	-	-

Table 3 above shows the IC_{50} value which was amounted to 684871.064 μg/mL. According to Balantye in Mardja et al (2016), IC_{50}> 1000 μg/mL is categorized as non-toxic. Referring to the results above, it may be inferred that the chitosan of haruan fish scales was not toxic to BHK-21 fibroblast cells.

DISCUSSION

The research on the toxicity test of haruan fish scale chitosan was successfully carried out using the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazo-liumbromide) assay method in vitro. This research was conducted to obtain the IC_{50} (Inhibitory Concentration) value, specifically the concentration that was able to inhibit cells by

B –B : Sample Concentration 25% 50% of the population.[17] The IC_{50} value may be[2] [6] used to determine the toxicity of chitosan of

C_2-C_6 : Sample Concentration50%

D_2-D_6 : Sample Concentration75%

E_2-E_6 : Sample Concentration100%

Table 2. Average Viability of Fibroblast Cells BHK-21

Haruan Fish Scale Chitosan Concentration	Average Viability of Fibroblast Cells BHK-21	Percentage of Decreased Cell Viability
25%	2,106%	97,894%
50%	12,01%	87,99%
75%	77%	23%
100%	80,194%	19,806%

The average percentage of viability of BHK-21 fibroblast cells after being treated with haruan fish scale chitosan at concentrations of 25%, 50%, 75%, and 100%. Having regard to the results above, it wasfound that every increase in the concentration of chitosan in fish scales was followed by an increase in the percentage of viability of BHK-21 fibroblast haruan fish scales to BHK-21 fibroblast cells.

The principle of the MTT assay is the reduction of the yellow colored MTT (3-(4,5-dimethylthiazole-2-yl)-2,5 diphenyltetrazoliumbromide) tetrazolium salt bythe reductase system. Living cells exposed to MTT reagent will produce formazan salts, particularly succinate tetrazolium in the mitochondrial respiration chain which produces dehydroginase enzymes, which then form purple formazancrystals and are insoluble in water. Therefore, the increasing intensity of the purple color produced may increase the number of living cells.[18,19]

Absorbance data of cells with five times replication at each concentration of chitosan of haruan fish scales and control samples to obtain variations in cell viability values. The data were used to calculate the average percentage of cell viability. The data generated by the calculation of the percentage of cell viability were entered into the SPSS application to calculate the IC_{50} value as

shown in Table 3.

The hypothesis of this research proved that the chitosan of haruan fish scales was capable of reducing the viability of BHK-21 fibroblast cells. The decrease in cell viability at a concentration of 25% was amounted to 97.894%, and decreased by 87.99% at a concentration of 50%, decreased by 23% at a concentration of 75%, and at a concentration of 100%, it was decreased by 19.806%. This is in line with a research conducted by Guo Zhanyoung et al (2011), which indicated that the lower concentration will lead to the relatively low antioxidant activity, while the increasing concentration will result in higher antioxidant activity.[20]

Chitosan has a positively charged NH2- amine group which will cause denaturation of the cell wall that may change the permeability and result in disruption of the flow of intracellular components, which will lead to cell death when interacting with negatively charged cell membranes. Positively charged amino groups in chitosan that interact with negatively charged cell membranes will also form a layer that is capable of inhibiting ion channel transformation and inhibiting the work of enzymes in cells, so that cells will lack any nutrients and may contribute to cell death.[15]

Haruan fish scale chitosan contains antioxidants that may reduce the activity of free radicals such as hydrogen peroxide, superoxide anions, and Cu2+ ions by binding to free radical ions. Antioxidants in chitosan have two types of mechanisms in inhibiting the oxidation process or stopping the reaction of free radicals. The first mechanism is carried out by removing electrons from antioxidants to bind to free radicals and turn them into stable molecules. The second mechanism is referred to as the process of releasing hydrogen ions from antioxidants that may bind to free radicals. The human body naturally has an antioxidant system, but if the free radicals in the body increase, the body will need additional antioxidants. A research conducted by Putri et al. (2020) indicated that the chitosan of haruan fish scales at concentrations of 20%, 25%, 30%, and 35% had antioxidant potential with a value of IC_{50}> 200 ppm which is classified as weak. Therefore, chitosan withhigher concentration is highly required to produce stronger antioxidant effect.[6]

The results of this research showed that the increasing concentration of chitosan of haruan fish scales would result in a darker purple color, which means that the viability of BHK-21 fibroblast cells would continuously increase with the addition of the concentration of chitosan of haruan fish scales. The more MTT reactions that are exposed to living cells, the more formazan salt or tetrazolium succinate which will produce dehydrogenase enzymes with a purple color and insoluble in water in living cells. The purple color of this dehydrogenase enzyme is defined as a reference to determine cell viability.[17,18]

The results of the research carried out by Saputra and Kroesnadi (2020) found that chitosan was not toxic to BHK-21 fibroblast cells.[15] Chitosan toxicity may be determined by identifying the degree of deacetylation, a degree of more than 35% will indicate low toxicity, whereas if the degree of deacetylation is below 35%, the toxicity depends on the dose of chitosan used. In regards to SNI, the value of the deacetylation degree of chitosan is 75%, while the chitosan in haruan fish scales has a degree of deacetylation of 85.25%, which indicates low toxicity.[6,19] Based on probit analysis, the value of IC_{50} > 1000 μg/mL was successfully obtained. Therefore, it can be concluded that haruan fish scale chitosan did not provide any toxicity to BHK-21 fibroblast cells. The IC_{50} value obtained in this research may be used as a partial reference for further research.

The biocompatibility of chitosan depends on the source of chitosan, the degree of deacetylation, the concentration of chitosan, and the method of manufacture. Chitosans from different sources have been found to have benefits in dentistry, specifically as antibacterial agents and as bone healing or regeneration materials..[21,22] Chitosan may be used as a biomaterial in bone graft scaffolds which contributes an important role in bone cell regeneration due to its biocompatible, biodegradable, and bioresorbable properties. Chitosan may also accelerate the growth and regeneration of new bone cells, because it has the same structure as the glycosaminoglycans and hyaluronic acid found in cartilage. A research conducted by Hutami et al (2020), Widyaningrum et al (2020), and Dania et al (2020) showed that fish scale chitosan was capable of inhibiting the growth and killing of Streptococcus sanguinis, Staphylococcus aureus, and Phorpyromonas gingivalis bacteria.[4,9,10] Chitosan has a chitin nanofibril structure similar to hyaluronic acid. This causes chitosan to be used as a cosmetic ingredient, particularly as an ingredient for repairing the skin barrier and effective anti-aging compounds.[3,23] Based on the research that had been carried out, it was concluded that the chitosan of Haruan fish scales at concentrations of 25%, 50%, 75% and 100% could reduce cell viability, and based on the IC_{50} parameter, chitosan of haruan fish scales was generally shown to have no toxic effect on BHK-21 fibroblast cells.

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