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TOXICITY TEST OF GARLIC EXTRACT ON VERO CELLS *IN VITRO*

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ABSTRACT

Background: *Denture stomatitis is an inflammation that occurs in the mucosa covered by dentures that is often attributed to increased colonization of the yeast Candida albicans that predisposes bacterial infections. Denture Stomatitis can occur due to the lack of awareness in maintaining denture hygiene. One alternative natural ingredient to serve as denture cleanser is garlic extract. Several studies have shown that garlic extract, contains flavonoids, essential oils, tannins and, mostly, allicin, exerts antibacterial and antifungal properties.* ***Purpose:*** *To investigate whether garlic extract (Allium Sativum L) is toxic to Vero cells using the MTT assay method.* ***Method:*** *This study was a purely experimental study, conducted using posttest-only with control group design. The samples were divided into 8 groups, consisted of several concentrations of garlic extract, including 0.5%, 1%, 2.5%, 5%, 7.5%, 10%, 12.5% and 15%, and 2 control groups, including control media and cell control. The absorbance was read using ELISA reader and the cell viability was calculated.* ***Result:*** *The value of Inhibitory Concentration (IC_{50}) of garlic extract on Vero cells was obtained from the spss probit test that resulted in a value of 18613.782 µg/mL.* ***Conclusion:*** *Garlic extract does not have a toxic effect on Vero cells as indicated by the IC_{50} value of >1000 µg/mL.*

Keywords: *allicin, garlic extract, toxicity test, Vero cell.*

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INTRODUCTION

Oral hygiene is one of the important factors preserving dental and oral health.[1] Poor oral health can lead to various oral diseases, including periodontal disease and dental caries. Diseases affecting the oral cavity cause pathological dental loss.[2] Dental loss must be replaced immediately using artificial denture as a preventive strategy for degenerative alterations attributed to untreated dental loss.[3]

Denture appliance will always make direct contact with saliva, food, as well as drink that predispose to plaque accumulation. Routine care and cleansing of the denture base are exceptionally necessary to wash out the plaques, calculi, and staining. The lack of awareness for regularly cleaning the dentures exhibits plaque accumulation that subsequently results in unpleasant odor, inflammation of the oral cavity, and the development of denture stomatitis.[4] Denture stomatitis is an inflammation occurred in the oral mucosa covered by implanted dentures, which is usually attributed to the colonization of yeast *Candida albicans* that increase the risk of bacterial infection.[5]

Denture stomatitis can be prevented by maintaining oral hygiene.[6] One of the popular materials used for denture cleanser is chemical polident, that is currently available on the market. As a denture cleanser, the effectiveness of polident relies on its active compound, hydrogen peroxide. This basic compound triggers physical alteration on the denture's surface. Recently, natural, herbal-based materials have been developed for being alternative options to traditional disinfectant and antiseptic agents.[7] One of the investigated material is garlic (*Allium sativum L*) extract. Garlic is known to contain number of beneficial compounds, such as allicin, flavonoid, essential oil, and tannin.[8]

According to Akinmusire et al., (2014), garlic has a lot of therapeutic activities, including antiviral, antifungal, antiparasitic, antibacterial, antioxidant, and antimicrobial activities. Zohra (2013), in her study, reported that garlic (*Allium*

sativum L) extract was found to inhibit the growth of *Candida albicans.*

Herbal plants are frequently used as therapeutic agents and its use has been limited only when the safety profile is confirmed through toxicity analysis, so that any severe adverse effect can be prevented. One of the most convenient methods used for assessing the safety of herbal plant is Methythiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay, as it is generally more sensitive, accurate, and less time-consuming.[10] MTT assay is an in vitro technique performed using Vero cell culture.

According to the aforementioned explanation, it is necessary to conduct research investigating whether garlic (*Allium sativum L*) extract is toxic to Vero cells using MTT assay. This study aims to demonstrate the toxicity level of garlic (*Allium sativum L*) extract on Vero cells.

MATERIALS AND METHODS

This study started after receiving an official statement of approval and ethical clearance from Research Ethical Committee of Faculty of Dentistry, Lambung Mangkurat University with reference No. 142/KEPKG-FKGULM/EC/I/2019. This true experimental study was performed using post-test-only with control group design. The study population included Vero cells population divided into 10 groups that comprised of 8 intervention groups receiving different concentrations of garlic extract (0.5%, 1%, 2.5%, 5%, 7.5%, 10%, 12.5% and 15%) and 2 control groups (medium and cell controls). The experiment was repeated 3 times for each group that was determined using federer formula.

Making Garlic Extract Using Blender Method

The first step in making garlic extract is chosen the fresh garlic, characterized by a hard tuber, clear skin, and hard texture. The skin was peeled and the garlic meat was then washed and followed by mixing it with aquadest for a total of 1 kg until even (homogeny) using a blender. The mixture was then centrifuged at 3000 rpm for 30 minutes and filtered using witmann filter paper no. 1. The content of the garlic extract can survive for 32 days when it is stored at 15^{o}C.[11] The filtration process resulted in an extract with 100% concentration that was diluted with DMSO solution until reaching the desired concentrations (0.5%, 1%, 2.5%, 5%, 7.5%, 10%, 12.5% and 15%) using the following formula: C1.V1 = C2.V2.[12]

Making the Vero Cells

Vero cells originated from kidney tissue of African green monkey (*Cercopithecus ethiops*) which, in a frozen state, was melted in a water bath at the temperature of 37^{o}C. Vero cells were cultured in a flask using M199 and FBS 10% medium and were incubated using incubator at 37^{o}C for 48 hours. After filling the flask wall, the M199 and FBS 10% solutions were removed and the colony was washed using PBS solution for disintegrating the cell clots. One millilitre of the trypsin-EDTA solution was added and the mixture was then incubated at 37^{o}C for 2-3 minutes until the reaction indicating cell detachment from the flask was seen. Lastly, M199 and FBS 10% solution was added to inactivate the trypsin-EDTA solution.[13]

Garlic Extract Toxicity Testing

Around 100μL of Vero cells were transferred into each microplate 96 well. During each filling process, re-suspension was performed to maintain its homogeneity. The solution was then incubated at 37^{o}C for a minimum of 4 hours (to let the cells recover after harvest). The experiment was performed once the cells regained its stability. The serial concentrations of the garlic extract were assigned for each group (including the cell and medium controls). Each of the assigned concentrations were poured into the wells and stored in a CO_2 incubator for 24 hours before experiment. MTT reagent was added into the wells, including the controls, and all were incubated again in the CO_2 incubator for other 4 hours. In addition, 0.1% stopper solution was added using DMSO to terminate the reaction between the MTT and the cells. Microplates were shaken for 5-10 minutes before being moved into the ELISA reader for assessing the cellular viability. Calculation of the cellular viability percentage was performed using the following formula:[13]

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

According to the calculation using the formula, the percentage of the viable cells of each extract concentration was obtained. The data was analyzed using probit analysis to determine the IC_{50} value using SPSS software. A material is considered non-toxic when the IC_{50} value exceeds 1000 µg/mL.[14]

RESULTS

During the extraction process, a total of 1 kg garlic was processed into 100 ml extract. In this study, the blender technique was used for garlic extraction by mixing it with aquadest as the solvent. Following the extraction process, 100% garlic extract was obtained. This extract appeared as a white-yellowish solution (Figure 1).

Figure 1. Garlic extract

Garlic toxicity test was performed using series of different concentrations, including 0.5%, 1%, 2.5%, 5%, 7.5%, 10%, 12.5%, and 15%, on the Vero cells, which was previously stored in the microplate 96 wells; the reaction between two components caused change in its color into violet that indicates viable Vero cells (Figure 2). From the calculation, it was noted that every increase in the garlic extract concentration causes a decrease in the meant percentage of viable cells. The higher the concentration of the garlic extract, the lower the percentage of cell viability of the Vero cells. Garlic extract toxicity is expressed in the percent of viable cells, presented as the mean value obtained from three experimentations (Table 1 and Figure 3).

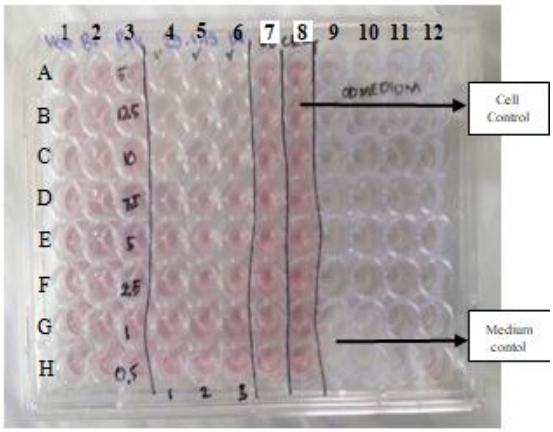

Figure 2. A_4, A_5, A_6 (Garlic Extract Concentration of 15%), B_4, B_5, B_6 (Garlic Extract Concentration of 12.5%), C_4, C_5, C_6 (Garlic Extract Concentration of 10%), D_4, D_5, D_6 (Garlic Extract Concentration of 7.5%), E_4, E_5, E_6 (Garlic Extract Concentration of 5%), F_4, F_5, F_6 (Garlic Extract Concentration of 2.5%), G_4, G_5, G_6 (Garlic Extract Concentration of 1%) H_4, H_5, H_6 (Garlic Extract Concentration of 0.5%), A-I_7, A-I_8 (Cell Control), I_9, I_{10}, I_{11} (Medium Control).

Table 1. Viability of Vero Cells

Concentration µg/mL	Viability (%)
0.5%	71.692%
1%	56.615%
2.5%	55.077%
5%	29.486%
7.5%	28%
10%	20%
12.5%	17.846%
15%	17.538%

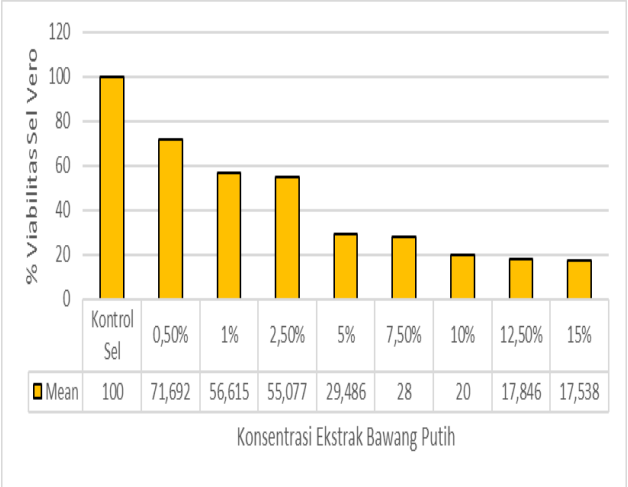

Figure 3. The Mean Number of Viable Vero Cells in Each Intervention

The results of the toxicity testing are expressed in the form of IC50 value. The IC50 value was enumerated based on the extract concentration and the percent of the viability of the Vero cells from three consecutive replications as presented in Table 1. The IC50 value was analyzed using probit analysis resulting in a value of 18613.782 µg/mL.

Table 2. Probit Analysis

Probability	**95% Confidence Limits for Concentration**		
	Estimate	Lower Bound	Upper Bound
.500	18613.782	7238.574	845703.562

According to Balantye in Mardja et al., (2016), if the analysis results in IC_{50} > 1000 µg/mL, the material is considered non-toxic; hence, it can be

concluded that garlic extract does not possess any toxic effects on Vero cells.

DISCUSSION

This study aimed to investigate garlic extract toxicity on Vero cells and determine the IC_{50} value after administration of garlic extract using MTT assay. The fundamental principle of MTT assay is the presence of reaction that causes reduction of the yellow formazan crystal into the bluish violet contents inside the mitochondria of viable cells. Viable cells, that experience active metabolism, convert MTT into a formazan product (tetrazolium crystal), which is purple in color and has maximum absorption capacity.[14] MTT is absorbed into the viable cells and is broken via a reduction reaction by a reductase enzyme in the respiratory chain reaction of the mitochondria. It is converted into water-insoluble formazan.[15]

The result showed that 0.5%, 1%, 2.5%, 5%, 7.5%, 10%, 12.5% and 15% garlic (*Allium sativum L*) extract did not exert any toxic effect of Vero cells, as reflected by the result from probit analysis showed the IC_{50} value of 18613.782 µg/mL. This study proved that the highest percent of viability of the Vero cells was found in the 0.5% concentration of the garlic (*Allium sativum L*) extract which was 71.69%, followed by 1% and 2.5% with the percent of viability of 56.615% and 55.077%, resepectively.

Decreased cell viability can be explained by the fact that high concentration produces more viscous solution, compare to the lower concentration, lead to an alteration in the cell membrane capacity and inhibition of cell proliferation as well as the disintegration of the cell membrane that eventually direct to the cell death. Dilution into a lower concentration makes the solution more soluble in the medium and enhance cell adaptation and growth.[16,17]

The compounds contained in garlic include allicin, flavonoid, essential oil, and tannin.[18] The allicin content of the garlic extract is one of the most active components and can reach as much as 82% of the total organic sulfur contained in garlic.[19] At several concentrations of garlic extract tested on Vero cells, the percent of viability were mostly below 50%. This might be due to the pro-oxidant nature of allicin that is found to be dominant at certain concentration. Allicin triggers apoptosis by producing ROS and free radical that are unstable in nature and lead to oxidative stress.[20] Stress-related conditions escalate the oxidation process and further cause cell damage. Loss of cellular integrity causes apoptosis and alteration in cell morphology.[21]

The cell will routinely produce ROS (Reactive Oxygen Species) and free radical, that will greatly affect the viability of a cell; therefore, antioxidant is extremely needed for a cell to survive.[22] Beside of having antifungal, antibacterial, and antiviral effects, garlic extract also exerts antioxidant effect that increase the level of antioxidant enzymes, named superoxide dismutase (SOD), catalase (CAT), and glutathione peroxide (GPx). This group of enzymes converts ROS and free radical into balanced compounds.[23,24] When a cell experiences oxidative stress, the allicin content in garlic activates Nrf2 that can translocate from Keap-1 to the cell nucleus and activate antioxidant response element (ARE) which capable in producing a variety of antioxidant enzymes, such as SOD, CAT, and GPx. SOD is worked by catalyzing superoxide into H_2O_2 (Hydrogen peroxide), a non-radical form of ROS that are reactive, and oxidant in nature and is subsequently re-catalyzed by CAT and GPx into $H_2O + O_2$ as its end-product balanced compounds and further direct cells to grow.[25] Therefore, allicin in garlic extract is contributed to the viability of Vero cells. According to the results of the IC_{50} analysis, it can be concluded that garlic (*Allium sativum L*) extract is not toxic to Vero cells, as indicated by the IC_{50} value of >1000 µg/mL, which was 18613.782 µg/mL.

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