IOP Conference Series: Earth and Environmental Science

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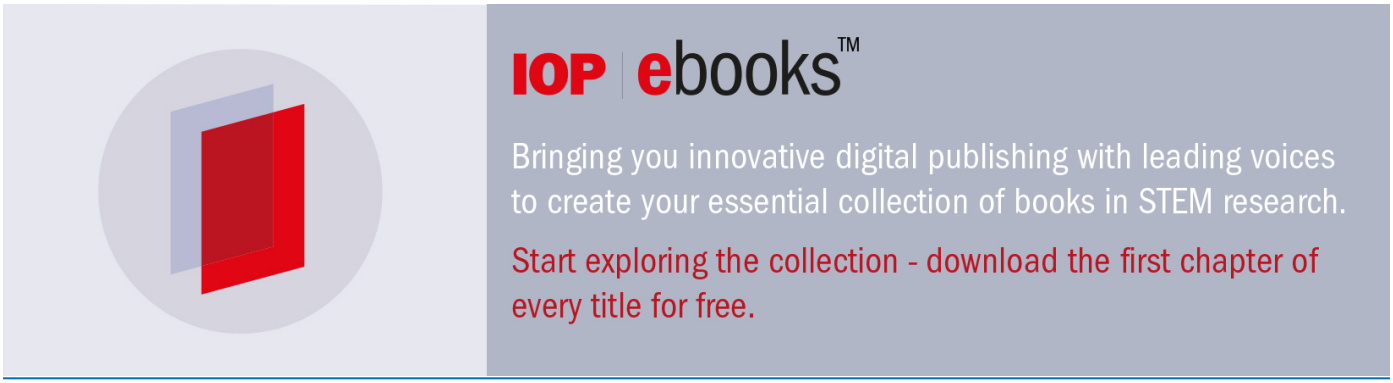
To cite this article: Sularsih *et al* 2018 *IOP Conf. Ser.: Earth Environ. Sci.* **217** 012030





# Cytotoxicity of Combination Chitosan with Different Molecular Weight and Ethanol Extracted *Aloe vera* using MTT Assay

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**Abstract.** The use of combination chitosan and ethanol extracted *Aloe vera* is considered to have activity to promote bone healing. The requirement dental material used in oral cavity include good biocompatibility, non toxic, non carcinogenic and nor allergenic response. The aim of this study was to examine the cytotoxicity combination chitosan with different molecular weight and ethanol extracted *Aloe vera* using MTT assay. 1 % chitosan gel (w/v) was made from chitosan powder with high (ChH) and low (ChL) molecular weight. 1 % chitosan gel was combined with ethanol extracted *Aloe vera* with concentration 50 % ($Av_1$) and 75% ($Av_2$). It divided five groups: control group, ChH-$Av_1$, ChH-$Av_2$, ChL-$Av_1$ and ChL-$Av_2$. Each of sample was immersed in eppendorf microtubes consist of media culture. After 24 hours, the immersion of media culture were used to investigate the cytotoxic effect to BHK-21 cell lines by MTT assay method. The density of optic formazan was indicated the number of living cells. All data were statistically analyzed by one-way Anova. The result showed that percentage of the living cells of all groups was uper 50 % using parameter $CD_{50}$. There was no significant difference among treatment groups. It can be concluded that combination chitosan with different molecular weight and ethanol extracted *Aloe vera* were non toxic for BHK-21 cell culture.

**Keyword :** Cytotoxicity, Chitosan, Ethanol, *Aloe vera*, MTT Assay

## 1. Introduction

Lidah buaya (Indonesian) or *Aloe vera* is natural herbal plant that easy grow in Batu, Malang, East Java, Indonesia. Batu's climate is classified as tropical. The average temperature in Batu is 21.4 °C and average annual rainfall is 3416 mm.[1] *Aloe vera* is called "the plants immortality" are proven use as herbal remedy in dentistry as anti inflamatory, antiviral, antibacteria and antioxidative.[2] It contain anthraquinones components namely aloin, aloe emodin, and barbaloin, carbohydrate, acetyl mannan glukomannan, alkaline phosphatase, bradikinase enzymes, healing hormones auxin, gibberellin, saponins and protein lectin that can enhance tissue and bone healing proces.[2; 3]

In the present dentistry study, *Aloe vera* stimulating PDLC proliferation, expression of Runx2, GDF-5, BMP-2, VEGF, type I collagen, ALPase activity, and mineral deposition. Runx2 is a transcription factor considered to be a regulator of osteoblast/cementoblast diff erentiation and function.[4] BMP-2, GDF-5 and VEGF are important growth factors for periodontal tissue and bone healing. Ethanol extracts *Aloe vera* have strongest antibacteria and antioxidative.[5; 6]

Chitosan is a linear polysaccharide derived from partial deacetylation of chitin. Chitosan have great utility because of their biological activities such as biocompatibility, biodegradability, non-toxicity and adsorptive abilities, as well as chemical applications, mainly in the medical and pharmaceutical fields. The characteristic properties of chitosan are related to its weight averaged molecular weight

(MW).[7] In vitro biological performance combination chitosan and *Aloe vera* offer good enviroment for hDF attachment, proliferation and viability. It has good celluler response, suitable mechanical and physical properties.[8]. In our preview study, the aplication of gel combination chitosan and ethanol extracted *Aloe vera* both concentration 50 % and 75 % increased fibroblast proliferation and collagen type 1 on wound healing process of traumatic ulser.

The requirement dental material used in oral cavity include good biocompatibility, non toxic, non carcinogenic and nor allergenic response. Therefore toxicity testing is needed.[9; 10]. The aim of this study is to examine the cytotoxicity combination chitosan with different molecular weight and ethanol extracted *Aloe vera* using MTT assay.

## 2. Experimental Method

The leaves of the plant *Aloe vera* L obtained from Batu, Malang, Jawa timur, Indonesia. Chitosan powder purchased from Sigma chemical, St. Louis, USA. The degree of deacetylation was more than 75 %. Chitosan powder with high molecular weight, 310.000 – 375.000 Da and chitosan powder with low molecular weight 50.000 – 190.000 Da.

The dried *Aloe vera* leaves were finely chopped and then soaked with 70% ethanol solvent in the macerator for 2 × 24 h. The macerate was thickened with a rotary evaporator and evaporated over a water bath to remove the residual solvent to obtain a thick viscous extract with a constant weight**.** This research using ethanol extracts *Aloe vera* with concentration was 50 % and 75 %. Chitosan gel was made from chitosan powder with high (ChH) and low (ChL) molecular weight. 1% chitosan gel (w/v) was made with diluted one gram of chitosan powder in 100 ml 2 % acetic acid. It added with NaOH solution to get neutral pH. The mixture was stirred until the gel was completely formed. 1% chitosan gel was combined with ethanol extracted *Aloe vera* with concentration 50 % ($Av_1$) and 75% ($Av_2$). Combination chitosan and extracted ethanol *Aloe vera* was made with ratio 50:50.

The sample divided five groups: control group, the combination of ChH-$Av_1$, ChH-$Av_2$, ChL-$Av_1$ and ChL-$Av_2$. The sample was then ready to be tested with cell culture. The control group was that each well plate had to be contained only with cells and culture media. BHK-21 cell culture in cell-line form was cultivated inside a bottle. After confluent, the cell culture was harvested by using trypsine versene solution. The harvesting result was taken out little bit and then cultivated again into Rosewell Park Memorial Institute (RPMI-1640) media containing with 10% bovine serum albumin incubated for 24 hours with temperature at 37° C. Those cells, afterwards, were put into small bottles with 2 × 105 cell/ml density as the testing samples. This toxicity testing used 96 well plates of cell culture with flat base (Figure 1). The testing was done based on standard protocol required for MTT assay. Each plate containing cells with 2 × 105 densities into 100 µl culture media. Before being tested, those samples had to be sterilized by ultra violet rays for 15 minutes. The samples were then put into well plates about 50 µl. Those well plates were then incubated for 20 hours with temperature at 37° C. After that, each plate was contained with 5 mg/ml MTT which had been diluted in 25 ml PBS, and those well plates were then incubated for 4 hours with temperature at 37° C. The next step, samples were taken from each well plate which was added with 50 ml DMSO and piped up and down in order to dilute crystals formed. Those well plates were then incubated for 5 minutes with temperature at 37° C. Those well plates was monitored by ELISA reader with 630 λ then shown in optical density (absorbents). The amount of absorbents in each well plate showed the number of viability cell in media culture. In order to analyze the percentage of living cell, the following formulation could be used. The percentage of the living cells that indicator it not toxix uper 50 % using parameter $CD_{50}$. [9,10]

$$\% \text{ living cell} = \frac{\text{Treatment + Media}}{\text{cell +Media}} \text{ x } 100\%$$

**Figure 1.** Cytotoxicity test on 96 well plates that was monitored by ELISA reader

The data result was then tabulated based on each groups, and tested by One-Way ANOVA with 5% level of significance. If there was significant difference, LSD test would be done.

### 3. Result and Discussion

The result of cytotoxicity testing of combination chitosan with different molecular weight and ethanol extracted *Aloe vera* on cell culture in BHK-21 evaluated depends on the number of living or viability cell shown as optical density can be shown in table 1. The graphic of optic density value and viability cell can be shown in figure 2. Figure 3 shown the viability cell of each group.

**Table 1.** Average formazan optic density value, percentage of living cell and standar deviation

| Group | Optic Density value X ± SD | Viability cell (%) X ± SD | n |
|---|---|---|---|
| Control | 0,503 ± 0,026 | 100 ± 0,00 | 8 |
| ChH-Av1 | 0,513 ± 0,034 | 93,087 ± 1,903 | 8 |
| ChH-Av2 | 0,426 ± 0,038 | 91,643 ± 5,006 | 8 |
| ChL-Av1 | 0,376 ± 0,098 | 88,568 ± 6,533 | 8 |
| ChL-Av1 | 0,386 ± 0,017 | 88,05 ± 4,461 | 8 |

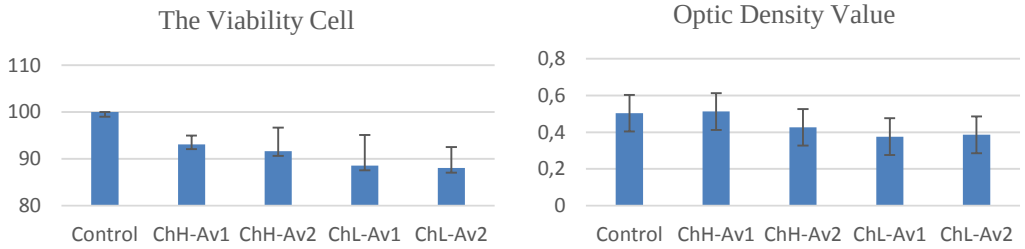


**Figure 2.** The graphic of optic density value and viability cell of control group, the combination of ChH-$Av_1$, ChH-$Av_2$, ChL-$Av_1$ and ChL-$Av_2$


**Figure 3.** The viability cell of control group (a), the combination of ChH-$Av_1$ (b), ChH-$Av_2$ (c), ChL-$Av_1$ (d) and ChL-$Av_2$ (e)

Based on table 1, the formazan optic density values of Ch-Av from chitosan with high molecular weight more higher than Ch-Av from chitosan with low molecular weight. The percentage of the

living cells of all groups was uper 50 % using parameter $CD_{50}$. Kolmogorof Smirnof Test shows that all groups have probability values more than 0.05 ($p > 0.05$). It means that all groups have normal distribution. In homogeneity test, however, the value obtained is about 0.130 ($p > 0.05$) which means that all groups are homogeny. Thus, one way Anova test was needed to be done.The result of One-Way ANOVA test shows that there is no significant difference of optic density and viability cell among treatment group with probability value ($p > 0.05$). The Combination chitosan with different molecular weight and ethanol extracted *Aloe vera* were non toxic for BHK-21 cell culture.

Cytotoxicity assays are based on the use of various parameters quantifying cell death or measuring the effect of the product on cell metabolism. This research using BHK-21 cell culture in cell-line by MTT assay method to measure the cytotoxicity combination chitosan with different molecular weight and ethanol extracted *Aloe vera.* BHK-21 cell from fibroblast of baby hamster's kidney is often used by the researchers for cytotoxicity testing of dentistry materials since it is the most important cell in the components of pulp, ligament periodontal and gingiva.[11] MTT assay is the most common methods used to determine cytotoxicity. The test was proved to be more accurate and time saving than other conventional haemocytometer counting methods. The well with the highest absorbance indicates the highest viability in the case of MTT assay.[12] The MTT assay is the most used in vitro method for determining cytotoxicity by measuring cell metabolism, using a reaction catalyzed by the succinate dehydrogenase enzyme from mitochondria of viable cells. Trypan blue is an assay that allows differentiation viable cells by microscopic evaluation, since the cells that present loss of membrane integrity allow the incorporation of this agent into the nucleus. The reparative materials evaluated by MTT and Trypan blue cytotoxicity assays demonstrated viability in all evaluated cell lines.[13]

The percentage of the living cells of all groups in this research was uper 50 % using parameter $CD_{50}$. There was no significant difference among treatment groups. It means that combination chitosan with different molecular weight and ethanol extracts *Aloe vera* were non toxic for BHK-21 cell culture. There is no toxicity on BHK-21 cell culture since chitosan does not have function and structure which can make chitosan and *Aloe vera* becomes toxic. The process of chitosan production, moreover, has passed deproteination phase which is a phase of separating or breaking bond between protein and chitin so that chitosan will not become toxic.[9] Combination chitosan with high molecular weight and ethanol extracted *Aloe vera* have high average formazan optic density value than combination chitosan with low molecular weight and ethanol extracted *Aloe vera*. The principal of MTT assay is to break tetrazolium MTT ring, (3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide), which has yellow color because of dehydrogenesis in active mitochondria, and to produce insoluble blue-purple formazan product. If the dehydrogenises are not active because of cytotoxic effects, the formazan is not produced. The formazan production can be measured by dissolving it and measuring optic density of the solution produced. The lower the percentage of optic density is, the fewer the number of active metabolic cells that can reduce MTT.[9; 14] The biological properties of chitosan depend on their physicochemical parameters, especially their solubility. The characteristic of chitosan is related with its molecular weight.[15] Chitosan with high molecular weight powder become chitosan gel with good physical characteristic and higher viscosity. Powder with high molecular weight has greater particle size, due to the larger size of the solute molecules solvent molecules surrounding more increasingly difficult to dissolve. It become tenuous bonds by the force of molecular attraction.[16] The molecular weight is also directly related to length of molecular chain, more longer the molecular chain, the viscosity more higher and more higher average formazan optic density value.[14; 16]

The result of this study, combination chitosan and ethanol extracted *Aloe vera* was not toxic. Chitosan is used in various biomedical applications due to its significant antibacterial activity, biodegradability, non toxicity, and biocompatibility. The synergistic association combination of chitosan and ethanol extracts *Aloe vera* have biological performance that offer good enviroment for viability of fibroblast cell. It regarding requirement for wound healing such as control inflamation, immunomudulatory activity, epithelialization and fibroblast proliferation.[17; 18]

**4.Conclusion**

Based on this research, the percentage of the living cells of group combination chitosan and ethanol extracted *Aloe vera* was uper 50 %. It shown that the combination chitosan with different molecular weight and ethanol extracted *Aloe vera* were non toxic with BHK-21 cell lines by MTT assay method.

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