World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

Check for updates

# Cytotoxicity of chitosan gelatin limestone-based carbonate hydroxyapatite composite scaffold after crosslinking

Devi Rianti *, Rahma Maulidya, Fayza Aldinafitri Anggraini, Maryama Aflaha, Anita Yuliati and Priyawan Rachmadi

*Department of Dental Materials, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia.*

World Journal of Advanced Research and Reviews, 2025, 25(02), 1909-1915

Publication history: Received on 11 November 2024; revised on 18 December 2024; accepted on 20 December 2024

Article DOI: https://doi.org/10.30574/wjarr.2025.25.2.3557

## Abstract

Background: The development of biomaterial scaffolds is used as one of the solutions for dealing with bone damage. The combination of materials in scaffolds before being applied to clinical practice therefore required several stages for testing the biocompatibility material. One of the material biocompatibility tests involves a cytotoxicity test on osteoblast 7F2 cells. Purpose: To demonstrates the percentage of viable cells for Chitosan-Gelatin:Carbonate HydroxyApatite (C G:CHA) scaffold with a ratio of 20:80, 30:70, and 40:60 (w:w) after crosslinking with 0.25% glutaraldehyde on 7F2 osteoblasts. Methods: MTT assay is a cytotoxicity test used in this research. The research data are analyzed by comparative tests using Shapiro-Wilk, Levene, and One-Way Anova test, post hoc Games-Howell. Results: Statistical tests from the percentage of live cells shows that between the control cell group and the C-G:CHA 20:80 crosslink group, the cell group and the C-G:CHA 20:80 group without crosslink, the C G:CHA group 20:80 crosslink and C-G:CHA group 30:70 without crosslink, C-G:CHA group 20:80 without crosslink and C-G:CHA group 30:70 without crosslink, there are significant differences. Conclusion: Scaffold C-G:CHA with a ratio of 20:80, 30:70, and 40:60 (w:w) after crosslinked with 0.25% glutaraldehyde was found not toxicity to osteoblast cells 7F2 because indirectly cytotoxicity test resulted a percentage of live cells of >50%.

**Keywords:** C-G:CHA; Crosslink; Glutaraldehyde; Cytotoxicity; MTT assay.

## 1 Introduction

Bone damage in the oral cavity is a common issue in dentistry [1]. One method for addressing bone damage is through bone tissue engineering [2]. Tissue engineering is a branch of engineering and biological science that remains under development and requires ongoing research and innovation. There are three foundational components for bone tissue engineering: biomaterials for scaffolds, cells with osteogenic potential, and growth factors [3].

Bone tissue engineering scaffolds must have specific requirements, including sufficient mechanical strength comparable to human trabecular bone, which has a strength of 1-12 MPa and an ideal degradation rate of around 2-3 months [4]. Previous research has focused on porous-structured scaffolds combining chitosan-gelatin and carbonate hydroxyapatite (C-G:CHA) [5]. Testing revealed that a C-G:CHA composite scaffold with a 40:60 (w/w) ratio achieved a compressive strength of 4.19 MPa and degradation of 20-25.98% at day 21, meeting the requirements for minimum compressive strength and an ideal degradation rate [4]. Additional enhancements in compressive strength and biodegradation may be beneficial for optimizing the use of C-G:CHA composite scaffolds in bone tissue engineering.

The scaffold is essential for achieving successful outcomes in tissue engineering. Scaffolds must have appropriate pore size, be free of harmful residues, and possess sufficient compressive strength to endure pressure. Crosslinking aims to

* Corresponding author: Devi Rianti

improve compressive strength, thus supporting scaffold performance during recovery and maintenance processes associated with the oral cavity [5].

Glutaraldehyde is commonly used in crosslinking materials, generally increasing compressive strength but potentially reducing cell viability, necessitating cytotoxicity testing. Glutaraldehyde concentrations such as 0.25% are recommended, as most aldehyde groups interact beneficially with osteoblast cells without cytotoxic effects [6].

Biocompatibility testing is required before scaffold use in clinical applications, one of which is cytotoxicity testing [7]. Cytotoxicity testing confirms the scaffold material's compatibility with body cells. One method for cytotoxicity testing is the MTT assay, which uses yellow tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) to evaluate cell proliferation and cytotoxicity. The assay involves the conversion of MTT to formazan crystals by living cells, indicating mitochondrial activity. Osteoblast cells 7F2 are used in this study due to their structural similarity to mature human osteoblasts, representing bone regeneration processes [8].

Cytotoxicity testing on C-G:CHA composite scaffolds crosslinking glutaraldehyde has not been conducted previously. Based on this background, a study on the cytotoxicity of chitosan-gelatin-hydroxyapatite carbonate scaffolds crosslinked with glutaraldehyde on 7F2 osteoblasts is needed.

## 2 Material and methods

### 2.1 The Procedure for Fabricating a Chitosan-Gelatin: Hydroxyapatite Carbonate Scaffold

The K-G: CHA scaffold with a ratio of 30:70 (w/w) was made by weighing 0.375 grams of chitosan powder, 0.375 grams of gelatin, and 1.75 grams of hydroxyapatite carbonate powder using an analytical balance. 2% acetic acid as much as 2 ml in a beaker was heated to a temperature of 50°C, then gelatin powder was added, then stirred using a magnetic stirrer until homogeneous. The chitosan powder was mixed little by little in a beaker glass that contained gelatin gel. Simultaneously, hydroxyapatite carbonate powder was mixed with 0.94 ml of distilled water in a different beaker glass and stirred until homogeneous. Hydroxyapatite carbonate was then mixed with chitosan-gelatin gel and stirred until homogeneous. The pH measurement of the scaffold was measured with litmus paper until it reached pH 7 (neutral). The K-G: CHA gel that had reached pH 7 was inserted into a well plate mold using a spatula and then compacted using a cement stopper so that there were no cavities.

### 2.2 Freeze-Drying Method

The scaffold that has undergone deep freezing is then placed in a freeze dryer. The freeze dryer is turned on by pressing the "COND" button, and it is left for 20-30 minutes until the temperature reaches -40°C. On the LED monitor, you can check that the condenser has reached -40°C or lower (between -90°C and -110°C) by observing the green indicator light. The vacuum process is then conducted for 48 hours by pressing the "VAC" button; ensure that the green light on the LED monitor is on before starting the vacuum process. The scaffold is observed periodically to check whether the freeze-drying process is complete, which is indicated when the outer surface of the tube shows no condensation and the scaffold appears dry. Once the process is complete, the vacuum and cooling machine are turned off, and the scaffold is removed. The "DEFR" button is pressed to begin the next stage, which is intended to melt the ice in the condenser, followed by cleaning and rinsing the condenser tube with a baking soda or detergent solution. The freeze-dried scaffold is then measured for height and diameter, as it undergoes shrinkage compared to its original size during freeze-drying. The scaffold is adjusted to the required dimensions by matching its height and diameter using a blade.

### 2.3 Crosslink Method

In this study, the scaffold crosslinking process uses an immersion method. The scaffold is rehydrated in a 0.05 M acetic acid solution for the first 15 minutes, followed by the crosslinking method by immersing it in a 0.25% glutaraldehyde solution diluted with double-distilled water at 4°C for 24 hours. The scaffold is then washed (10 minutes x 5 times) and freeze-dried for 24 hours [9].

### 2.4 Sample Preparation

The scaffold samples are sterilized for 30 minutes using an ultraviolet sterilizer. The sterilized samples are then placed into wells and immersed in DMEM media. The samples are sterilized again to prevent contamination. The immersed samples are placed in an incubator at 36.4°C [9].

### 2.5 MTT Assay Testing Procedure

CHA toxicity examination was performed using the MTT test on 7F2 osteoblasts. Cultured 7F2 osteoblast cells and sterile 96-well microplates were prepared in laminar flow. Wells in column A of the Microplate were filled with DMEM culture media as a control media of 100 µl in each well. Wells in columns B to H of the microplate were filled with 50 µl of 7F2 osteoblast cell suspension in each well and 100 µl of DMEM 3 culture media was added to each well to a density of 3x10. The sample soaking solution for each treatment was previously filtered using a 0.20 µm millipore and added to each well as much as 50 µl each in columns C to H. The 96-well microplate was incubated in a 5% CO2 incubator at 37°C for 24 hours. The culture media was taken from each well using a syringe and 7F2 osteoblast cells were left in the well. The cells left in the wells were refilled with PBS as much as 100 µl. MTT was added as much as 10 µl for each well. The 96-well microplate was incubated in a 5% CO2 incubator at 37°C for 4 hours. DMSO was added to dissolve the formazan crystals as much as 50 µl per well and the microplate was shaken for 5 minutes. The OD or absorbance value of the formazan crystals was read using an ELISA reader with a wavelength of 540 nm [10].

$$\%\ of\ living\ cells\ = \frac{OD\ Treatment - OD\ Media\ x100\%}{OD\ Cell - OD\ Media} \quad (1)$$

The data was then analyzed using the Shapiro-Wilk test to determine the normality of the data, then a homogeneity test was performed using the Levene test. If the data is normally distributed (p-value > 0.05), it is continued with the parametric one-way ANOVA and Post Hoc Tukey HSD tests. If the data is not normally distributed (p-value < 0.05), it is continued with non-parametric statistical tests with Kruskal Wallis and Mann-Whitney-Wilcoxon U.

## 3 Results and discussion

### 3.1 Scaffold C-G:CHA Synthesis

The results of the synthesis of the C-G:CHA scaffold with a ratio of 20:80 (w:w), 30:70 (w:w), 40:60 (w:w) after crosslinking with 0.25% glutaraldehyde or without crosslinking.

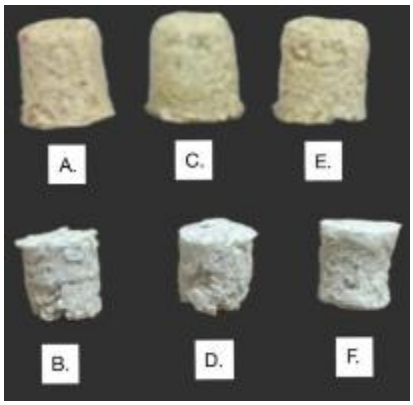


**Figure 1** Scaffold synthesis results (a) comparison of C-G:CHA ratio with 20:80 (w:w) after crosslinking, (b) comparison of C-G:CHA ratio with 20:80(w:w) without crosslinking (c) comparison of C-G:CHA ratio with 30:70 (w:w) after crosslinking, (d) comparison of C-G:CHA ratio with 30:70 (w:w) without crosslinking, (e) comparison of C-G:CHA ratio with 40:60 (w:w) after crosslinking, (f) comparison of C-G:CHA ratio with 40:60 (w:w) without crosslinking

The scaffold in this study is derived from a combination of chitosan, gelatin, and hydroxyapatite carbonate materials. This combination is used to produce both organic and inorganic components resembling bone structure. The organic component of the bone structure comes from gelatin and chitosan, while hydroxyapatite carbonate produces the inorganic component of the bone structure [11].

### 3.2 Osteoblast Culture 7F2 after MTT Essay Test

Results of 7F2 osteoblast cell culture on 96 well microplate.

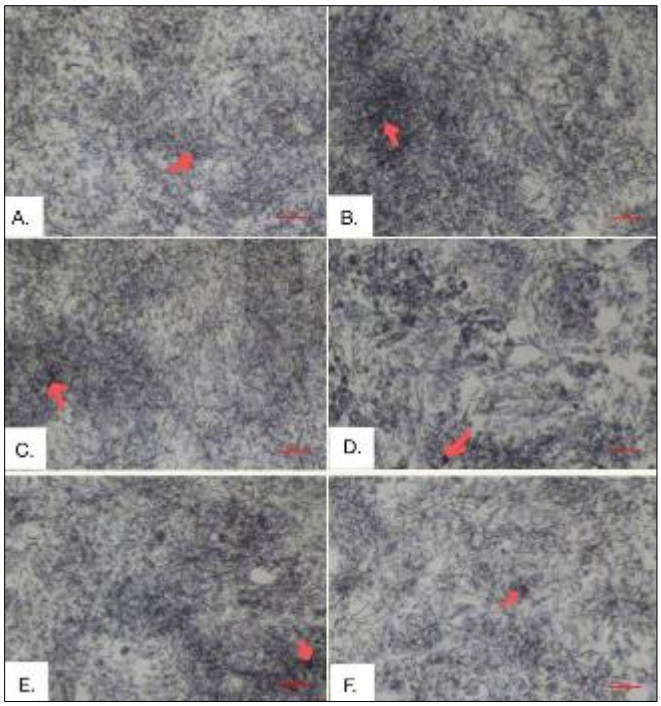


**Figure 2** Osteoblast cell culture 7F2 with 10x microscope magnification (a) Formazan crystals (green arrows) formed in the group after 0.25% glutaraldehyde crosslinking with a C-G:CHA ratio of 20:80 (w:w) (b) Formazan crystals (green arrows) formed in the group without crosslinking with a C-G:CHA ratio of 20:80 (w:w) (c) Formazan crystals (green arrows) formed in the group after 0.25% glutaraldehyde crosslinking with a C-G:CHA ratio of 30:70 (w:w) (d) Formazan crystals (green arrows) formed in the group without crosslinking with a C-G:CHA ratio of 30:70 (w:w) (e) Formazan crystals (green arrows) formed in the group after 0.25% glutaraldehyde crosslinking with a C-G:CHA ratio 40:60 (w:w) (f) Formazan crystals (green arrows) formed in the group without crosslinking with a C-G:CHA ratio of 40:60 (w:w)

The image above shows the formazan formed after performing the MTT assay on a 96-well microplate. The image shows 7F2 osteoblast cells that remain viable after exposure to the C-G:CHA scaffold.

### 3.3 MTT Essay Test

The MTT assay results show the percentage of live cells in the media group, cell group, C-G:CHA crosslinked group, and C-G:CHA non-crosslinked group. With a minimum sample size of 5 for each group, the results are presented in Table 1 and Figure 1 as follows.

**Table 1** The results show the percentage of live cells in each group

| NO. | Media | Cell | Crosslink | | | Non-Crosslink | | |
|---|---|---|---|---|---|---|---|---|
| | | | 20:80 (w:w) | 30:70 (w:w) | 40:60 (w:w) | 20:80 (w:w) | 30:70 (w:w) | 40:60 (w:w) |
| 1 | 0.00 | 100.00 | 70.62 | 88.54 | 79.04 | 71.55 | 95.16 | 64.75 |
| 2 | 0.00 | 100.00 | 62.20 | 85.02 | 66.67 | 76.34 | 87.44 | 128.91 |
| 3 | 0.00 | 100.00 | 86.23 | 109.72 | 79.01 | 78.37 | 103.10 | 83.99 |
| 4 | 0.00 | 100.00 | 73.46 | 96.90 | 126.28 | 84.49 | 109.36 | 87.89 |
| 5 | 0.00 | 100.00 | 71.17 | 85.83 | 104.36 | 72.78 | 93.45 | 114.94 |
| Average | 0.00 | 100.00 | 72.74 | 93.20 | 91.07 | 76.71 | 97.70 | 96.10 |

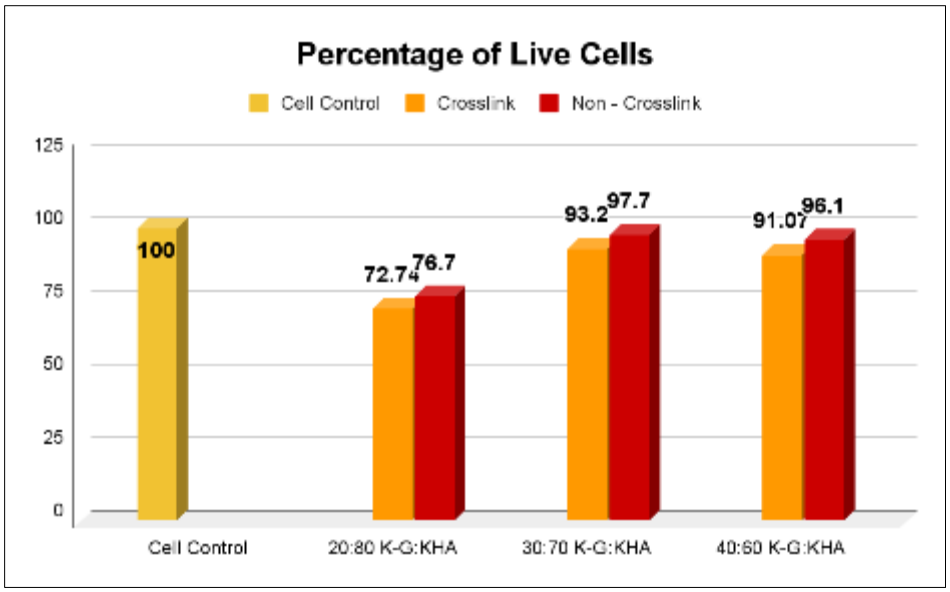


**Figure 3** Bar chart of percentage of living cells.

Based on Table 1 and Figure 3, it is known that the highest percentage of living cells is on the C-G:CHA 30:70 (w:w) scaffold and the lowest percentage of living cells is on the C-G:CHA 20:80 (w:w) scaffold. The C-G:CHA 20:80 (w:w), 30:70 (w:w) and 40:60 (w:w) scaffolds showed that without 0.25% glutaraldehyde crosslinking, they were higher than with 0.25% glutaraldehyde crosslinking.

The principle of the MTT assay involves the reduction of yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by the reductase system. Succinate tetrazolium, which is part of the respiratory chain in living cell mitochondria, forms purple, water-insoluble formazan crystals. The addition of a stopper reagent (detergent) dissolves the colored crystals, which are then measured for absorbance using an ELISA reader. The intensity of the purple color formed is proportional to the number of live cells. The greater the purple intensity, the higher the number of live cells [12]. The MTT assay data is obtained from the number of living cells after reading with an ELISA reader, represented as absorbance or optical density (OD). A wavelength of 540 nm was used in the study as it provides accurate absorbance readings on the ELISA reader [13]. The higher the OD value, the greater the number of living osteoblast cells. The results are considered non-toxic if > 50% of the cells remain alive.

The results of the MTT assay are based on the ability of the enzyme succinate dehydrogenase in cells to convert tetrazolium compounds into an insoluble purple substance. The insoluble purple substance in formazan generates differences in absorbance levels across the experiments. Solvents such as DMSO solution are added to dissolve the residue produced in formazan crystals [14]. A previous study by Budiatin et al. (2022, p. 4975) highlighted the use of glutaraldehyde at a low concentration of 0.25% due to its non-toxic properties, as indicated by a cell viability percentage >60%. The viability of BHK-21 fibroblast cells was higher in samples without glutaraldehyde compared to those with 0.25% glutaraldehyde. A similar observation was made in a study by Yuangga (2022, p. 31) on 7F2 osteoblast cells. The MTT assay results, with a minimum sample size of 5, demonstrated that C-G:CHA ratios of 20:80 (w:w), 30:70 (w:w), and 40:60 (w:w) had higher cell viability in samples without glutaraldehyde crosslinking compared to those with 0.25% glutaraldehyde crosslinking. The average cell viability percentages with a minimum sample size of 5 showed that the C-G:CHA ratio of 30:70 (w:w), both cross linked and non-crosslinked, was higher compared to the C-G:CHA ratios of 20:80 (w:w) and 40:60 (w:w), both cross linked and non-crosslinked.

The K-G:KHA scaffold at ratios of 20:80 (w:w), 30:70 (w:w), and 40:60 (w:w), after being crosslinked with 0.25% glutaraldehyde with a minimum sample size of 5, showed that the percentage of live cells met the CD50 parameter [15]. The results from the combination of these materials demonstrate non-toxic properties when tested for cytotoxicity indirectly on 7F2 osteoblast cells, making it a potential candidate scaffold for bone tissue engineering.

## 4 Conclusion

The C-G:CHA scaffold at a ratio of 20:80, 30:70, 40:60 (w:w) after being crosslinked with 0.25% glutaraldehyde showed

that the percentage of living cells was achieved according to the CD50 parameter, proving that it was non-toxic when an indirect cytotoxicity test was testing on 7F2 osteoblast cells. This result indicates that the scaffold is capable of being one of the scaffold candidates in bone tissue engineering.

## Compliance with ethical standards

*Acknowledgements*

The authors are thankful to Research and Innovation Institute and Faculty of Dental Medicine of Universitas Airlangga Surabaya for the kind support.

*Disclosure of Conflict of interest*

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

*Statement of ethical approval*

This research met ethical requirements and was approved by the UNAIR Faculty of Dentistry Ethics Committee No. 1184/HRECC.FODM/X/2023.

## References

[1] Ghassemi T, Shahroodi A, Ebrahimzadeh MH, Mousavian A, Movaffagh J, Moradi A. Current concepts in scaffolding for bone tissue engineering. In Arch Bone Jt Surg. 2018; 6(2):90-9.

[2] Mittwede PN, Gottardi R, Alexander PG, Tarkin IS, Tuan RS. Clinical Applications of Bone Tissue Engineering in Orthopedic Trauma. In Current Pathobiology Reports. 2018; 6(2):99–108.

[3] Battafarano G, Rossi M, De Martino V, Marampon F, Borro L, Secinaro A, Fattore A. Strategies for bone regeneration: from graft to tissue engineering. In International Journal of Molecular Sciences. 2021; 22(3):1–22.

[4] Kim T, See CW, Li X, Zhu D. Orthopedic implants and devices for bone fractures and defects: Past, present and perspective. Engineered Regeneration. 2019; 1:6–18.

[5] Rianti D, Purnamasari AE, Putri RR, Salsabilla NZ, Faradillah, Munadziroh E, Agustantina TH, Meizarini A, Yuliati A, Syahrom A. The compressive strength and static biodegradation rate of chitosan-gelatin limestone-based carbonate hydroxyapatite composite scaffold. Dental Journal. 2023; 56(3):160–5.

[6] Pinto RV, Gomes PS, Fernandes MH, Costa MEV, Almeida MM. Glutaraldehyde-crosslinking chitosan scaffolds reinforced with calcium phosphate spray-dried granules for bone tissue applications. Materials Science and Engineering C. 2022; 109:1-13.

[7] Tite T, Popa AC, Balescu LM, Bogdan IM, Pasuk I, Ferreira JMF, Stan G. Cationic substitutions in hydroxyapatite: Current status of the derived biofunctional effects and their in vitro interrogation methods. MDPI In Materials. 2018; 11(11):1-62.

[8] Ahmad NB, Bharatham H, Abdul HZ, Zulkipli NZ. Cytotoxicity and oxidative stress evaluation of alginate/cockle shell powder nanobiocomposite bone scaffold on osteoblast. Jurnal Sains Kesihatan Malaysia. 2017; 15(1):97–104.

[9] Raharjo, S. I. Y. Cytotoxicity of Limestone-Based Hydroxyapatite Composite Scaffold on 7F2 Osteoblast Cells after Crosslinking [Thesis]. 2022: 1-68.

[10] Putri, D. S. 2012, Uji Sitotoksisitas Ekstrak Kulit Biji Coklat Terhadap Fibroblas BHK-21 dengan Metode MTT Assay., 25–26.

[11] Rianti, D., Kristanto, W., Damayanti, H., S. Putri, T., Dinaryanti, A., Syahrom, A., & Yuliati, A 2022, 'The Characteristics and Potency of Limestone-based carbonate hydroxyapatite to Viability and Proliferation of Human Umbilical Cord Mesenchymal Stem Cell', Research Journal of Pharmacy and Technology, pp. 2285-2292.

[12] Ibrahim, M. S., El-Wassefy, N. A., & Farahat, D. S 2017, 'Biocompatibility of dental biomaterials' , Elsevier Health Science, pp. 117–140.

[13] Grela, E., Kozłowska, J., & Grabowiecka, A 2018, 'Current methodology of MTT assay in bacteria', Elsevier Health Science, vol. 120, no. 4, pp. 303-311.

[14] Kuete, V., Karaosmanoğlu, O., & Sivas, H 2017, 'Anticancer Activities of African Medicinal Spices and Vegetables. In Medicinal Spices and Vegetables from Africa', Elsevier Health Science, pp. 271–297.

[15] Sularsih, S., Soetjipto, S., & Rahayu, R. P 2019, 'Cytotoxicity of Combination Chitosan with Different Molecular Weight and Ethanol Extracted Aloe vera using MTT Assay', IOP Conference Series: Earth and Environmental Science, vol. 217, no.1, pp. 1-5.