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)

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Biocompatibility of crab shell-derived graft sterilized by ultraviolet light towards human gingival fibroblast cell culture

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World Journal of Advanced Research and Reviews, 2023, 17(03), 728–735

Publication history: Received on 07 February 2023; revised on 15 March 2023; accepted on 18 March 2023

Article DOI: https://doi.org/10.30574/wjarr.2023.17.3.0438

Abstract

Background: *Portunus pelagicus* crab species meat is generally taken, and their shells that is thrown away becoming untapped waste. Their shells contain so many useful minerals such as calcium carbonate which can be processed into hydroxyapatite (HA). HA has been extensively used in the medical field for bone grafting, to repair damaged in bone structures.

Purpose: To determine the biocompatibility of hydroxyapatite from *Portunus pelagicus* crab's shells extract after sterilized using UV on human gingival fibroblast cell**.**

Methods: This research is an experimental laboratory research using post-test only control group design. The shells were first heated using furnace. Once hydroxyapatite was obtained, it was sterilized using ultraviolet (UV) with certain concentration (25 ppm, 50 ppm, and 100 ppm) to kill the microorganisms. MTT assay was done to test the biocompatibility of the sterilized hydroxyapatite on human gingival fibroblast cell.

Results: The highest viability of fibroblast cells was the group with 50 ppm UV (98.10%), followed by 100 ppm UV (97.93%), and 25 ppm (93.28%).

Conclusion: Hydroxyapatite from *Portunus pelagicus* crab's shells extract sterilized using UV is biocompatible towards human gingival fibroblast cell.

Keywords: Biocompatibility; UV light; Hydroxyapatite; *Portunus pelagicus*; Human gingival fibroblast cell; Medicine

1. Introduction

Scylla serrata and *Portunus pelagicus* crab species are one of Indonesia's seafoods commodities that are often exported to the United States. In 2012, the export value of the two marine animals reached 9% and occupied the third place in the export of Indonesian seafood products after shrimp and tuna. About 60% of the volume of crabs exported is in processed form (canned crab) [1]. The consumption of crab meat from the domestic community also contributes to shell waste. The by-product of crab meat processing in the form of shell waste reaches about 40-60% of the total weight of crabs. This waste has not been put to good use and is not efficient, in fact most of it is a waste that pollutes the environment [2,3].

Crab shell waste contains organic and inorganic compounds, including protein (15.60% - 23.90%), $CaCO_3$ (53.70 - 78.40 %), and chitin (18.70% - 32.20%) [4]. Minerals contained in crab shells can be utilized and processed into

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hydroxyapatite (HA) and can be synthesized in many ways. HA is widely used in orthopedic and biomedical field applications. The structure of HA has a similar structure with human bones which makes HA have the potential and innovation as a manufacture of synthetic bones (bone graft). In dentistry, bone graft is used to increase the alveolar bone volume, remodeling the jawbone, and reshaping the alveolar crest [5,6].

To be used as a graft material, hydroxyapatite graft from crab shells (*Portunus pelagicus*) must meet biocompatibility requirements. Biocompatibility relates to the nature of the material that demonstrates biologic compatibility or is acceptable to the body without providing a harmful local or systemic response. This property is very important for synthetic bones (bone graft) because it is related to the success of osteoconductivity, osteoinduction and osteogenesis. Osteoconductive and osteoinductive are paramount to resorbable biomaterials to direct and encourage tissue growth formations [5,7].

In the manufacture of hydroxyapatite, a heating / calcination process has been carried out which can kill various microorganisms but at the time of heating there is a possibility of exposure to the external environment so that additional sterilization process is needed [8]. Sterilization is a process that destroys all forms of life. From a microbiological point of view, a sterile object is free from living microorganisms. In the sterilization process, bacterial spores are the most resistant among all living organisms. Currently, there are various sterilization equipment with different working mechanisms, each of which has its own limitations in its practical application The method chosen usually depends on the nature of the material to be sterilized [9]. Sterilization of ultraviolet (UV) light is one method that can emit rays with wavelengths between 200-400 nm. A wavelength of 200-300 nm is usually referred to as germicidal because UV light of this wavelength is absorbed by DNA and RNA from microorganisms. Photons absorbed from these UV rays will cause changes in DNA and RNA structure, resulting in microorganisms not being replicable [10]. Based on research by Nishikawa (2003) another effect that can be produced from UV light sterilization on hydroxyapatite is the formation of free radicals (OH and O_2^-) due to the breakdown of H_2O and O_2 molecules which can cause decomposition of organic pollutants [11].

To find out the biocompatibility of a material towards human body, a toxicity test is conducted so that it is known how far the adverse effects can be caused, so as not to harm the body. In this study, human gingival fibroblast (HGF) cell culture was used to get fast results and to expose cell cultures directly with experimental materials. Cell cultures are also more sensitive to toxic materials. Thus, not only toxicity can be measured quantitatively, but the response to living cells can be seen. Fibroblasts are often used by researchers to test dental materials, because fibroblasts are one of the constituent components of the tissue around the tooth [12].

Based on the description above, the purpose of this study is to determine the biocompatibility of hydroxyapatite of *Portunus pelagicus* crab shells that have been sterilized with UV light against HGF cell cultures.

2. Material and methods

2.1. *Portunus pelagicus* crab shell preparation

This study used a post-test only control group design. The crab population used comes from Semedu Sari, Grati, Pasuruan District. Cleaned crab shells were soaked with 5.25% sodium hypochlorite (Bayclin, SC Johnson) and fed into a furnace engine with an initial temperature of ±50 °C. The temperature was raised by 5 °C/min until it reaches a temperature of 1000 °C for 2 hours. The next process was to sift the powder using a sifting machine to obtain hydroxyapatite powder measuring less than 150 µm particle. The transparent plastic tubes with an additional layer of polyethylene plastic were used as a container to keep hydroxyapatite so that it can be sterilized with UV rays and not contaminated. Hydroxyapatite was sterilized with a UV sterilizer for 2 x 30 minutes by turning it over.

2.2. Human gingival fibroblast cell culture preparation

Human gingival fibroblast cell cultures were given Phosphate-Buffered Saline (PBS) of 2.5 mL to separate or remove the media in the culture, then given the enzyme trypsin as much as 2 mL to release cells in the medium. After that, cell cultures were incubated at a temperature of 37 ^{0}C, CO_2 levels of 5% for 5 minutes. Observations were made to make sure all the cells were detached. Cells were taken using a pipette and incubated again for 5 minutes. Cells that have been detached were given alpha MEM and trypsin medium in a ratio of 1:1 (2 mL each). Then, cells were inserted into the conical tube so that the cell would stick to the tube wall. Several washes were carried out using trypsin and alpha MEM so that the monolayer cells became single cells in the conical tube. Centrifugation of 1800 rpm was carried out for 5 - 6 minutes. The medium and enzymes were removed using a pipette, then alpha MEM was given up to 50 mL. The vibrating

process was carried out using a tube mixer and cells were poured into a microplate of 96 well as much as 100 µL with a density of 3 – 5x10^3 and incubated for 24 hours with a temperature of 37°C.

2.3. Experimental procedure

This study consisted of 4 groups, control group (without hydroxyapatite, consist of control of media and control of HGF cell), group with 25 ppm of *Portunus pelagicus* crab shell, 50 ppm, and 100 ppm. Each group consisted of 5 samples, was poured into microplate with HGF cells. This microplate underwent UV sterilization for 60 minutes. After UV sterilization, that was underwent incubation for next 24 hour inside the incubator with a temperature of 37 °C.

2.4. MTT assay

Tetrazolium salt (MTT) was dissolved in 5 mg/mL PBS. MTT was added directly to the plate containing 10 µl of culture medium, then re-incubated for approximately 4 hours at 37 °C. The whole medium inside the well was taken out. Then, each well was added DMSO (Dimethylsufoxide) of 50 µl. The plate was stirred mechanically with a plate shaker until the formazan crystals dissolved for 5 minutes. Living fibroblast cells will be stained with formazan (turning into blue colour), while the dead cells do not form blue colour. Formazan was read by spectrophotometry with an ELISA reader at a wavelength of 620 nm. The amount of absorbance indicated the number of living cells in the media culture. The calculation of the viability of fibroblast cells in each group was carried out with the formula below:

$$cell\ viability\ = \frac{OD\ of\ treatment\ group\ + OD\ of\ control\ media\ group}{OD\ of\ control\ cell\ group\ + OD\ of\ control\ media\ group} \times 100\%$$

Notes:

- Cell viability: percentage of living cells after experimental procedure
- OD refers to optical density.
- All variables were taken from mean value of each group

Data obtained was analyzed with Kolmogorov-Smirnov, Levene test, and one-way ANOVA test with confidence level of 95%.

3. Results

Precursor compound found in the *Portunus pelagicus* crab shell that had been used in this study has been observed and the results were shown below:

Table 1 The result of assessment of calcium carbonate in *Portunus pelagicus* crab shell

Main molecule	Percentage
Calcium carbonate	61.82%
Chitin	28.60%
Phosphate	13.86%

On the SEM micrograph observation, the fine structure of hydroxyapatite crystal could be seen along with its low porosity, clustered, and the similar size (Figure 1).

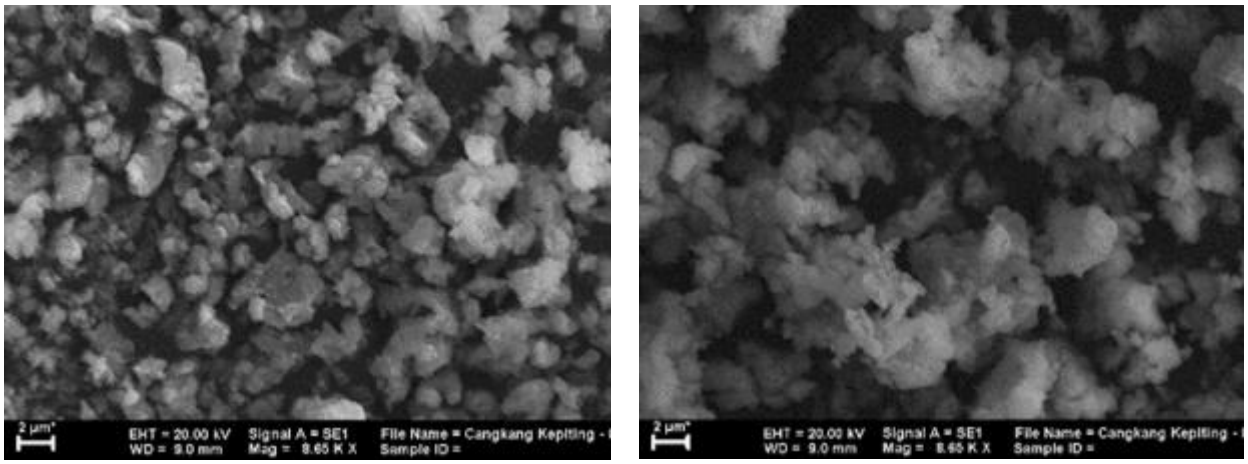

Figure 1 SEM micrograph samples of hydroxyapatite *Portunus pelagicus* shell with an enlargement of 8650x with two different field of view

It could be seen the smoothness and homogeneity of the hydroxyapatite structure because the sifting process has been carried out using a sifting machine so that a size of less than 150 µm was obtained (Figure 2)

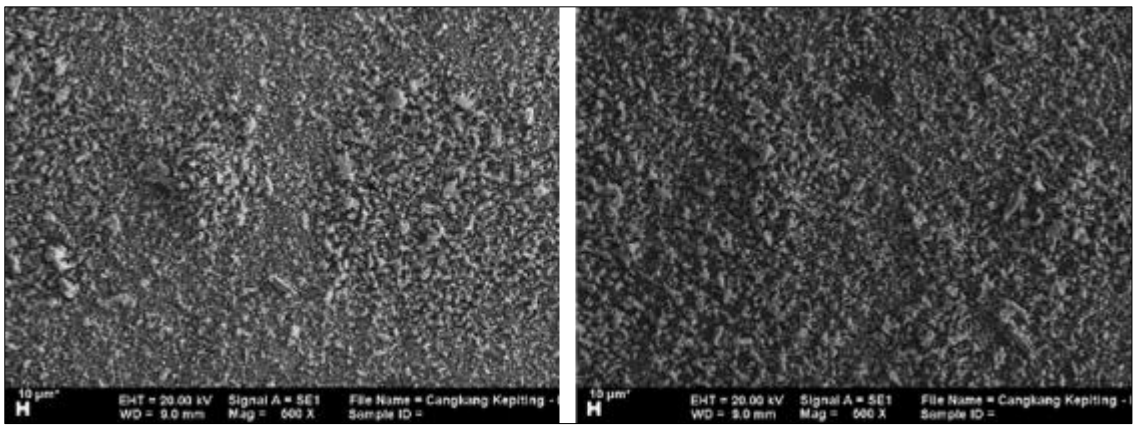

Figure 2 SEM micrograph samples of hydroxyapatite *Portunus pelagicus* shell with an enlargement of 500x with two different field of view

The composition of hydroxyapatite was dominated by oxygen (O) 67.59±6.9%, followed by calcium (Ca) 29.16±0.7%, and phosphorus (P) 3.25±0.1% for normalized weight and oxygen (O): 83.54±6.9%, calcium (Ca): 14.39±0.7%, and phosphorus (P): 2.08±0.1% for the calculation of the number of atoms (Table 2, Figure 3).

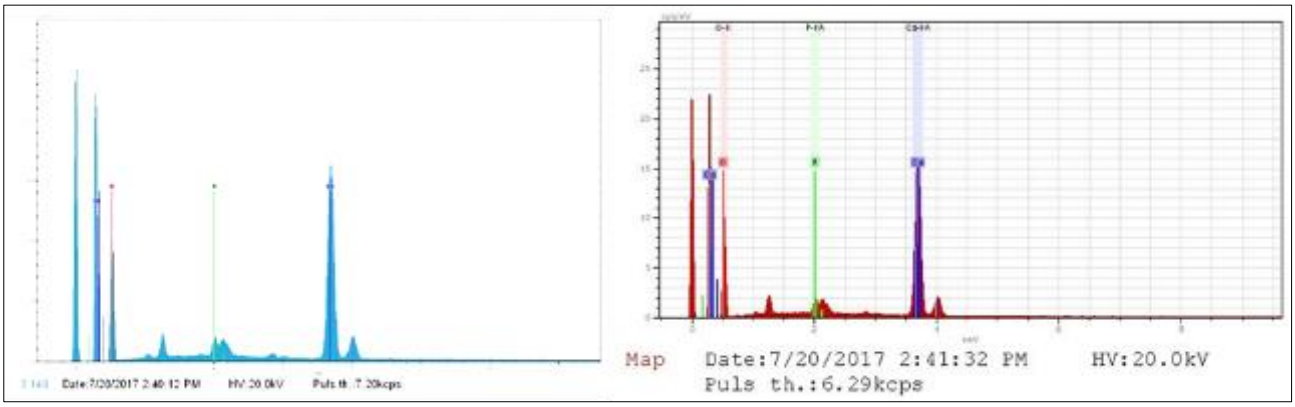

Figure 3 The EDX spectrum samples of hydroxyapatite from *Portunus pelagicus* shell with three main constituent elements dominated by Ca, O, and P

Table 2 Weight measurement of not normalized, normalized, and atom measurement from three main constituent elements dominated by Ca, O, and P

Element	Atom number	Weight measurement (wt. %)		Atom measurement (at. %)	Error (%)
		Not normalized	normalized		
O	8	56.23	67.59	83.54	6.9
Ca	20	24.25	29.16	14.39	0.7
P	15	2.71	3.25	2.08	0.1
Total		83.19	100.00	100.00	

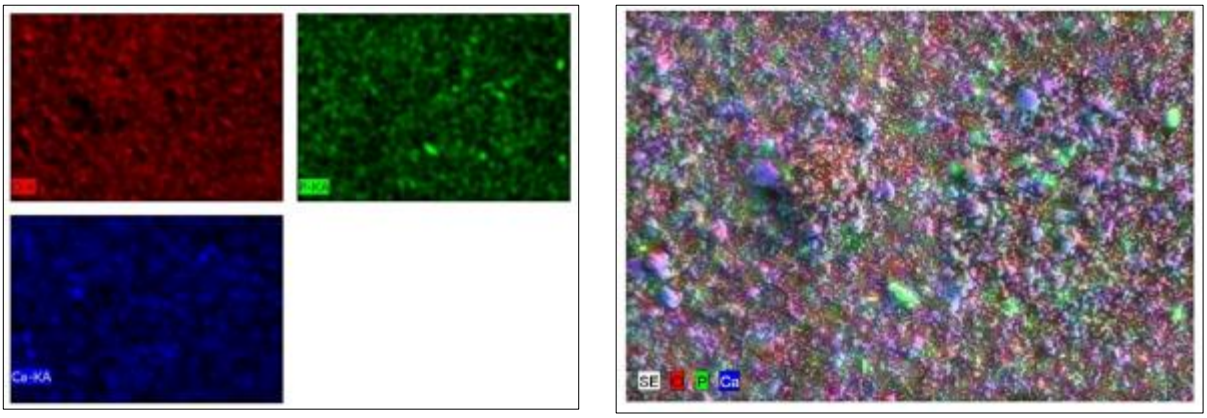

Figure 4 Left: mapping hydroxyapatite elements using EDX. Right: Combining the three mappings of hydroxyapatite elements using EDX

Figure 4 showed the mapping of Ca, O, and P atomic elements and combination of three of them using EDX on hydroxyapatite. The Ca atom was shown in blue, the O atom was shown in red, and the P atom was shown in green.

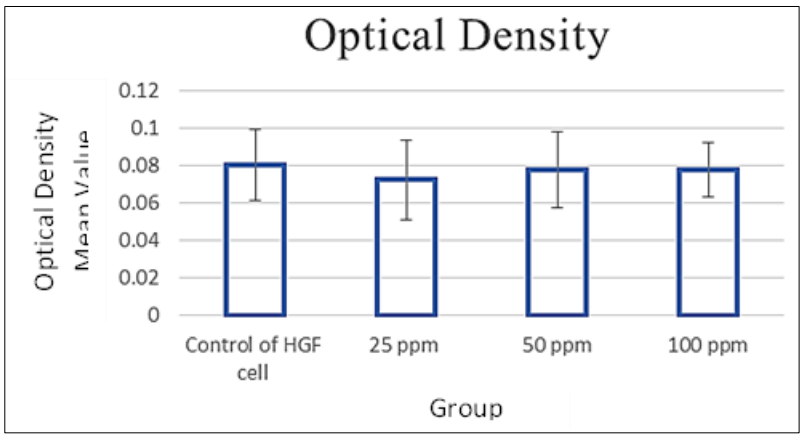

Figure 5 Diagram of optical density mean value from each group along with standard deviation

From the data represented by Figure 5 and Table 3, it could be seen that other than control group, the 100 ppm group showed higher living cells, followed by 50 ppm and 25 ppm group. ANOVA test also showed that there was no significant difference between each group.

Table 3 Percentage of living cells from each group. The numbers below were obtained from the formula mentioned on Material and methods section

Group	N	Viability (%)
Control of HGF cell	5	100%
25 ppm	5	93.28%
50 ppm	5	98.10%
100 ppm	5	97.93%

4. Discussion

Hydroxyapatite (HA) is the main mineral component of human hard tissues (especially bones and teeth). It is a storage area to control the absorption and release of calcium from the human body. HA belongs to the class of calcium phosphate-based biochemistry with the chemical formula $Ca_{10}(PO_4)_6(OH)_2$.[13] HA can be used as a bone substitute in orthopedics and dental care due to its good biocompatibility and osteoconductive properties [14].

Crab shells are rich in inorganic and organic materials that can be processed into useful materials. The results of component on *Portunus pelagicus* crab shells originating from the population of Semedu Sari, Grati, Pasuruan Regency: 61.82% CaCO3, 28.60 chitin, and 13.86% PO_4. $CaCO_3$ and PO_4 are precursor materials for hydroxyapatite [13]. Based on this study, it showed that the shell of *Portunus pelagicus* has the potential to be extracted into hydroxyapatite.

Characterization of hydroxyapatite samples using SEM-EDX, where the SEM micrograph images (Figure 4) show the formation of small-sized crystals (<150 μm), smooth surfaces with low porosity [15]. The small particle size is good for filling bone because it is quickly absorbed, has a larger surface area, and increases osteogenesis [16]. On examination of the EDX spectrum (Table 2) the hydroxyapatite sample was dominated by oxygen (O): 67.59 ± 6.9%, calcium (Ca): 29.16 ± 0.7 %, and phosphorus (P): 3.25 ± 0.1 % for normalized weight and oxygen (O): 83.54 ± 6.9 %, calcium (Ca) : 14.39 ± 0.7 %, and phosphorus (P): 2.08 ± 0.1 % for calculation of the number of atoms. The distribution map (Figure 4) of atoms also shows the evenness of O, Ca, and P atoms in the hydroxyapatite sample. The composition of these three elements confirms the hydroxyapatite compound.

Hydroxyapatite extract from *Portunus pelagicus* shell was sterilized with UV light to kill microorganisms that can harm surrounding tissues by releasing toxic substances that can damage them. UV light with a wavelength of 200-300 nm is usually referred to as germicidal because UV light with this wavelength is absorbed by the DNA and RNA of microorganisms. Photons absorbed from UV rays will cause changes in the structure of DNA and RNA.[10] Photon energy causes the breaking of hydrogen bonds in nitrogenous bases in DNA or RNA, resulting in chemical modifications of nucleoproteins and causing cross-links between adjacent thymine molecules by covalent bonds. This relationship can cause misreading of the genetic code in the process of protein synthesis, which will produce mutations that will further damage or weaken the vital functions of the organism. The existing electrons will be transferred to the surrounding oxygen and followed by the formation of O_{2^-} or superoxide radicals. This reaction occurs due to the mechanism of photoactivation. These superoxide radicals will oxidize organic molecules and react with water molecules and OH^- ions, produce hydrogen peroxide (H_2O_2) The H_2O_2 will further separate and produce OH radicals which oxidize organic molecules (microorganisms) during sterilization. UV sterilization also has the following advantages, namely relatively inexpensive, easy to operate, no chemicals needed, and fast sterilization [10].

According to Akhila *et al*., 2007, the determination of a material as toxic or non-toxic was based on Lethal Dose 50 (LD50) which is the first step for assessment and evaluation [17]. It was said that a material is non-toxic or biocompatible if it has cell viability greater than 50%. Of the three results, the percentage of cell viability was above 50% which indicated that the hydroxyapatite graft extract from *Portunus pelagicus* shell which was sterilized by UV light had good biocompatibility towards human gingival fibroblast cells. The treatment with a concentration of 50 ppm had the highest cell viability at 98.10%. The highest cell viability in certain groups can be influenced by differences in the ability of hydroxyapatite receptors on fibroblast cells. In a study conducted by Kasaj et al., it is proven that hydroxyapatite plays a role in stimulating the proliferation of human periodontal fibroblast cells. This is related to the activation of the epidermal growth factor receptor (EFGR) and its subsequent targets ERK1/2 and Akt. Activation of pYFAK 397 also occurs, this can be seen from the increased cellular attachment. The reason for the increased attachment of human periodontal fibroblast cells to hydroxyapatite is an increase in the activation of α5β1 integrin. Increased expression of α5β1 integrin will cause an increase in fibroblast cell proliferation. Integrins act as attachment receptors

for extracellular matrix proteins transducing signaling pathways through FAK phosphorylation and lead to ERK1/2 activation. This is related to the activation of the epidermal growth factor receptor. Another mechanism that enhances proliferation is activation of the Akt pathway. After the epidermal growth factor receptor is activated, this receptor is phosphorylated at tyrosine 1173, so that Akt increased. The high ability of the epidermal growth factor receptor and the expression of a5ß1 integrin on fibroblast cells in group 2 (50 ppm) caused an increase in fibroblast cell proliferation so that this group had a high number of living fibroblast cells. This is supported by the statement of Yuan et al. that the active epidermal growth factor receptor signaling is responsible for the proliferation of fibroblast cells [18].

5. Conclusion

Hydroxyapatite from *Portunus pelagicus* crab's shells extract sterilized using UV is biocompatible towards human gingival fibroblast cell.

Compliance with ethical standards

Acknowledgments

The authors would like to say thank you to Jennifer Widjaja for her assistance to review and editing this manuscript thoroughly.

Disclosure of conflict of interest

No conflict of interest.

References

[1] Supartono W, Rakhmadhani NR P. Analysis of Rejection of Indonesian Export Products Blue Swimming Crab (*Portunus pelagicus*) and Crab (*Scylla serrata*) in the United States for the Period of 2002 -2013. Prosiding Seminar Agroindustri Dan Lokakarya Nasional FKPT-TPI 2015:27–32.

[2] Rochima E. Characterization of Chitin and Chitosan from Crab Waste in Cirebon, West Java. Indonesian Fisheries Processing Journal 2007;10. https://doi.org/10.17844/JPHPI.V10I1.965.

[3] Rahayu L, Purnavita S. Optimization of Chitosan Manufacture from Chitin Crab Shell Waste (*Portunus pelagicus*) for Mercury Metal Ion Adsorbents. Reaktor 2017;11:45. https://doi.org/10.14710/REAKTOR.11.1.45-49.

[4] Asni N, Saadilah MA, Saleh D. Optimization of Chitosan Synthesis from Crab Shell as Adsorbent of Heavy Metal Pb(II). Spektra: Jurnal Fisika Dan Aplikasinya 2014;15:18–25.

[5] Khiri MZA, Matori KA, Zainuddin N, Abdullah CAC, Alassan ZN, Baharuddin NF, et al. The usability of ark clam shell (Anadara granosa) as calcium precursor to produce hydroxyapatite nanoparticle via wet chemical precipitate method in various sintering temperature. Springerplus 2016;5. https://doi.org/10.1186/S40064-016-2824-Y.

[6] Drismayanti I, T S, B AU, Ruslin Muh, Jubhari EH. Ratio of successful rate of dental implant between distraction osteogenesis with autogenous bone graft. Journal of Dentomaxillofacial Science 2012;11:180. https://doi.org/10.15562/JDMFS.V11I3.335.

[7] Ichsan MZ, Siswanto S, Hikmawati D. Synthesis of Macropores Collagen - Hydroxyapatite Composite as a Candidate for Bone Graft. Jurnal Fisika Dan Terapannya 2013.

[8] Troy D, Beringer P. Remington: The Science and Practice of Pharmacy. 21st ed. Philadelphia: Lippincott Williams & Wilkins; 2006.

[9] Adji D, Zulianti Z, Larashanty H. Comparison of the Effectiveness of 70% Alcohol, Infrared, Autoclave and Ozone Sterilization against the Growth of Bacillus subtilis Bacteria. Jurnal Sain Veteriner 2007;25. https://doi.org/10.22146/JSV.275.

[10] Bolton JR, Cotton CA. The Ultraviolet Disinfection Handbook. 1st ed. American Water Works Association; 2008.

[11] Nishikawa H. Radical generation on hydroxyapatite by UV irradiation. Mater Lett 2004;58:14–6. https://doi.org/10.1016/S0167-577X(03)00396-3.

[12] Ariani MD, Yuliati A, Adiarto T. Toxicity testing of chitosan from tiger prawn shell waste on cell culture. Dental Journal (Majalah Kedokteran Gigi) 2009;42:15–20. https://doi.org/10.20473/j.djmkg.v42.i1.p15-20.

[13] Zhang S. Biological and Biomedical Coatings Handbook: Applications - Google Books. Taylor & Francis; 2011.

[14] Mucalo MR. Hydroxyapatite (HAp) for Biomedical Applications. Hydroxyapatite (HAp) for Biomedical Applications, Elsevier Ltd; 2015, p. 1–381. https://doi.org/10.1016/C2013-0-16440-9.

[15] Srivastava A, Jain VK, Srivastava A. SEM-EDX analysis of various sizes aerosols in Delhi India. Environmental Monitoring and Assessment 2008 150:1 2008;150:405–16. https://doi.org/10.1007/S10661-008-0239-0.

[16] Higashi T, Okamoto H. Influence of particle size of hydroxyapatite as a capping agent on cell proliferation of cultured fibroblasts. J Endod 1996;22:236–9. https://doi.org/10.1016/S0099-2399(06)80139-1.

[17] Akhila JS, Shyamjith D, Alwar M. Acute Toxicity Studies and Determination of Median Lethal Dose. Curr Sci 2007;93:917–20.

[18] Kartono GS, Widyastuti W, Setiawan HW. Biocompatibility of Hydroxyapatite Graft from Blood Mussel Shells (Anadara granosa) Against Fibroblast Cell Culture. Denta Jurnal Kedokteran Gigi 2014;8:1–8. https://doi.org/10.30649/DENTA.V9I2.8.