

Synthesis and Characterizations of Hydroxyapatite Derived Blood Clam Shells (*Anadara granosa*) and Its Potency to Dental Remineralizations

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Abstract

Blood clam shells (*Anadara granosa*) was used as a raw material. Synthesis of hydroxyapatite was utilized for dental remineralization. Calcination was carried out to clam shell species *Anadara granosa* at 1100°C for 5 hours. The calcination result was reacted with $(\text{NH}_4)_2\text{HPO}_4$, then dried at 110 °C for 5 hours. Sintering was conducted on dry precipitates that produced at 800 °C for 2 hours. The size of the obtained Hydroxyapatite was 7.66507 nm. Hydroxyapatite was then tested for its effectiveness for the remineralization of tooth using acetate buffer pH 5.0 with acetic acid 1 M with variations of immersion time and the hydroxyapatite addition. The results showed that the rate of dental remineralization in acetate buffer has increased significantly with the provision of hydroxyapatite into a solution in which the greater the addition of hydroxyapatite, the greater the rate increase of tooth remineralization.

Keyword: clam shells, hydroxyapatite, remineralization.

INTRODUCTION

Nanoparticle synthesis of hydroxyapatite (HAp) has been known for decades [1,2]. HAp nanoparticle synthesis can be undertaken in several ways, one of which is a deposition method. Deposition method is chosen as the most efficient method of synthesis due to the large quantities of Hap product without using organic solvents and inexpensive cost [1].

Hydroxyapatite can be obtained by extraction from natural sources [3,4]. Some sources HAp including egg shells [5], clam shell mussel [4], a chicken bone [6] corals [3,7] and bone [8]. HAp can be synthesized from the shells of blood (*Anadara*

granosa) [9,10]. Nano-sized hydroxyapatite obtained and might be utilized in repairing bone [10]. Hydroxyapatite also can be employed as bone implants [11]. Hydroxyapatite could also be harnessed for tooth remineralization. This is due to the bones and teeth that have similar structures containing hydroxyapatite [1].

Hydroxyapatite can be acquired through synthesis by reacting with the calcium phosphate [12]. Hydroxyapatite can be synthesized from the precursors of calcium and phosphate through precipitation method at 40 °C [13]. The precursor calcium might be from calcium oxide (CaO) that derived on the calcination of calcium carbonate (CaCO₃) contained natural material which is then reacted with a precursor phosphate such as diammonium hydrogen phosphate [(NH₄)₂HPO₃] [14].

The mussels blood contains approximately 98.7% CaCO₃ [15]. The content of calcium carbonate in the shells of blood is a source of calcium (CaCO₃) which can be used as a natural material synthesis of hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂]. This compound is the major inorganic component in bones and teeth [16]. In addition, the abundant availability and the price is highly reasonable. The amount of blood shellfish produced in Indonesia as many as 34382 tonnes/year, but this commodity generates waste in the form of a shell that utilization is not optimal [17]. This material is one of the active ingredients that can be added to toothpaste products for protection against tooth demineralization [18].

MATERIALS AND INSTRUMENTS

The materials that used in this research were the tooth sample, taken from the shell *Anadara granosa* at the coast of Tanjung Bayam Makassar, (NH₄)₂HPO₄, CH₃COOH 100%, CH₃COONa.3H₂O, (NH₄)₆Mo₇O₂₄.H₂O, NH₄VO₃, HNO₃ pa, NaF, distilled water, aluminum foil, gauze, tissue, Whatman filter paper number 42.

The tools that would be used in this study were common glass equipments in the laboratory, Buchner flask, Buchner funnel, vacuum pump Sargent-Welch Co. the model 1400, hotplate Idealife, furnace 6000-Barnstead Thermolyse, desiccator, thermometer, oven SPNISOSFD, Spectrophotometer 20D +, X-ray diffraction Shimadzu models 6000 (XRD), Spectrophotometer UV-vis US-110PC, Scanning Electron Microscope (SEM) Tescan Vega 3 and Fourier Transform Infra Red (FTIR) Prestige-21 Shimadzu.

METHODS

Synthesis Ca₁₀(PO₄)₆(OH)₂ from Shell of blood cockle *Anadara granosa* [Yahya (2014) and Iswar (2015)]

Waste shells were cleaned with double distilled water and dried at room temperature. Then, samples were calcined at 1100 °C for 5 hours. Calcium oxide (CaO), which was the calcination result was suspended in 100 mL of double distilled water with Ca 1 M. After that, the suspension was added with 100 mL (NH₄)₂HPO₄ solution 0.6 M drop by drop while stirring with a magnetic stirrer at 40 °C. Subsequently, the precipitate was allowed to stand at room temperature for 24 hours. The precipitate was filtered through a piece of Whatman filter paper and dried at 110 °C for 5 hours.

Furthermore, sintering was performed on the dry precipitate to gain $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ compound at 800 °C for 2 hours. Eventually, it was characterized by X-ray diffraction, FTIR, and SEM.

Preparation of Acetate Buffer solution pH 5.0 with 1 M Acetic Acid Concentration

Acetic acid (CH_3COOH) glacial 100% was pipetted as much as 17.2 mL, then diluted with a distilled water until the proper volume of 50 mL (solution I). Then, sodium acetate trihydrate ($\text{CH}_3\text{COONa}\cdot 3\text{H}_2\text{O}$) was weighed again as much as 38.9142 grams, then dissolved and diluted with distilled water until the proper volume of 250 mL (solution II). The solution I and II were then mixed and the pH was measured by using a pH meter.

Preparation of Color Reagent Phosphate Solution

A total of 2 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$ was weighed and dissolved in 20 mL of distilled water that had been boiled beforehand (reactant I). Furthermore, a total of 0.1 grams of NH_4VO_3 was dissolved in a mixture of 14 mL HNO_3 and 200 mL of distilled water that had been boiled previously (reactant II). Reagent I and II were mixed (this reagent could not be used more than one night).

Preparation of Phosphate Standard Solution

1000 ppm phosphate mother liquor was prepared by weighing 0.1394 g $(\text{NH}_4)_2\text{HPO}_4$ and then diluted to 100 mL using distilled water, after that made the standard series of 25 ppm, 50 ppm, 100 ppm, 200 ppm and 400 ppm from the mother liquor 1000 ppm.

Determination of the Effect of Adding $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ synthesis results on Acetate Buffer to Rate Enhancement Remineralization Teeth

Each of Acetate buffer solution pH 5.0 1 M (150 mL) was incorporated into five beakers sized 250 mL. Then four acetate buffer solution were added with NaF 10 ppm and another acetate buffer solution was treated without adding NaF solution. Three of acetate buffer that contain NaF were treated with the adding of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ in various concentrations of 50 ppm, 150 ppm, 200 ppm, 250 ppm, 300 ppm. Then each solution was measured the phosphate contents by using UV-Vis spectrophotometer. Tooth sample that had been cleaned and weighed, was inserted respectively into the acetate buffer 5. After the time setting from 2 h, 4 h, 8 h, 16 h, 32 h and 64h, the samples were removed and measured phosphate levels by utilizing UV-vis to observe the effect of adding $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ to the increasing rate of the remineralization of teeth, In addition, before soaking the teeth, SEM analysis was undertaken to determine the morphology of the teeth.

RESULT AND DISCUSSION

Synthesis $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ from Shell of blood cockle *Anadara granosa* [Yahya (2014) and Iswar (2015)]

Synthesis of hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ is initiated by calcination at the shells. Calcination is carried out at 1100 °C with the aim for eliminating organic components and converting the compound of calcium carbonate (CaCO_3) to calcium oxide (CaO) and CO_2 (carbon dioxide) [19].

Moreover, calcination results of CaO was measured the calcium contents using X-Ray Fluorescence (XRF) in order to obtain calcium levels after calcination at 99.47%. These results are used for calculating the amount of calcination that needed to react with a precursor phosphate $(\text{NH}_4)_2\text{HPO}_4$ then produced $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ as the reaction product between CaO contained in the result of calcination with $(\text{NH}_4)_2\text{HPO}_4$ and $\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$ as a byproduct of CaCO_3 reaction on the sample of the calcination results that was not undergoing a conversion reacted with $(\text{NH}_4)_2\text{HPO}_4$. Samples of dried precipitate result were subsequent sintered at 800 °C for 2 hours. Sintering treatment aims to remove impurities. Sintering results were then characterized using XRD, FTIR, and SEM.

FTIR Spectroscopy

Figure 1 shows that the hydroxyapatite is the dominant compound formed. The spectrum shows absorption of PO_4^{3-} in the region at 960.55 cm^{-1} ; 472.56 cm^{-1} ; 1095.57 cm^{-1} ; 603.72 cm^{-1} ; and 563.21 cm^{-1} . The sharp absorption is also found in the area 3421.72 cm^{-1} indicating the presence of the OH group as a constituent of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. In addition, the uptake in the area of 1448.54 cm^{-1} indicates the presence of carbonate groups (CO_3^{2-}) that identifies the presence of $\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3$ and $\text{Ca}_{10}(\text{PO}_4)_6\text{CO}_3(\text{OH})_2$ which has not been transforming into $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ during the sintering process.

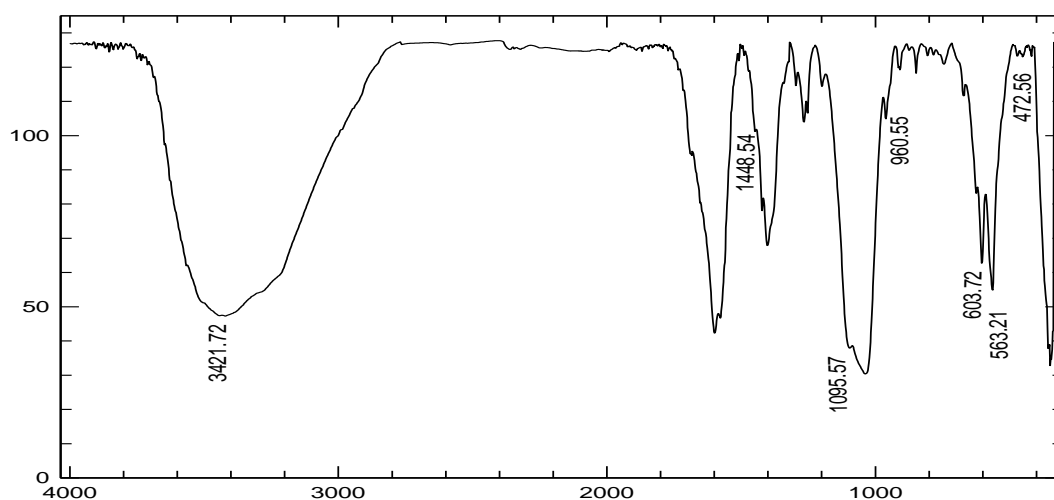


Figure 1. Spectra FTIR HAp Synthesis

Table 1. Results of FTIR Analysis hydroxyapatite compound

Uptake Cluster	Number of waves (cm ⁻¹)		
	Synthesis	Theoretical	
PO ₄ ³⁻	ν ₁	960,55	970-962
	ν ₂	472,56	472-466
	ν ₃	1095,57	1093-1041
	ν ₄	603,72 ; 563,21	603 ; 570
OH ⁻	3421,72	3650-3000	
CO ₃ ²⁻	1448,54	1440-1456	

FTIR results reinforce the notion that overall dominant compound formed is HAp. PO₄³⁻ absorption spectrum for stretching vibration (ν₁) is in the region 970 - 962 cm⁻¹ absorption, vibration symmetric bending (ν₂) is in the region 472 - 466 cm⁻¹ absorption, asymmetric stretching vibration (ν₃) is in the region 1093 - 1041 cm⁻¹, whereas for asymmetric bending vibration (ν₄) is indicated with the existence of a split absorption band in the region 603 and 570 cm⁻¹ indicating the presence of HAp crystals. The existence of free OH⁻ group in the absorption range is 3650 - 3000 cm⁻¹ [20].

X-ray Diffraction

X-Ray Diffraction is used for phase identification and characterization of the crystal structures and nanoparticles [21]. The X-rays penetrate into the nanomaterial and the resulting diffraction pattern is compared with a standard to obtain structural information [22]. XRD diffractogram of the synthesis result is disclosed in Figure 2. XRD analysis results reveal that the highest intensity of 2θ value, namely 32, 4043 °; 29.2000 °; and 26.1890 °.

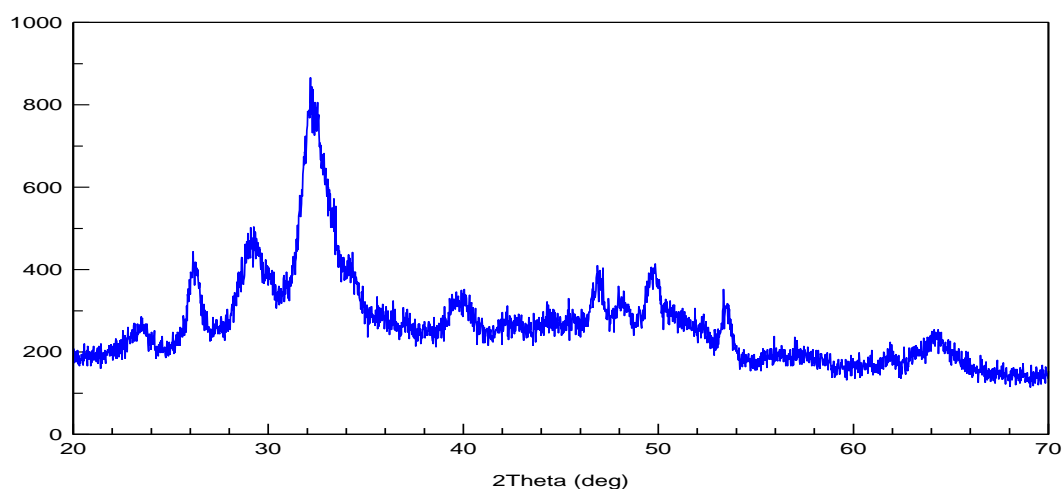


Figure 2. XRD pattern HAp Synthesis

Figure 2 presents that the result synthesis of the research is hydroxyapatite which characterized by the similarity in the data JCPDS (*Joint Committee on Powder Diffraction Standards*) number 96-900-2219. Moreover, the results of the synthesis of HAp can also be characterized by the orientation value of the crystal planes (hkl). In Figure 2 we can see that the hkl value of HAp synthesized in this research indicates a similar value to hkl HAp of the ICDD data (*International Committee Diffraction Data*) number 01-076-0694 are (121), (112) and (300).

Addition to identifying a sample, XRD results also may indicate the size of the sample. XRD patterns in figure 2 reveals the strong peaks on the spectrum with a distance value of 2θ from 20° to 70° . The average diameter of the crystal lattice of hydroxyapatite nanoparticles is 7.66507 nm that acquired from FWHM peak with high intensity.

Table 2. Diameter Calculation of HAp Crystal Lattice of Synthesis Results

Summit	2θ ($^\circ$)	d (\AA)	FWHM ($^\circ$)	D (nm)
5	32,4043	2,76066	1,63140	5,50015
3	29,2000	3,05590	1,54660	5,79129
	26,1890	3,40002	0,75800	11,70379
Average				7,66507

Scanning Electron Microscope (SEM)

Figure 3 describes that at 500 times magnification of HAp samples were not uniformly rounded shape. At a magnification of 10000 times, hydroxyapatite sample morphology is uneven and non-porous. The morphology of commercial hydroxyapatite is granules that form aggregates, the granule size is uneven and has delicate structures [23].

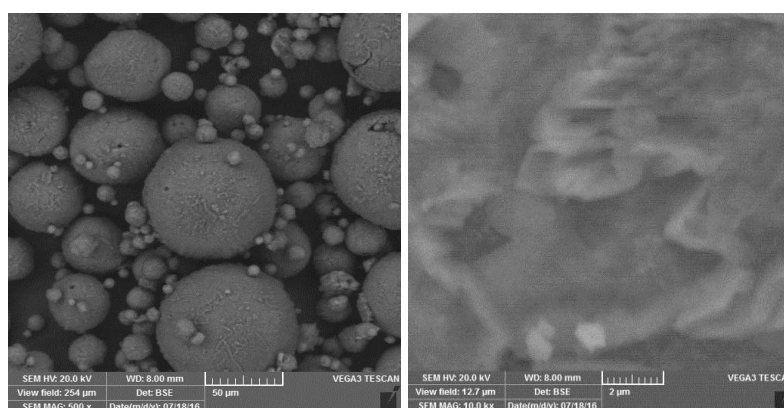


Figure 3. Morphology HAp Synthesis results Zoom Magnification 500 times and 10000 times

EDS instrument is one device which is arranged on the SEM appliance. EDS analysis generates qualitative and quantitative information about the composition of the locations on the sample with a diameter of several micrometers [24].

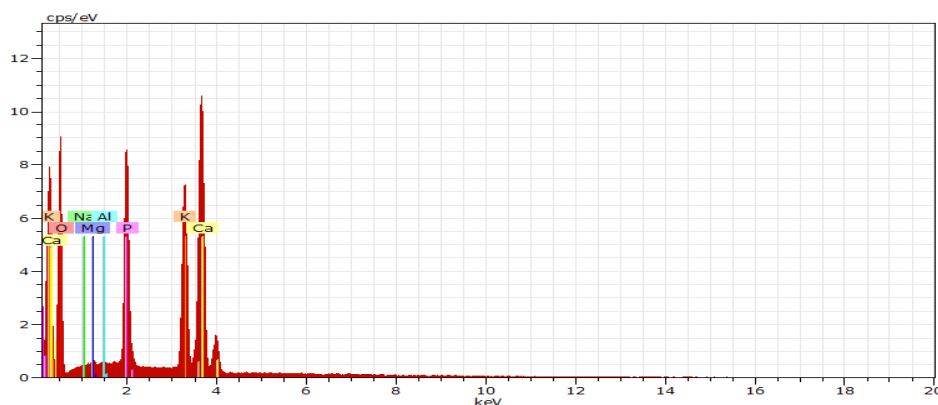


Figure 4. Results of EDS HAp Synthesis

The results of EDS analysis of the atomic compositions and HAp compound of resulted synthesis can be seen in Table 3,

Table 3. Atomic Compositions and HAp compounds of resulted synthesis

Atom	Composition (%)	Compound	Composition of the ompound (%)
O	21,83		0,00
Na	0,35	Na ₂ O	0,76
Mg	0,24	MgO	0,65
Al	0,05	Al ₂ O ₃	0,15
P	8,40	P ₂ O ₅	30,72
Ca	21,28	CaO	47,52
K	10,51	K ₂ O	20,21

The results of EDS analysis shows the HAp compositions of synthesis result are dominated by oxygen 21.83%, 21.28% calcium and 8.40% phosphorus. These compositions are in accordance with the Hap compositions.

Determination of the Effect of Adding Ca₁₀(PO₄)₆(OH)₂ synthesis results on Acetate Buffer to Rate Enhancement Remineralization Teeth

Demineralization is the loss of a part or all of mineral enamel because it dissolves in acid. The lower the pH, it will increase the hydrogen ion which will damage the enamel hydroxyapatite. Demineralization process can be restored if the pH is neutralized and there are sufficient ions of Ca²⁺ and PO₄³⁻ in the oral environment. The existence of ions will replenish the room of crystals that have been demineralized later called remineralization.

Figure 5 shows tooth sample of SEM results at magnification 1000 times and 4990 times. SEM results reveals the sample morphology of the teeth where there are cavities in the demineralization and remineralization.

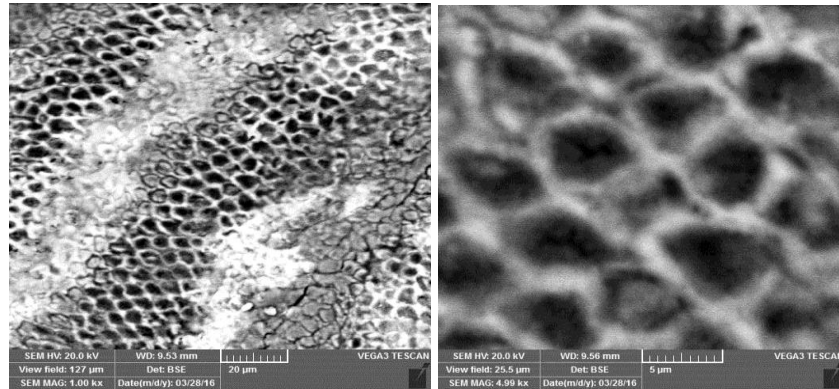


Figure 5. Morphology of tooth sample at a magnification of 1000 times and 4990 times

Figure 6 describes the relationship between the tooth immersion time and the adding level of PO_4^{3-} ions in solution where the teeth was soaked. The rate of remineralization is enhancing with the increasing adding of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ into acetate buffer. The results also showed that the longer the immersion time, the more levels but elevated the content of PO_4^{3-} ions with the addition of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ was lower than without the addition. This proves that the $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ synthesized from blood shells effective for protection against demineralization of the tooth.

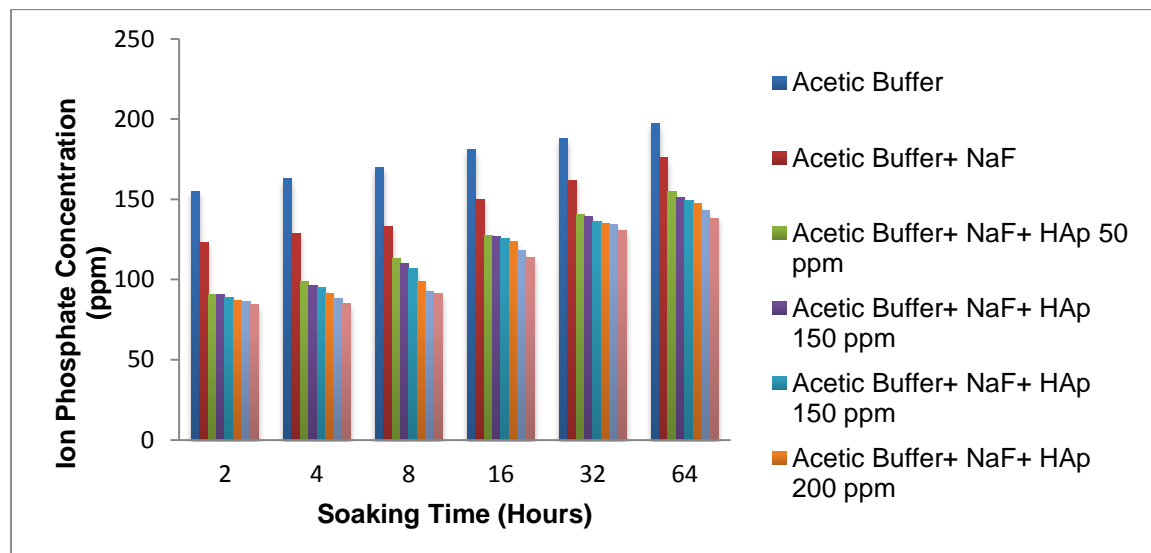


Figure 6. Relationship Between Time Immersion of Tooth Versus Levels of PO_4^{3-} Ion in the Solution where Dental Soaked

Increasing the rate of remineralization of the tooth with the addition of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ can also be observed through the mass loss of teeth soaked in eight variations of acetate buffer solution (Table 4). The larger the addition of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ in the acetate buffer solution, the mass of soluble teeth soaked in a solution of the acetate buffer is becoming smaller.

Table 4. Percentage of Dental Mass Remained On Soaking In The Acetate Buffer Solutions With Certain Various Treatments During 64 Hours

Sample		Initial mass (g)	Final mass (gram)	Percentage (%)
Acetate Buffer	Tooth 1	1,4684	1,4205	96,7379
	Tooth 2	0,5608	0,5458	97,3252
	Average	2,0292	1,9663	96,9002
Acetate Buffer +NaF	Tooth 1	1,3324	1,2907	96,8703
	Tooth 2	0,6659	0,6483	97,3569
	Average	1,9983	1,9390	97,0325
Acetate Buffer + NaF + HAp 50 ppm	Tooth 1	1,3720	1,3310	97,0117
	Tooth 2	1,1415	1,1099	97,2317
	Average	2,5135	2,4409	97,1116
Acetate Buffer + NaF + HAp 100 ppm	Tooth 1	1,7566	1,7050	97,0625
	Tooth 2	0,8851	0,8604	97,2093
	Average	2,6417	2,5654	97,1117
Acetate Buffer + NaF + HAp 150 ppm	Tooth 1	1,1024	1,0780	97,7866
	Tooth 2	0,7296	0,7136	97,8070
	Average	1,8320	1,7916	97,7947
Acetate Buffer + NaF + HAp 200 ppm	Tooth 1	1,4881	1,4563	97,8630
	Tooth 2	0,5460	0,5405	98,9927
	Average	2,0341	1,9968	98,1663
Acetate Buffer + NaF + HAp 250 ppm	Tooth 1	0,8510	0,8442	99,2009
	Tooth 2	0,7506	0,7429	98,9741
	Average	1,6016	1,5871	99,0947
Acetate Buffer + NaF + HAp 300 ppm	Tooth 1	0,9921	0,9875	99,5363
	Tooth 2	0,9690	0,9669	99,7832
	Average	1,9611	1,9544	99,6584

CONCLUSIONS

Based on these results it is concluded as follows:

1. Hydroxyapatite was synthesized from blood clam shells *Anadara granosa* with nano-sized of 7.66507 nm.
2. $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ was synthesized in vitro from waste shells of crabs effectively inhibited the demineralization of the tooth where the greater the addition of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ in solution, it was increasingly inhibiting demineralization of the tooth or the smaller the rate of the tooth demineralization in solution.

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