IJPSR (2019), Volume 10, Issue 6 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 07 October 2018; received in revised form, 25 December 2018; accepted, 31 December 2018; published 01 June 2019

HYDROXYAPATITE AND Zn-HYDROXYAPATITE SYNTHESIS USING CALCIUM FROM LAKE MANINJAU PENSI SHELLS AND RESISTANCE TEST ON BACTERIA

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Keywords:

Shells, Hydroxyapatite, Zn-Hydroxyapatite, Antibacterial, Sol-gel

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ABSTRACT: HAp and Zn-HAp Synthesis with several concentrations of Zn (5%, 10%, 15%, and 20%) w/w using Ca precursor from Maninjau Lake pensi shells was done with the sol-gel method. XRF for three hours obtained 96.525% Ca content in the form of CaO. XRD result for the synthesized samples showed the same diffraction pattern as the standard HAp (ICSD: 154493) phase which the intensity decreases with the addition of Zn concentration. In Zn-HAp 15% and 20% which were sintered at 600 °C for two hours, it was found that the impurity peaks were TCP [$Ca_3(PO_4)_2$] and [$Zn_3(PO_4)_2$] on the XRD pattern. In this study, the maximum concentration of Zn substitution in the HAp structure was obtained in Zn-HAp 10%. SEM result showed that Zn-HAp 10% had a better homogeneity than Zn-HAp 15% which produced impurities. Good antibacterial properties were also shown by Zn-HAp 10% for both tests of bacteria *i.e. Staphylococcus aureus* and *Escherichia coli*.

INTRODUCTION: Hydroxyapatite (HAp, $Ca_{10}(PO_4)_6(OH)_2$ is a bioceramic which is chemically similar to the main mineral component of bone and tooth tissue. Hydroxyapatite in bone vertebrates amounts to 65% of the total bone mass, with the remaining mass formed from organic and water. HAp is widely applied in the medical materials use as in orthopedic applications of bone and dental implants, bone fillers, bioactive coatings, soft bone repair, drug/ protein/ gene preparation, *etc.* because it has good biocompatibility properties, osteoconductive, bio-active, non-toxic, and not immunogenic [1, 2, 3, 4, 5]. Various methods have been widely applied for HAp synthesis such as sol-gel method, hydrothermal, precipitation, *etc.* [4, 5, 6,]

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.10(6).2993-97

The article can be accessed online on www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.10(6).2993-97

Each method has its strengths and weaknesses, both in the products, costs, and availability of tools. The sol-gel method provides certain advantages such as mixing homogeneous molecules, using low temperature during processes, and its ability to produce nanosized particles and nanocrystalline powders [5]. Calcium (Ca) precursor used in the synthesis of HAp is found in nature such as limestone, shell waste, cow bone waste, and many others [5, 7, 8].

Utilizing natural materials as precursors can minimize the existing waste and provide more valuable benefits. West Sumatra has various types of shell, one of which is pensi shells which are spread in Lake Maninjau, Lake Singkarak, Lake Diatas, and Alahan Panjang, Pensi shells waste containing large CaO which has not been maximally utilized. Therefore, this is one of a potential solution for the synthesis of HAp by utilizing CaO from pensi shells [9, 10]. Every year more than 2.2 million people worldwide require bone implant surgery, bone replacement, and bone grafting to repair bone defects arising from

accidents, trauma or disease (such as osteoporosis, tumor resection, *etc*.) which are common problems in orthopedics [11].

Post-implantation bacterial contamination has dire consequences in surgical and orthopedic practice. Although, the incidence rate is low, infections caused by the *Staphylococcus aureus* or *Escherichia coli* bacteria are the cause of general clinical problems each year [14]. HAp is the solution to this problem because it has good biocompatibility, osteoconductive and bioactive properties. It has been reported that the incorporation of Zn^{2+}, Ag^{+}, Mg^{2+}, Mn^{2+} cations, *etc*. into the structure of HAp can improve the properties of HAp, crystallinity, and also its antimicrobial properties. Zn ions can stimulate bone mineralization pathological detoxification because of its presence in biological apatites [1, 14]. It has also been determined that hydroxyapatite doped with Zn ions has a strong inhibitory effect on various bacterial growths [12].

Characterization of the shell is done by XRF (X-Ray Fluorescence) to determine the chemical composition of a material. Characterization of HAp and Zn HAp samples carried out with XRD (X-Ray Diffractometer) and SEM (Scanning Electron Microscope).

EXPERIMENTAL:

Materials: Materials used in this study were mortar and pestle, glassware, sieve, filter paper, crucible, analytical balance, oven, furnace, incubator, autoclave, laminar chamber. Characterization instruments used were XRF (PANalitytical Epsilon 3), XRD (Philips X'pert Powder), and SEM (JEOL JSM-IT-300).

Reagents: The chemical used are pensi shell as a source of calcium, aquades, NH_4OH (Brataco) as a pH controller solution, HNO_3 (Brataco) as solvent, $(NH_4)_2HPO_4$ (Brataco) as a source of phosphate, Zn $(NO_3)_2.4H_2O$ (Brataco) as a Zn metal source of HAp modifier, NA (Bataco) media, and MHA media.

Methods:

Ca Preparation from Pensi Shell of Lake Maninjau: The pensi shell as a Ca precursor was washed with clean water to remove the sludge, then dried. The pensi shells are finely ground using mortar and pestle. The sample was then calcined in the furnace for five hours at 900 ºC. Cooled and obtained CaO.

Synthesis of Hap with Sol-Gel Method: A total of 4.2 g of CaO from the calcination of pensi shells were dissolved in 2 M HNO_3, a stirred at 85 ºC for 15 min and filtered. The filtrate $Ca(NO_3)_2$ was added to NH_4OH to pH 10 and obtained $Ca(OH)_2$ sol. Sol $Ca(OH)_2$ added with 250 ml of 0.18 M $(NH_4)_2HPO_4$ solution, stirred and pH was adjusted to 10 with NH_4OH. White sol is heated at 85 ºC temperature for 5 h. White sol was left overnight, decanted and filtered with Whatman filter paper to obtain HAp gel. The HAp gel is dried at 110 ºC, homogenized with mortar and pestle. HAp powder sintered at 600 ºC for 2 h to increase the crystallinity of HAp material.

Synthesis of Zn-HAp with Sol-Gel Method: A total of 4.2 g CaO from calcination were dissolved in 2 M HNO_3, stirrer at 85 ^{o}C for 15 min and filtered. The filtrate $Ca(NO_3)_2$ was added to NH_4OH to pH 10 and $Ca(OH)_2$ sol was obtained. Sol $Ca(OH)_2$ was added with 250 ml of 0.18 M $(NH_4)_2HPO_4$ solution andstirred. Before the whole 0.18 M $2HPO_4(NH_4)_2$ solution was mixed, $Zn(NO_3)_2.4H_2O$ was added (5%, 10%, 15%, and 20%) w/w, and the pH of the solution was adjusted to 10 with NH_4OH. White sol was heated at 85 ^{o}C for 5 h. White sol was left overnight, decanted and filtered with whattman filter paper to obtain HAp gel. This gel was dried at 110 ^{o}C, homogenized with mortar and pestle. HAp powder was sintered at 600 ^{o}C for 2 h to increase the crystallinity of the Zn-HAp material. Materials obtained were 5% Zn-HAp, 10% Zn-HAp, 15% Zn-HAp, and 20% Zn-HAp.

RESULTS AND DISCUSSION:

Composition Analysis of Pensi Shell Using XRF: The chemical compositions of Maninjau pensi shells calcined at 900 ^{o}C for three hours were analyzed by using X-Ray Fluorescence (XRF). The results were obtained in **Table 1**.

From the results, it can be seen that the Maninjau pensi shell has a very high CaO content namely 96.525%. Based on these results it is possible to use pensi shell as the basic material for the production of hydroxyapatite as calcium precursor.

Meanwhile, *pensi* also contains other metal oxides in small amounts as presented in **Table 1**.

TABLE 1: COMPOSITION OF PENSI MANINJAU SHELL SHELLS

Compounds	Composition (%)
Al_2O_3	0.623
SiO_2	0.7
SO_3	1.062
K_2O	0.026
CaO	96.525
TiO_2	0.015
MnO	0.009
Fe_2O_3	0.206
ZnO	0.003
Rb_2O	0.001
SrO	0.154
ZrO_2	0.002
Ag_2O	0.651
CdO	0.037
BaO	0.025
Nd_2O_3	0.001

Analysis of XRD: The results of X-ray diffraction was used to determine whether HAp and Zn-HAp were formed from synthesis known by comparing with the HAP standard (ICSD: 154493) [16]. This data was also used to find out how substituted Zn affect the composition of the HAp phase.

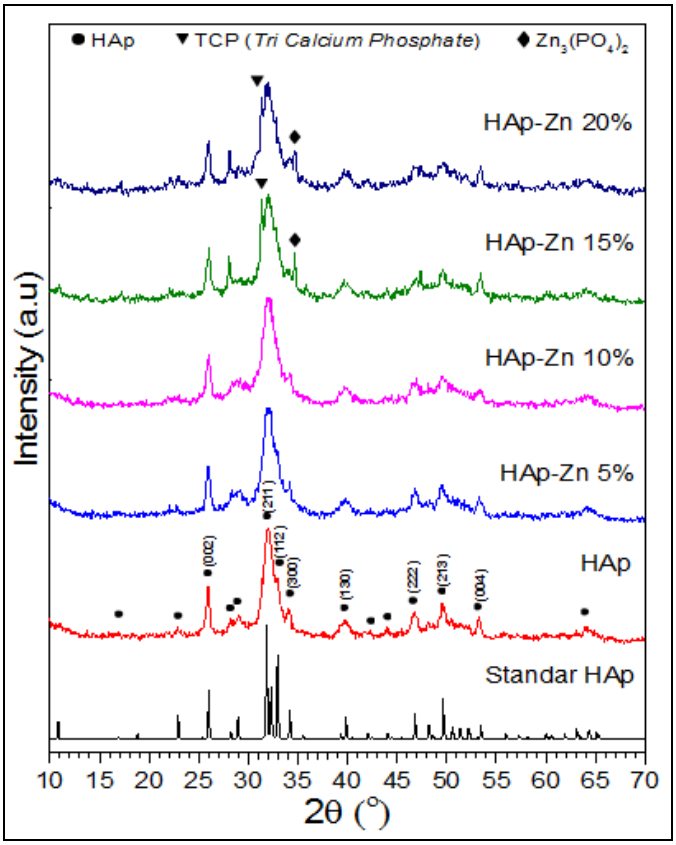

FIG. 1: STANDARD XRD PATTERNS OF HAp, HAp, AND Zn-HAp

In **Fig. 1**, the XRD patterns of HAp and Zn-HAp with several variations of Zn concentration in w/w% inform that HAp and Zn-HAp are successfully synthesized by utilizing Ca precursors from Maninjau pensi shells with the sol-gel method. XRD diffraction patterns generally show good correlation with HAp stoichiometry (ICSD: 154493). It has been reported in reference [1] that the Zn^{2+} ion can replace Ca^{2+} ions in HAp lattice. Zinc affects the structural parameters when replacing Ca^{2+} ions in the HAp crystal structure as shown in the XRD pattern **Fig. 1**. The XRD patterns of Zn-HAp 15% w/w and Zn-HAp 20% w/w show the presence of an impurity peak in the form of TCP (Tri-calcium phosphate) which is a derivative of apatite due to sintering temperature and impurity peak [$Zn_3(PO_4)_2$] because Zn ion is extracted from the HAp structure to form this phase. Increasing Zn ions will slow the growth of HAp [17, 18].

TABLE 2: BASIC DATA FOR ONE OF THE STRONGEST PEAKS OF EACH TEST SAMPLE

Sample	Peak 2θ (Deg)	FWHM (Deg)	Intensity (Counts)
HAp	32,1126	1,30170	119
Zn-HAp 5%	32,1667	1,46000	101
Zn-HAp 10%	32,1400	1,51330	96
Zn-HAp 15%	32,0768	1,88000	94
Zn-HAp 20%	32,0368	1,72000	93

Table 2 shows a decrease in the intensity of the diffraction peak where the intensity is lower with increasing concentration of Zn ion substitution [1, 13, 16, 17, 18]. The decrease in intensity also indicates that the Zn^{2+} ion is substituted into the structure of HAp to replace several Ca ions in the HAp crystal structure without changing the phase of the HAp.

SEM (Scanning Electron Microscope) Analysis: SEM analysis was performed on 10% Zn-HAp samples and 15% Zn-HAp in order to compare the morphology of the two samples as shown in **Fig. 2**. From the figure, it can be seen that the morphology of the two samples looks different.

Differences in the morphology of the 10% Zn-HAp and 15% Zn-HAp samples are seen according to the results of the XRD characterization of these two samples. The increase in Zn^{2+} ion concentration in the HAp structure affects the crystallinity of HAp which shows impurities in the XRD pattern in the form of TCP (Tri-calcium phosphate) and [$Zn_3(PO_4)_2$].

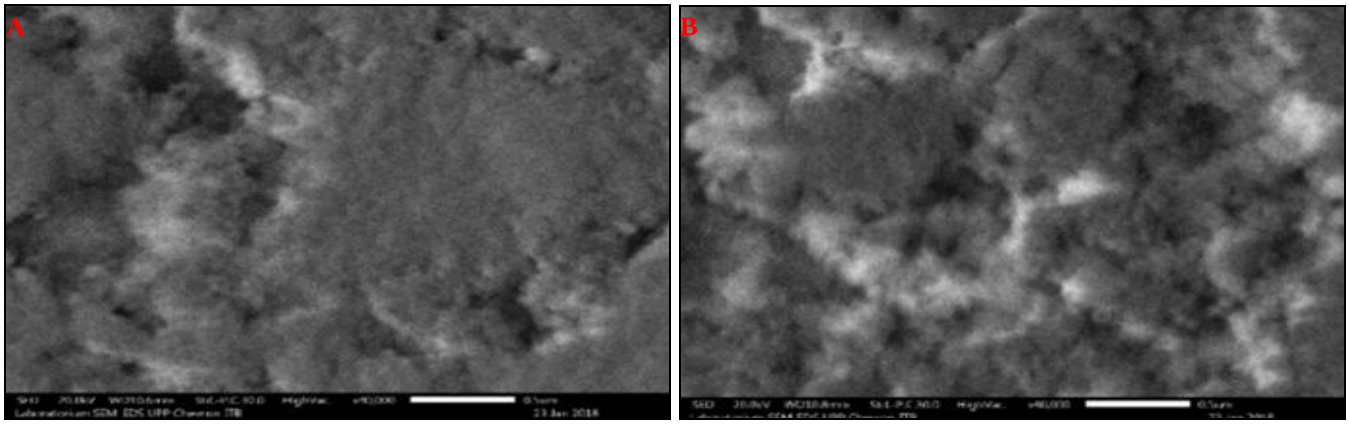

FIG. 2: SEM (A) 10% Zn-HAp, (B) 15% Zn-HAp AFTER CALCINATION AT T = 600 ºC FOR 2 h

Anti-bacterial Test of HAp and Zn-Hap: Antibacterial tests were conducted on HAp samples (as anegative control), 5% Zn HAp, 10% Zn-HAp, 15% Zn-HAp, 20% Zn-HAp, and amoxicillin as apositive control. In this antibacterial test, two types of bacteria were used namely gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*. The inoculum was prepared from fresh culture after incubation for 24 h at 37 °C. Anti-bacterial activity was observed using the disc diffusion method, the minimum inhibition zone was measured in millimeters as shown in **Table 3**. From the results of theantibacterial test in **Table 3**, it can be seen that the increase in Zn concentration increases the activity of the HAp biomaterial **Fig. 1**.

TABLE 3: INHIBITION ZONE OF ANTIBACTERIAL ACTIVITY IN mm

Type of bacteria	The diameter of the inhibitory zone (mm)					
	Negative control	Zn-HAp 5%	Zn-HAp 10%	Zn-HAp 15%	Zn-HAp 20%	Positive control
S. aureus	8	11	13.5	15	12.5	11
E. coli	7.75	8	14.25	9.1	8.25	14

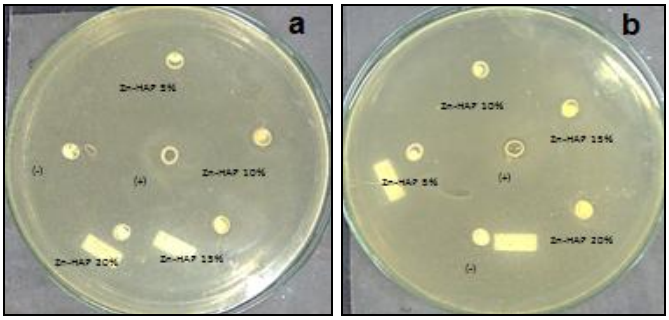

FIG. 3: TEST RESULTS OF ANTIBACTERIALS (a) *E. COLI*, AND (b) *S. AUREUS*

The highest inhibition zone is found in 15% Zn-HAp in gram-positive bacteria *S. aureus* but in XRD analysis, this sample shows different phases of HAp which will be used as bone and dental implants. It can be concluded that 10% Zn-HAp has better antibacterial activity than pure HAp and HAp-Zn at other concentrations. The suggested mechanism is associated with the ability of Zn ions to form strong bonds with functional groups (carboxylates, imidazoles, thiols, and amines) from proteins which present in bacterial cell membranes (serious damage to membranes because metal ions touch the membrane) [1, 15].

As a consequence, nutrients and other components of the cytoplasm come out of the cell causing death in microorganisms. Structural changes in the membrane cause permeability to increase as a result of collapsing cell transport systems and dead microorganisms. Anti-bacterial activity is caused by three factors namely metal concentrations, metals interaction with bacteria and the cell membrane structure that is different from gram positive and gram negative.

CONCLUSION: This research has succeeded in synthesizing HAp and Zn-HAp with various concentrations of Zn in w/w% by utilizing Ca precursors from Maninjau pensi shells containing 96.525% CaO with the sol-gel method. The maximum substituted Zn concentration was obtained at 10% w/w at 600 °C sintering for two hours. In the results of 15% Zn-HAp XRD and 20% Zn HAp, there were obtainedimpurities in the form of TCP [$Ca_3(PO_4)_2$] and [$Zn_3(PO_4)_2$]. The greatantibacterial properties that have beenshown by 10% Zn-HAp generally have the largest

inhibition zone for both types of *Staphylococcus aureus* and *Escherichia coli*.

ACKNOWLEDGEMENT: The authors thankthe Ministry of Research, Technology and Higher Education, Republic of Indonesia, since part of this work is supported by DP2M DIKTI No. 06/UN.1.17PPP.PBK.EET/LPPM/2018 under Penelitian berbasis kompetensi Research Grant.

CONFLICT OF INTEREST: The authors declare that there is no conflict of interest.

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How to cite this article:
Putra WA, Agustien A and Jamarun N: Hydroxyapatite and Zn-Hydroxyapatite synthesis using calcium from Lake Maninjau pensi shells and resistance test on bacteria. Int J Pharm Sci & Res 2019; 10(6): 2993-97. doi: 10.13040/IJPSR.0975-8232.10(6).2993-97.