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SYNTHESIS AND CHARACTERIZATION OF Mg, Cu-HAp USING CALCIUM FROM LOKAN SHELL (*Geloina expansa*) AND ITS ANTIBACTERIAL ACTIVITY

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ABSTRACT

Hydroxyapatite (HAp) is the primary inorganic constituent found in bones and teeth. It possesses biocompatibility and bioactivity within the human body, while its mechanical qualities are somewhat inferior. Magnesium is a viable alternative to calcium in the composition of bone minerals. Copper is both an antibacterial agent and a vital metal within our bodies. This study aims to produce Mg, and Cu-HAp by employing the sol-gel process in situ utilising Lokan shell (Geloina. Expansa) as the source material. Additionally, the antibacterial properties of Mg and Cu-HAp will be evaluated. The lokan shell is utilized as a calcium source, containing 94.626% calcium oxide (CaO), for synthesizing hydroxyapatite (HAp) with a calcium-to-phosphorus ratio of 1.67. The X-ray diffraction (XRD) analysis revealed a distinct pattern at a specific angle of 2θ, following the ICDD (No.96-101-1243) standard. The FT-IR spectra suggest that the bending vibration of the PO_4^{3-} functional group occurs at a wave number of 610 cm^{-1}, whereas the asymmetrical stretching occurs at wave numbers between 1010-1036 cm^{-1}. These observations suggest the presence of HAp content. The antibacterial test results demonstrated that Mg, Cu-HAp3, and Mg, Cu-HAp4 exhibited good antibacterial activity, as evidenced by inhibition zone diameters of 0.9 mm for Escherichia coli and 0.8 mm for Staphylococcus aureus. These findings indicate that Mg and Cu-HAp effectively possess antibacterial properties.
Keywords: Lokan Shells, HAp, Mg, Cu-HAp, Sol-Gel, Antibacterial.

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INTRODUCTION

HAp is also known as calcium phosphate which has a high compatibility level with the human body due to its calcium and phosphorus content and is often used in some parts of the body as implants.[1] In addition, HAp is also used as a coating for metal prostheses in maxillofacial surgery, dentistry, and orthopedics, such as bone graft materials and particle fillers for bone defects.[2] Hydroxyapatite (HA) is a compound with a chemical formula $Ca_{10}(PO_4)_6(OH)_2$ and hexagonal symmetry in the spatial group P63/m, lattice parameters a=0.95 nm and c=0.68 nm.[3,4] HAp can come from various natural (biomaterials) sources with applications like adsorbents and catalysis. HAp biomaterials include animal bones, cuttlebone[5], eggshells[6], scallop shells[7], blood cockle shell[8], and bamboo shell[9]; Lokan HAp can also made in the laboratory through several methods, including the precipitation method, sol-gel method, and electrodeposition method.[10] HAp plays a crucial role in its use as a substitute for dentin for filling teeth. HAp as a bioceramic insert in teeth has several advantages, such as reducing the amount of composite dental filling material and reducing polymerization shrinkage.[11] Because it has excellent biocompatibility and high affinity with polymers[12], HAp is better used as a dental filling material than amalgam because it is safer and reduces the risk of failure due to breakage and wear.[13] Although synthetic HAp has good biocompatibility and bioactivity, the mechanical properties of synthetic HAp are lower than those of human bone or teeth. In previous studies, Mg, Cu-doped HAp synthesis has been carried out by maintaining the Cu ion concentration of 0.4 mol% to provide high antimicrobial properties and maintain a decrease in biocompatibility with the addition of Mg ions, where the source of calcium HAp used comes from commercial $Ca(NO_3)_2{\cdot}4H_2O$ and synthesized hydrothermally. Magnesium (Mg) is the fourth most prevalent positively charged ion in the human body, comprising 0.44-1.23% of its weight. It replaces calcium in bone minerals because its chemical structure is identical to bone Ca. Mg can mineralize bones and proliferate osteoblastic-like cells, increasing bioactivity

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and biocompatibility.[2] When Mg ions partially replace Ca ions in the hydroxyapatite structure, it can affect the crystallization and thermal stability of hydroxyapatite, causing the formation of β-tricalcium phosphate (β-TCP) and producing biphasic calcium phosphate (BCP). In addition, the incorporation of Mg^{2+} stabilizes the β-TCP phase and increases its transition temperature to α-TCP above 1125°C. Copper (Cu) is an antimicrobial agent and an essential element in the body. In addition to its bactericidal effects, Cu has a role in cross-linking collagen and bone elastin.[2] In this study, the synthesis and characterization of Mg, Cu-HAp using the Sol-gel method and testing the antibacterial activity of Mg, Cu-HAp were carried out. HAp is used from natural sources, namely the shell of the Lokan clam (*Geloina expansa*) waste. The shell of the Lokan clam is a potential source of calcium. In addition, almost 95% of the main composition of the Lokan clam is in the form of CaO.

EXPERIMENTAL

Materials

The materials used in this study are the shells of *Geloina expansa* obtained from the coast of Padang, West Sumatra. HNO_3 pa (Merck), $(NH_4)_2HPO_4$ p.a (Merck) as a phosphate source, $Mg(NO_3)_2.6H_2O$ p.a (Merck) as Mg metal source, $Cu(NO_3)_2.3H_2O$ p.a (Merck) for Cu metal source, NH_4OH as a pH controller or base-giver in HAp synthesis, Nutrient agar (NA), and isolates of *S. Aureus* and *E. Coli* bacteria.

Preparation of CaO Powder from Lokan Shells (*G. expansa*)

The Lokan shell samples are cleansed of debris using water and then pulverized to acquire shell powder. The shell powder undergoes calcination at 900°C for 5 hours, producing CaO powder. The CaO powder acquired is analyzed using X-ray fluorescence (XRF).[8]

Synthesis of Bionano-HAp Composite by Sol-Gel Method

4.2 grams (0.075 moles) of CaO powder was dissolved in a beaker containing 70 milliliters of 2 molar HNO_3. The solution was agitated for 15 minutes at 65°C, forming a $Ca(NO_3)_2$ solution, which was then filtered. The filtrate was heated and stirred at 110ºC for 5 hours. NH_4OH p.a solution was added drop by drop until a pH of 10 was obtained, forming a $Ca(OH)_2$ sol. Then, 250 mL of $(NH_4)_2HPO_4$ 0.18 M was slowly added while maintaining the pH of the solution; HAp gel was formed from the $Ca(NO_3)_2$ sol, which was then allowed to stand for 24 hours and filtered and dried at a temperature of 110ºC, followed by calcination at a temperature of 900ºC for three hours.[14]

Mg, Cu-HAP Composite Synthesis by Sol-Gel Method

CaO powder was dissolved in a beaker with 70 mL of 2 M HNO_3. The solution was stirred using a magnetic stirrer for 15 minutes at 65°C with a stirring speed of 250 rpm, forming a $Ca(NO_3)_2$ solution, which was then filtered. The filtrate was heated and stirred at 110ºC for 5 hours. NH_4OH p.a solution was added drop by drop until a pH of 10 was obtained, forming a $Ca(OH)_2$ sol. Then, 250 mL of $(NH_4)_2HPO_4$ 0.18 M was slowly added while maintaining the pH of the solution, $Mg(NO_3)_2$ and $Cu(NO_3)_2$ were added according to the composition in Table-1, forming HAp gel that had been doped with Mg, Cu, then allowed to stand for 24 hours and filtered and dried at a temperature of 110ºC, followed by calcination at 900ºC for 3 hours. Mg, Cu-Hap is created with variations in Mg composition as follows 0 mol, 0 mol, 0,0005 mol, 0,0025 mol, and 0,00375 mol and Hap as follows Mg, Cu-HAp0 (0,075 mol, 0,075 mol, 0.0743 mol, 0,0723 mol and 0,07105 mol that have been synthesized were characterized by XRD and FT-IR.

Table-1: Oxide Composition of Lokan Shell

Compound	Composition (%)
Al_2O_3	0.411
P_2O_2	0.548
CaO	94.626
Cr_2O_3	0.004
MnO	0.02
Fe_2O_3	0.461
CuO	0.003
SrO	0.13

Mg, Cu-HAp0 and Mg, Cu-HAP Antibacterial Activity Test

The antimicrobial efficacy of Hydroxyapatite (HAp) and Magnesium-Copper Hydroxyapatite (Mg, Cu-HAp) was assessed against Staphylococcus aureus and Escherichia coli germs, alongside the positive control Amoxicillin, using the disc diffusion or Kirby Bauer method. Bacteria were cultivated using a nutrient agar medium. The test bacteria were incubated for 24 hours in an incubator. After that, the antibacterial testing was carried out by incubating the bacteria into the NA medium in a petri dish. HAp (negative control), Mg, Cu-HAp, and Amoxicillin (positive control) were added 5 mg each to each disk placed on the test medium filled with bacteria. Then, it was allowed to stand for 24 hours in an incubator at 37ºC, then observed, and the diameter of the clear zone formed was measured as the diameter of the inhibition zone of HAp and Mg, Cu-HAp antibacterial activity.[15]

Instrumentation

The instruments used in this study are XRF (PANalytical minipal 4 type), XRD (Bruker D8 Advance), and FT-IR (Shimadzu IR Prestige 21 type).

RESULTS AND DISCUSSION

Preparation of Lokan shell

The preparation of Lokan is first cleaned from dirt and remaining attached meat. Then, it undergoes sieving to standardize the sample size and increase the surface area. The calcination of Lokan shells aims to break down organic groups, such as $CaCO_3$ which will be transformed into CaO.[10] The amount of calcium oxide formed will affect the dissolution process; perfect calcium oxide dissolution will produce an optimum amount of calcium ions.[16]

Analysis of Lokan Shell Using XRF

Table 1 shows that the Lokan shell contains 94.626% calcium oxide (CaO); 0.461% hematite (Fe_2O_3) and 0.411% alumina (Al_2O_3) as well as several other oxides. According to the provided data, Lokan shell can be utilized as a precursor or substitute material for CaO in the synthesizing of hydroxyapatite as its main component.[9] It can also be observed in Table-1 that, besides CaO, there are other compound contents and oxides in the lokan shell; however, this will not affect the results for the synthesis of HAp from CaO as their concentrations are minimal.

Results of Synthesis of Mg, Cu-doped HAp Composite (Mg, Cu-HAp)

The synthesis of HAp from the Lokan shell is initially performed using the sol-gel method with HNO_3 as the solvent. HNO_3 will dissolve the metal residues from the calcination process CaO that are not needed for the synthesis. Subsequently, stirring is done for 15 minutes to achieve a homogeneous solution. The reaction of CaO dissolution with HNO_3 is as follows:

$$CaO_{(s)} + 2HNO_{3(l)} \rightarrow Ca(NO_3)_{2(aq)} + H_2O_{(l)} \quad (1)$$

The filtrate solution, consisting of $Ca(NO_3)_2$ and NH_4OH, is then added until a sol is formed with a pH of 10. Then, the formed sol is supplemented with $(NH_4)_2HPO_4$, $Mg(NO_3)_2$, and $Cu(NO_3)_2$ until it transforms into a white to bluish-white HAp gel. This process is referred to as condensation. The doped HAp is then left for 24 hours and undergoes calcination for 3 hours to evaporate any remaining solvent. The synthesis of Mg and Cu-doped HAp is conducted with various concentration variations based on the Ca/P ratio of 1.67 to investigate the influence of doping on Mg, and Cu-HAp as antibacterial agents.

X-ray diffraction (XRD) Analysis of HAp and Mg, Cu-HAp

Figure-1 (b) Shows the XRD diffraction peaks of Mg, Cu-HAp0, Mg, Cu-HAp1, Mg, Cu-HAp2, Mg, Cu-HAp3, and Mg, Cu-HAp4. The diffraction peaks of the Mg, Cu-HAp were found at the 2θ peaks, which corresponds to the hydroxyapatite standard (ICDD no. 96-101-1243) 27.87º, 31.12º, 32.51º, and 34.45º with index main (hkl): (002), (211), (300), and (202). The Mg, Cu-HAp0, Mg, Cu-HAp1, Mg, Cu-HAp2, Mg, Cu-HAp3, and Mg, Cu-HAp4 have different diffraction peaks (0,8881 nm, 0,9048 nm, 1,0059 nm, 0,9853 nm, and 0,8129 nm) a crystal size measured using the Scharrer method with the Debye-Scharrer equation this indicate that Mg and Cu-added HAp affect both the size and the degree of crystallinity.

$$L = \frac{k\lambda}{\beta \cos\theta} \qquad (2)$$

With L as the crystal size (nm); k as the constant (0,9), λ as the wavelength of X-rays, β as the FWHM (full width at half maximum) at 2θ (π=180); θ_B = Bragg angle.[17]

Fourier Transform Infra-Red Spectroscopy (FT-IR) Analysis of HAP and Mg, Cu-HAp

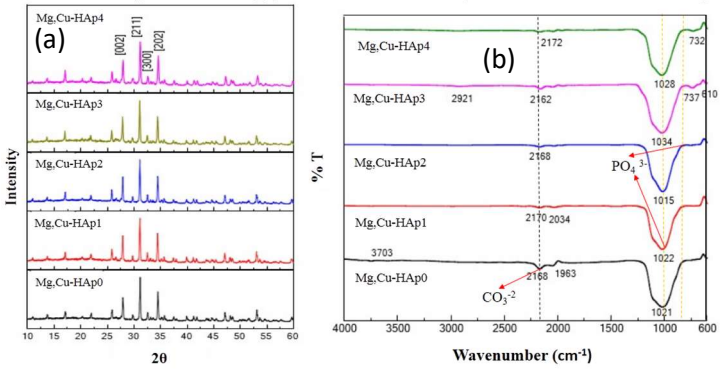

Fig.-1: (a) XRD Patterns of the Mg, Cu-HAp0, Mg, Cu-Mg, Cu-HAp1, Mg, Cu-Mg, Cu-HAp2, Mg, Cu-Mg, Cu-HAp3 and Mg, Cu-Mg, Cu-HAp4 (b) FTIR Analysis of Mg, Cu-HAp0, Mg, Cu-Mg, Cu-HAp1, Mg, Cu-Mg, Cu-HAp2, Mg, Cu-Mg, Cu-HAp3 and Mg, Cu-Mg, Cu-HAp4

The typical HAp peaks of the FT-IR spectrum commonly observed are the PO_4^{3-}, CO_3^{2-}, and OH^- functional groups. At a wavenumber of 610 cm^{-1}, there is a vibrational bending of PO_4^{-3}. The PO_4^{3-} peak at wavenumbers 1010-1036 cm^{-1} is an asymmetric stretching or an asymmetric stretching of the P-O bond, which results in weak absorption.[18] The FT-IR spectrum also shows a wavenumber of 2160-2170, the peak of CO_3^{-2}. The carbonate peak is formed due to the reaction of HAp with CO_2 in the HAp synthesis process. At a wavenumber of 3703 cm^{-1}, which is the vibrational stretching of the O-H group of $Ca(OH)_2$, indicating the presence of absorbed water on the surface of HAp. In Mg, Cu-HAp, the molecular vibrations are the same as stoichiometric HAp, indicating that Mg, Cu has replaced some of the Ca and formed Hap.[19,20]

Antibacterial Testing of HAp and Mg, Cu-HAp

Antibacterial testing was conducted on Mg, Cu-HAp0 samples used as negative controls, Mg, Cu-HAp1, Mg, Cu-HAp2, Mg, Cu-HAp3, Mg, Cu-HAp4, and amoxicillin as positive controls. Amoxicillin can inhibit the growth of both gram-positive and gram-negative bacteria. The experiment involved utilizing two distinct species of bacteria: gram-positive *Staphylococcus aureus* and gram-negative *Escherichia coli*. The inoculum was prepared using recently grown cultures that were incubated for 24 hours at a temperature of 37°C. Antibacterial activity was observed by measuring the minimum inhibition zone in millimeter units, as shown in Table-2.

Table-2: Antibacterial Activity Inhibition Zone

Bacteria	Inhibition zone diameter (mm)					
	(-) Control	Mg, Cu-HAp1	Mg, Cu-HAp2	Mg, Cu-HAp3	Mg, Cu-HAp4	(-) Control
E. coli	0	0.2	0.5	0.9	0.7	1.2
S. aureus	0	0.4	0.4	0.7	0.8	1

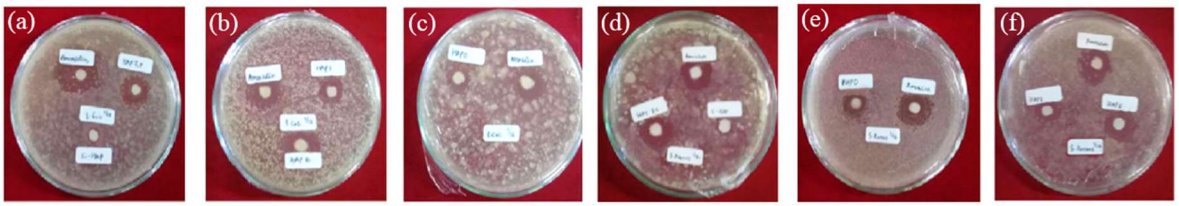

Fig.-3: Aktivitas Antibakteri Dari Mg, Cu-HAp0, and Mg,Cu with *Escheria coli* dan *Staphylococcus aureus*

Table-2 presents the antibacterial activity of Mg, Cu-HAp, with Mg, Cu-HAp3 demonstrating the most potent antibacterial characteristics against E. coli bacteria and Mg, Cu-HAp4 exhibiting the highest effectiveness against S.aureus bacteria. These findings indicate that Mg, Cu-HAp is a very efficient antibacterial agent and can be utilized for its antibacterial qualities. The antibacterial activity of Cu-doped HAp is attributed to its capacity to establish robust bonds with thiol, imidazole, amine, and carboxylate protein groups.[21] This interaction leads to structural alterations and enhanced permeability, resulting in malfunction of membrane transport and, ultimately, cell death.[22]

CONCLUSION

HAp and Mg, Cu-HAp were successfully synthesized using the sol-gel method with Ca precursor from a Lokan shell containing 94.626% in CaO with Mg, and Cu concentration variations. The XRD results were consistent with the HAp standard (ICDD no. 96-101-1243), indicating that the crystal structure of HAp is hexagonal with maximum peaks at 2θ of pure HAp, HAp1, HAp2, HAp3, and HAp4 are 31.12º, 31.08º, 31.07º, 31.07º, and 31.16º, respectively. The FT-IR results showed the presence of functional groups of HAl, namely PO_4^{3-}, CO_3^{2-}, and OH^-. Good antibacterial activity of HAP and Mg, Cu-HAp was found in HAp3 with a DDH of 0.9 mm for *E. coli* and HAp4 with a DDH of 0.8 mm for *S. aureus*.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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