Synthesis of Calcium Phosphate Bioceramics Based on Snail Shells: Towards a Valorization of Snail Shells from Republic of Benin

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Synthesis of Calcium Phosphate Bioceramics Based on Snail Shells: Towards a Valorization of Snail Shells from Republic of Benin

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Abstract In this study, we used *Lanistes varicus* and *Achatina achatina* snail shell powders as a calcium source for the synthesis of calcium phosphate bioceramics by using coprecipation method and microwave irradiation method. X-Rays Diffraction Analysis, Thermal Analysis (TGA), Acid-Base back titration method and FTIR analysis of the powders of the snail shells revealed that they contain more than 98% of calcium carbonate $CaCO_3$ with aragonite as the major phase. Structural characterization and elucidation of synthesized bioceramics were done using X-Rays diffraction analysis, Scanning Electron Microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR) analysis. The results have revealed that synthesized calcium phosphate bioceramics contain a mixture of Hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) and apatitic Tricalcium Phosphate (TPa: $Ca_9(HPO_4)$ $(PO_4)_5(OH)$ whatever the method of synthesis. and the variety of snail shell used. The study of the antibacterial activity of synthesized bioceramics has shown that only those obtained with *Lanistes varicus* powder significantly inhibit the growth of *Staphylococcus aureus* with a lasting effect. On the other side, inhibition of growth of *Klebsiella oxytoca* is partial and a resistance to the antimicrobial activity of bioceramics was noticed. Thus bioceramics synthesized from *Lanistes varicus* snail shell powder has antibacterial property and should be used against the growth of pathogens causing dental cavities.

Keywords Bioceramic, Hydroxyapatite, Apatitic Tricalcium Phosphate, Snail shell, Benin

1. Introduction

The increase of traffic accidents that occurred recently led to high demand for material that can repair damaged bones. An estimated 1.2 million people die each year in traffic accidents around the world, and the number of seriously injured could be 50 million, the combined population of the five largest cities in the world Planet [1]. Fortunately, with the development of surgery, the man has managed to repair or replace damaged parts (tissues or organs) such as cracked or fractured bones recorded during these accidents. The substitutes used were of various origins and the consequences were not always the most glowing for the patients. Implantation in the damaged bone is a good treatment in restoring the bone function. Some calcium phosphates are part of biomaterials used in bone substitutes because of their resemblance to the major mineral phase of vertebrate hard tissue. Indeed since 1926, De Jong, using X-ray diffraction, showed the analogy of bone mineral with calcium phosphate minerals possessing an apatitic structure [2].

Hydroxyapatite (HA, $Ca_{10}(PO_4)_6(OH)_2$) is one of the calcium phosphates that has been used as bone substitute to promote bone repair and regeneration because of its similarity to the mineral fractions in human bone and its good biocompatibility in living tissue [3, 4]. Recently, natural materials such as corals, fish bone, eggshells, snail shell, etc., have been selected as a source for the synthesis of calcium phosphates due to their advantage of biological origin as well as recycling of bio-waste [5]. A few methods were developed to prepare calcium phosphates including hydrolysis hydrothermal or precipitation methods dry process, freezing method, hydroxylation of calcium

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phosphate, spray pyrolysis, gel diffusion, microwave synthesis and sol-gel technique [6, 7].

The various high purity calcium phosphates synthetic chemical procedures utilize commercial precursors such as calcium carbonate, calcium nitrate or calcium hydroxide as the source of ions calcium and ammonium hydrogen phosphate $(NH_4)_2HPO_4$, phosphoric acid H_3PO_4 or sodium hydrogenphosphate Na_2HPO_4 as a source of phosphate ions. calcium phosphates obtained under these conditions and commercially available is relatively expensive because of the use of high purity reagents which are very expensive [8]. The use of bio-waste as a calcium source for the synthesis of calcium phosphates can reduce the amount of waste to be disposed and reduce the costs from the requirement of using expensive and high purity calcium reagents to synthesize calcium phosphates.

Indeed, the flesh of the giant African snails is appreciated by many African populations; it contains protein between 27 and 51% of the dry matter and represents the "bushmeat" the most popular and most consumed. An estimated 300 tons per year of snail meat consumption in Benin [9]. Thus after consumption the shells are released into the wild without any measure and therefore constitute an environmental threat for their resistance to atmospheric weather. Thus the national availability of snail shells especially in southern Benin, its natural biological origin, very low cost and its high calcium content are important characteristics which classify these shells as an ideal source for the preparation of calcium phosphate for use in the biomedical field.

This study aims to valorize Benin's snail shells, released after consumption, through the synthesis of calcium phosphate bioceramics usable for biomedical applications.

2. Experimental Section

2.1. Materials and Method

2.1.1. Collection of Samples

Achatina achatina and *Laniste varicus* snails were collected from the local market and their shell covering was removed carefully. Shells were washed with water followed by distilled water to remove the mud, sand and other impurities. Then, cleaned shells were sun dried during 24 hours and ground to a fine powder.

2.1.2. Synthesis of Calcium Phosphate Bioceramics

For coprecipitation method, 6 grams of each snail shell powder were suspended in a few drops of ethanol and 120 mL of a 1M hydrochloric acid solution in order to completely dissolve all the powder and facilitate the release of all the calcium in solution. In order to obtain the Ca/P molar ratio as 1.6; 2.4 mL of the commercial orthophosphoric acid solution was added to the contents of the beaker. The ammonia solution was added to make the reaction medium basic condition of a precipitation of calcium ions by the phosphate ions. A white precipitate is obtained which is heated to 80℃ under stirring during 8h. The powders were removed from the liquid by centrifugation and dried overnight at 100℃. The product is then calcined at 700℃, 900℃ and 1100℃ in a hot air oven for 4 hours.

For products obtained by microwave method, the experimental procedure and the amounts of reagents used remain the same as before until the white precipitate. The resulting mixture was stirred for 20 min and immediately transferred to a domestic microwave oven and irradiated at 450 W energy of frequency continuously during 30 min.

After the irradiation, the powders were removed from the liquid by centrifugation and dried overnight at 100℃. The product is then calcined at 700℃, 900℃ and 1100℃ in a hot air oven for 4 hours.

2.2. Characterization Techniques

X-ray powder diffractometry (p-XRD) measurements were performed on a D8 Advance BRUKERX-ray diffractometer, in Bragg-Brentano configuration, using Cu-Kα radiation (1.79021 Å). The data were collected with 2θ value from 5° to 80°.

Back titration method was used to measure the mass percent of calcium carbonate in the powdered shell samples. 1 g of powdered shell samples was weighed and added into each of 250 mL conical flasks. Few drops of ethanol were added to flasks; it acts as a wetting agent and helps the hydrochloric acid dissolve the $CaCO_3$ according to the reaction showed in equation (1).

$$CaCO_3 + 2(H_3O^+ + Cl^-) \rightarrow (Ca^{2+} + 2Cl^-) + CO_2 + 3H_2O. \quad (1)$$

100 mL of HCl solution (1.0 M) were added to each of the labeled conical flasks, swirled well to wet all the solids. The solutions in the flasks are heated until it begins to boil and the shell samples dissolves completely. 3-4 drops of phenolphthalein indicator were added to flask. Excess of acid was titrated against standardized NaOH solution (2 M), until barely pink color appears and persists for 30 seconds and fades slowly. Repeat 3 times the titration to obtain concordant values. The mass percent of calcium carbonate in the powdered shell samples can be determined by equation (2)

$$\%(CaCO_3) = [m(CaCO_3)/m(\text{snail shell powders})]x100. \quad (2)$$

Chemical bonding was analyzed by transmission infrared spectroscopy, using a Perkin Elmer 100 Series spectrometer (FTIR). Samples were prepared by mixing and pressing under $1.33*10^{-2}$ N/m^2, the sands with potassium bromide (1/100 by weight) in 13 mm diameter pellets.

Thermal analysis, i.e. thermogravimetry (TG) was performed by using a SETARAM LABSYS system, from ambient to 1200℃, using a 10℃/min heating rate and a 40 ml/min oxygen flow. Each measurement was realized on about 10 mg of snail shell powder in a 100 µL alumina crucible.

The particle morphology of the synthesized products was observed by a JEOL JSM-6510LV scanning electron

microscope (SEM).

2.3. Application of Calcium Phosphate Bioceramics

The antibacterial activity of the products of synthesis is evaluated using the well diffusion method described by Gheni et *al* [10]. Indeed, the suspension of each synthetic product is used as an antimicrobial agent to fill the wells made in the appropriate media previously inoculated with the inoculum of a pure strain of the bacterium to be tested. After incubation, the media are examined and the zones of inhibition surrounding the wells are measured to evaluate the effect of each synthetic product on the bacteria.

Transplanting bacterial strains.

The different bacterial strains, collected at the stomatology department of CHD-Borgou (Parakou, Benin), were sub-cultured on CHAPMAN medium and then incubated in an oven at 37°C for 24 hours in order to obtain isolated strains. Isolated colonies were used to prepare the inoculum.

Antibiogram technique

Microbiological tests are very sensitive tests. Thus for our manipulations, we used the Bunsen burner to create a sterile atmosphere located within a radius of twenty centimeters around the flame and to sterilize the instruments by passing them in the flame.

Preparation of the inoculum

The inoculum of each strain was prepared according to the Mc Farland concentration of 0.5 CFU / mL. Young colonies isolated after 24 hours of incubation were used. One or two colonies of *Staphylococcus aureus* or *Klebsiella oxytoca* were removed using a platinum loop and homogenized in 5 mL of sterile distilled water.

Achievement of the antibiogram

The inoculum of each prepared strain is used to flood Muller Hinton solid culture media cast in petri dishes. Then the dishes are tilted for 5 minutes to remove the excess of the inoculum. After the elimination time of the excess of the inoculum, the 0.6 cm wells are made in the medium using small sterile cones. The suspensions obtained from the bioceramics are first brought to room temperature and then centrifuged at 3000 rpm for 5 minutes to obtain the pellet which is then used to fill the wells. A witness has been made. This time the wells were filled with sterile distilled water instead of the suspension. Finally the dishes were incubated in an oven for 24 hours.

Reading

After the incubation time, the reading was made by measuring the inhibition diameter around the wells using a graduated ruler.

3. Results and Discussion

3.1. Characterization of Snail Shell Powders

XRD characterization of snail shell powders

Fig. 1 showed the similar XRD patterns of two varieties of snail shell powders. The characteristic peaks of aragonite (JCPDS-File N°: 05-0453) appear as being widely in the majority, confirming the important content in crystallized calcium carbonate of studied snail shell powders.

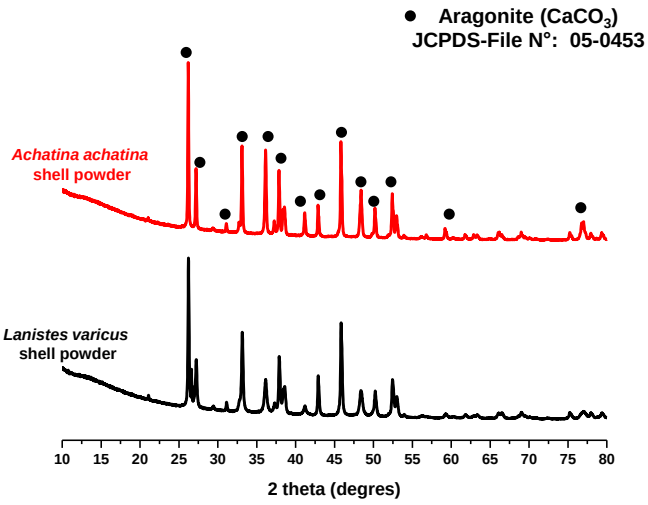

Figure 1. XRD patterns of snail shell powders

FT-IR spectroscopy characterization of snail shell powders.

Fig. 2 showed similar profiles FT-IR analysis spectra of two varieties of snail shell powders.

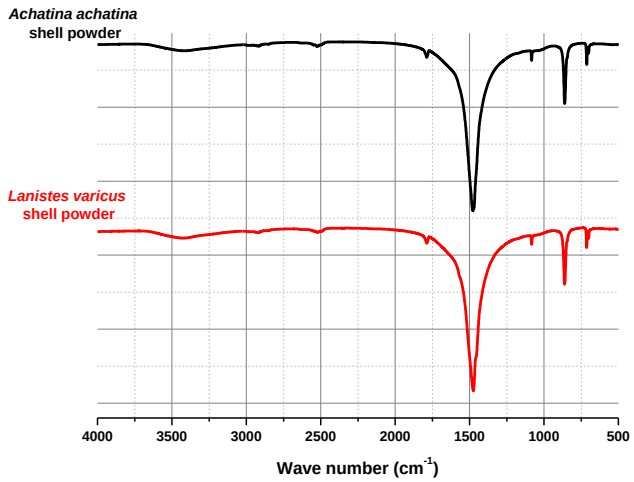

Figure 2. FT-IR spectra of snail shell powders

The weak broad O─H stretching bands around 2500 cm^{-1} and 3375 cm^{-1} proved the adsorption for H_2O molecules. The main bands of absorption observed at 875 cm^{-1} and 1480 cm^{-1} can be assigned to C─O vibrations of carbonate group. These observations are very similar to the ones already discussed in previous works [11, 12].

TG characterization of snail shell powders.

The TG curves (Fig. 3) show the thermal decomposition of the snail shell powders in order to estimate the amount of calcium carbonate. Results obtained showed that the weight loss of snail shell powders below 500°C, due to the burning

of the organic matter is about 2.5%. The weight loss between 500 and 800℃ in two samples is about 42.5% and can be attributed to the decomposition of aragonite according to the reaction (3).

$$CaCO_3 \leftrightarrow CaO + CO_2 \quad (3)$$

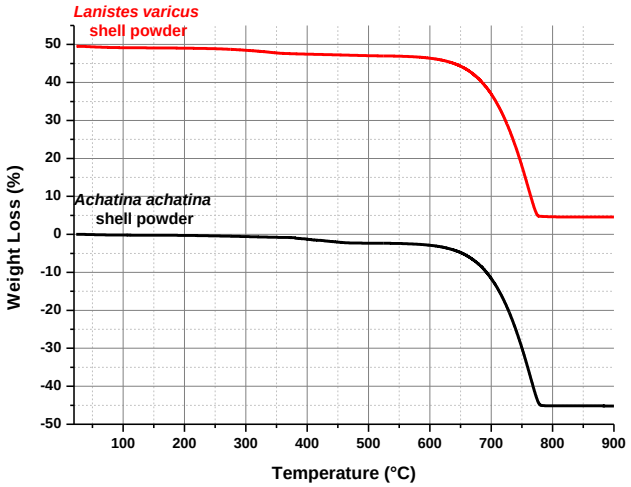

Figure 3. TG curves snail shell powders

Estimation of calcium carbonate using back titration method

The average percentage of calcium carbonate in snail shell powders was 98.5% and 98.75% respectively for *Achatina achatina* and *Laniste varicus* snails. These results confirmed that the two studied varieties of snail shells content in majority calcium carbonate and can be used as calcium precursors in calcium phosphate bioceramics synthesis.

3.2. Characterization of Synthesized Calcium Phosphate Bioceramics

XRD characterization of synthesized calcium phosphate bioceramics

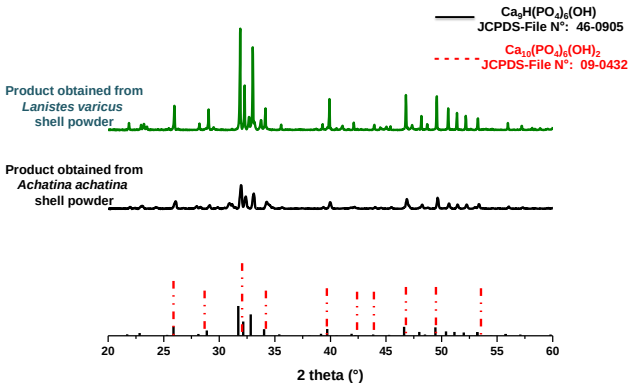

Figure 4. XRD patterns of products obtained by microwave irradiation method

Fig. 4 shows the XRD patterns of the obtained products by using 2 studied varieties of snail shell as a calcium source by microwave method. Patterns were all similar and matched well with JCPDS data for Hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) (JCPDS-File N° 09-0432) and apatitic Tricalcium Phosphate (TPa: $Ca_9(HPO_4)$ $(PO_4)_5(OH)$ (JCPDS-File N° 46-0905), indicating that samples are composed of mixture of phases of HA and TPa. Moreover, XRD analysis showed that product obtained from *Achatina achatina* snail shell powder is less crystallized than those obtained with *Lanistes varicus* snail shell powder.

For precipitation method, Fig.5 showed also the similar patterns for product obtained from 2 varieties of snail shell. These peaks matched well with JCPDS data for Hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) (JCPDS-File N° 09-0432) and apatitic Tricalcium Phosphate (TPa: $Ca_9(HPO_4)$ $(PO_4)_5(OH)$ (JCPDS-File N° 46-0905).

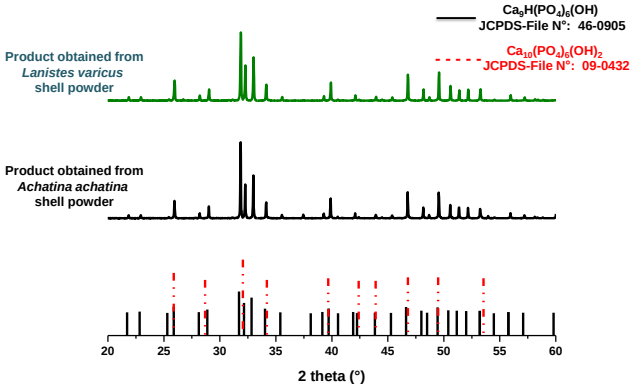

Figure 5. XRD patterns of products obtained by precipitation method

These results revealed that both of 2 varieties of snail shell can be used to elaborate calcium phosphate bioceramics.

FTIR characterization of synthesized calcium phosphate bioceramics

The FT-IR spectrum of the synthesized calcium phosphate bioceramics from *Lanistes varicus* snail shell is shown in Fig.6. Similar profiles were observed for all samples. The characteristic peaks corresponding to stretching vibrations of PO_4^{3-} ions at 1050 cm^{-1} and the peaks around 565 cm^{-1} are assigned to the deformation of PO_4^{3-} ions in HA. The peaks at 3590 cm^{-1} and 3650 cm^{-1} are due to stretching vibration of OH group of HA. The broad band extending from 3000 to 3500 cm^{-1} is attributed to the adsorption of water molecules. The band at 870 cm^{-1} could be attributed to P─O(H) stretching mode of hydrogenophosphate groups. The corresponding absorbance bands confirmed the presence of mixture of Hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) and apatitic Tricalcium Phosphate (TPa: $Ca_9(HPO_4)$ $(PO_4)_5(OH)$ in the synthesized bioceramic products. These observations are very similar to the ones already discussed in previous works [13, 14].

The two bands observed around 1450 cm^{-1} designate the existence of carbonate in trace level. These two bands can be ascribed to B-type carbonate substitution on phosphate ion sites. Moreover, carbonate groups observed in this article closely matched those of A- and B-type carbonates. It is believed that the synthesized bioceramic products are chemically and structurally similar to biological apatite in natural bone, which contains carbonate ions in significant amount from about 4–6 wt% [15]. The carbonated calcium phosphate has excellent bioactivity with hard tissues when

used for bone and dental tissue reconstitution [16].

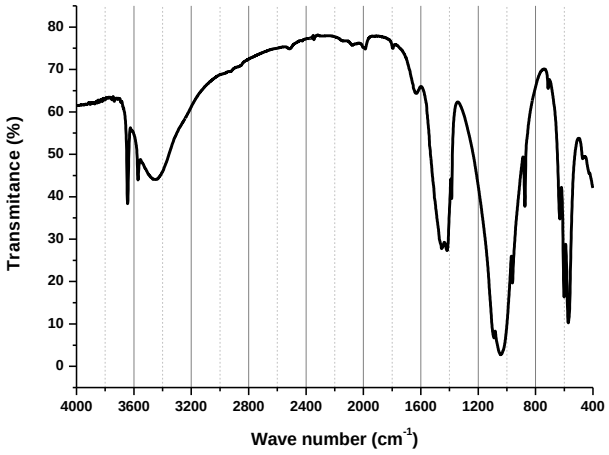

Figure 6. FT-IR spectrum of obtained product from *Lanistes varicus* snail shell

SEM characterization of synthesized calcium phosphate bioceramics

Fig. 7 shows the Scanning Electron Microscopy images of the synthesized products. We observed that the particles obtained from *Achatina achatina* shell powder are hexagonal and cubic forms of average size 2 µm whereas with the snail powder of the *Lanistes varicus* variety, the particles are in cubic forms with average size of 1 µm.

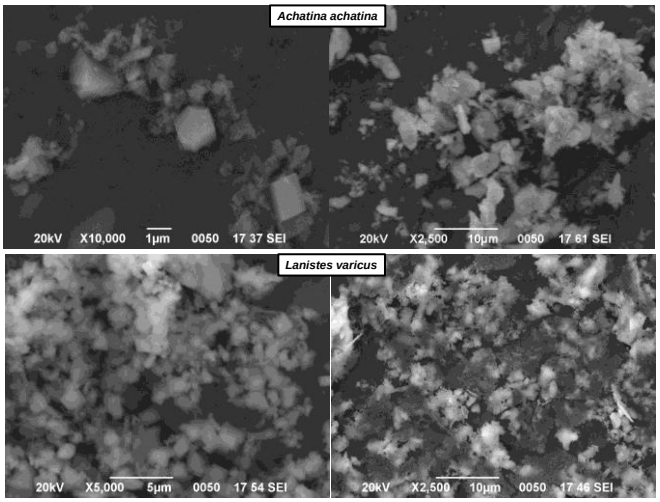

Figure 7. SEM images of synthesized bioceramics using microwave irradiation method

3.3. Application of Synthesized Calcium Phosphate Bioceramics

Antibacterial activity is assessed by measuring the width of the inhibition zone around each well. The results are shown in the Table 1.

Table 1. Anti-bacterial activity using synthesized calcium phosphate bioceramics

	Organisms	Product obtained from *Lanistes varicus* snail shell Powder			Product obtained from *Achatina achatina* snail shell Powder		
		Calcined at 700°C	*Calcined at 900°C*	*Calcined at 1100°C*	*Calcined at 700°C*	*Calcined at 900°C*	*Calcined at 1100°C*
Zone of inhibition (mm)	***Staphylococcus aureus***	*22*	*28*	*20*	00	00	00
	Klebsiella oxytoca	*16*	*22*	*15*	00	00	00

The results in Table 1 show that calcium phosphate bioceramics synthesized from *Lanistes varicus* snail shell powder inhibits the growth of *Staphylococcus aureus* with a lasting effect. On the other hand, inhibition of growth of *Klebsiella oxytoca* is temporal, and it is possible to note a certain time after regrowth of the bacteria, which therefore have resistance to the antimicrobial activity of the calcium phosphate bioceramics obtained. Products obtained from *Achatina achatina* snail shell powder have no antimicrobial effect on both strains of bacteria. Moreover, the calcination temperature is a factor that influences the efficiency of the synthesized bioceramics which knows its optimum value at 900°C. These results are similar to those obtained by Anchana et al [17]. Finally bioceramics synthesized from *Lanistes varicus* snail shell powder can be used against the growth of pathogens causing dental cavities.

4. Conclusions

Lanistes varicus snail shells and *Achatina achatina* snail shells are abundant in Republic of Benin. These shells are composite materials consisting of calcium carbonate and organic matter. The use of this waste as a calcium source for the synthesis of calcium phosphate bioceramics can reduce the amount of waste to be disposed and reduce the costs from the requirement of using expensive and high purity calcium reagents to synthesis of calcium phosphate bioceramics. By using coprecipation method and microwave irradiation method, we synthesized in this study calcium phosphate bioceramics which content a mixture of Hydroxyapatite (HA: $Ca_{10}(PO_4)_6(OH)_2$) and apatitic Tricalcium Phosphate (TPa: $Ca_9(HPO_4)$ $(PO_4)_5(OH)$. Only bioceramics synthesized from *Lanistes varicus* snail shell powder can be used against the growth of pathogens causing dental cavities. This antibacterial property should be used to good advantage as a bioactive biomaterial in dental applications.

REFERENCES

[1] OMS.: 2017. Chapitre 1 intitulé Données fondamentales. Rapport mondial sur la prévention des traumatismes dûs aux accidents de la circulation.

[2] De Jong W.F.: 1926. La substance minérale dans les os. Rec. Trav. Chim. Pays-Bas 45, 445-448.

[3] Mondal S., Bardhan J., Mondal B., Dey A., Mukhopadhyay S.S., Roy S., Guha R., and Roy, K.: 2012. Synthesis, characterization and in vitro cytotoxicity assessment of hydroxyapatite from different bioresources for tissue engineering application. Bulletin Material Science 35(4), pp: 683–691.

[4] Zakaria S.M., Sharif S.H., Othman M.R., Yang F., Jansen J.A.: 2013. Nanophase Hydroxyapatite as a Biomaterial in Advanced Hard Tissue Engineering: A Review. Tissue Eng. Part B-Rev 19, 431-441.

[5] Sadat-Shojai M., Khorasani M.T., Dinpanah-Khoshdargi E and Jamshidi A.: 2013. Acta Biomater 9, 7591–7621.

[6] Foroughi M.R., Karbasi S., Ebrahimi R.: 2012. Physical and mechanical properties of poly-3-hydroxybutyrate-coated nanocrystalline hydroxyapatite scaffold for bone tissue engineering. Journal of Porous Materials 19, 5, 667-375.

[7] Vijayalakshmi U., Rajeswari S.: 2006. Preparation and characterization of microcrystalline hydroxiapatite using sol gel method, Trends Biomater Artificial Organs, 19(2): 57-62.

[8] Britel O., 2007. Modélisation et optimisation par la méthodologie des plans d'expériences de la synthèse: de l'hydroxyapatite phosphocalcique du phosphate tricalcique apatitique du phosphate de calcium apatitique carbonate, manuscrit de Thèse, Université Mohamed V, faculté des sciences de Rabat, p158.

[9] Sodjinou E., Biaou G., Codjia J.C.: 2002. Caractérisation du marché des escargots géants africains (achatines) dans les départements de l'Atlantique et du Littoral au Sud-Bénin. TROPICULTURA 20, 2, 83-88.

[10] Gheni A.I., Noori Y., Al-shekhany M.H. O.: 2016. Smail, Inhibitory Effects of Aqueous Garlic Extract (AGE) against multidrug resistant from Staphylococcus aureus and Escherichia coli. International Journal of Advanced Research 4, 1, 928- 933.

[11] Tin M.M., Gopalakrishnan V., Samsuddin A.R., Al-Salihi K.A., Shamsuria O.: 2007. Antibacterial property of locally produced hydroxyapatite. Archives of Orofacial Sciences 2, 41-44.

[12] Antoniac I., Lesci I.G., Blajan A.I., Vitioanu G., Antoniac A.: 2015. Bioceramics and biocomposites from marine sources. Key Engineering Materials 672, 276-292.

[13] Murugan R., Ramakrishnan S.: 2005. Aquesous mediated synthesis of bioresorbable hydroxyapatite. J.Cryst. Growth 274: 209-213.

[14] Raya I., Mayasari E., Yahya A., Syahrul M., Andi Ilham L. A.: 2015. Synthesis and Characterization of Calcium Hydroxyapatite Derived from Crab Shells (Portunus pelagicus) and Its Potency in Safeguard against to Dental Demineralizations. International Journal of Biomaterials, 469176.

[15] Olszta M.J., Cheng X., Jee S.S., Kumar R., Y.Y. Kim Y.Y., Kaufman M.J., Douglas E.P., Coger L.B.: 2007. Bone structure and formation: a new perspective. Mater. Sci. Eng. R 58, 77–116.

[16] Redey S.A., Razzouk S., Rey C., Bernache-Assollant D., Leroy G., Nardin M., Cournot G.: 1999. Osteoclast adhesion and activity on synthetic hydroxyapatite, carbonated hydroxyapatite and natural calcium carbonate: relationship to surface energies. J. Biomed. Mater. Res 45, 140–147.

[17] Anchana C., Perumal P.: 2016. Synthesis & Application of Hydroxyapatite Bioceramics from Different Marine Sources. Quest Journals Journal of Research in Environmental and Earth Science 2, 11 pp: 07-15 ISSN (Online): 2348-2532.