



Nano Scale Hydroxyapatite from *Crassostrea virginica* (Oyster Seashells) and its Modification with *Azadirachta Indica* (Neem) Extract

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Abstract

Hydroxyapatite (HAp) is a widely explored biomaterial for bone and tooth replacements. Most of the research studies are being carried out in terms of novel routes of synthesis and fabrication in the nano scale to suit its applications. The present research study is unique in two aspects one being the use of seawaste namely *Crassostrea virginica* (Oyster) seashells as the source of calcium carbonate along with phosphoric acid and in the alkaline pH to synthesize nano sized HAp and secondly to check the enhanced activity of HAp if any, upon the addition of *Azadirachta indica* Extract (neem leaf and neem stem) by wet precipitation method. The structural activity relationship of as synthesised HAp sample was compared with that of the modified HAp using *Azadirachta indica* extract as medium. The presence of Hexagonal phase (JCPDS no-09-0432) was identified in all HAp samples. The crystallite size of 38nm was observed in the HAP prepared from the seashell in comparison with the crystallite of 49 nm (neem leaf) and 52nm (neem stem) modified HAP. The presence of spherical morphology and elemental composition of Ca,P in HAp and modified HAPs were identified from Scanning Electron Microscopic images. The toxicity studies of as synthesised HAp and modified HAp with *Azadirachta indica* against *Candida albicans* and *Streptococcus mutans* were compared and the results were encouraging. This study ultimately provided a simple, cost effective green method of converting seawaste into high value biomedical product of nanoscale hydroxyapatite with enhanced antimicrobial activity.



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Introduction

Hydroxyapatite (HAp) $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ is one of the widely used replacement bioceramics in terms of bone and tooth substituent due to its high corrosion resistance, better compressive strength, porosity, low density and low weight.¹ Porous morphology of HAp and β -TCP (Tri calcium phosphate) are attractive for bone regeneration and good growth property. HAp is the best alternative for bone and tooth replacement because of its similarity in terms of chemical structure, crystallography, morphology and Ca/P ratio of 1.67 with that of humans.^{2,3,4,5} HAp is also used for non-medical application in terms of packing column in chromatography, gas sensors, and catalysts.⁶

The nature of the precursor, additives, method of synthesis is used to determine the composition, crystal structure, size, shape and morphology of the resulting Hydroxyapatite HAp. In today's context, there is a need to prepare multifunctional HAp and hence several synthetic approaches like sol gel, solvothermal, wet chemical precipitation, are carried out with variation in precursors, experimental parameters, additives and modifiers. The precursors of Calcium carbonate HAp exists in hexagonal crystal structure and their natural resources being corals, sea urchins, oyster shell, and egg shell. Among them oyster shells (*Crassostrea virginica*) are abundant in the coast of Bay of Bengal in India.⁷ The present study aimed in tapping the natural sea waste and convert into valuable bio material and further modifying the material with the components of potent medicinal plant extract (stem and leaf) of neem (*Azadirachta indica*).

The leaves, twigs, and seeds of plant of Neem (*Azadirachta indica*) have been widely used for medicinal purposes due to their immense anti-bacterial, anti-fungal, anti-viral, anti-diabetic properties. More importantly they play a significant role as dentalcare products as they contain several anti-microbial compounds belonging to diterpenoids family namely margolone, margolonone and iso margolonone. They are reported to prevent dental plaque formation mostly caused by streptococcus mutans bacteria and candida albicans fungi. Also, the demineralization of enamel is found to be reversed by treating with Hydroxyapatite. The present study is also aimed at combining the unique Osteo-

dental property of Hydroxyapatite and anti microbial activity of neem plant extract.⁸ In the study, the chemical constitution, micro structure and the anti-microbial activity of the synthesized Hydroxyapatite after modification with neem extract was explored.

Results

FT-IR Spectrum of *Crassostrea virginica* Seashell after Calcination

The purity of the sea waste (sea shell) in terms of calcium carbonate was evaluated using FT IR spectrum. In the FT-IR spectrum of calcined *Crassostrea virginica* seashell powder used as the precursor in the present study, a peak was observed at 1038cm^{-1} due to symmetric stretching C-O (ν_1) of calcium carbonate which correlated with the report.⁶ The peak was observed at 1419cm^{-1} (ν_3) which is due to doubly degenerate asymmetric stretching which is prominent peak of C-O in calcium carbonate.^{7,8,9,10} The peak observed at 467cm^{-1} due to stretching vibration of Ca-O bond was also identified in the calcined seashell. A peak was observed at 3697cm^{-1} due to O-H stretching which is more prominent in oyster shell.^{9,11}

FT-IR Spectrum of Hydroxyapatite Obtained from *Crassostrea virginica* Seashell

In the FT-IR spectrum of hydroxyapatite as obtained from calcined *Crassostrea virginica* shell as shown in the figure 2, there was a peak at 463cm^{-1} due to Ca-O bond, which is correlated with the report.⁹⁻¹¹ A peak was observed at 584cm^{-1} due to asymmetric bending vibration of PO_4^{3-} , which is well correlated with the reports.¹⁰⁻¹² The peaks observed at 1051cm^{-1} due to asymmetric stretching vibration of P-O bond and the broad band in the region 3435cm^{-1} , 1636cm^{-1} due to stretching vibrations of -OH group in hydroxyapatite also agreed with the available reports.¹¹⁻¹³

FT-IR Spectrum of Hydroxyapatite Modified with *Azadirachta indica* (leaf, stem) using *Crassostrea virginica* Seashell as Precursor

The FT-IR spectra of Hydroxyapatite modified with *azadirachta indica* extract obtained from *Crassostrea virginica* shell as shown in the figure 3. showed a peak at 476cm^{-1} (with neem leaf) and 454cm^{-1} (with neem stem) due to Ca-O bond.⁹⁻¹¹ The peaks observed at 586cm^{-1} (with neem leaf) and 590cm^{-1} (with neem stem) are due

to asymmetric bending vibration of PO_4^{3-} , and well correlated with the reports.¹⁰⁻¹² The peak observed at 1043 cm^{-1} (neem leaf) and 1056 cm^{-1} (neem stem) due to asymmetric stretching vibration of P-O

bond and the broad band in the region 3429 cm^{-1} , 1637 cm^{-1} (neem leaf) and 3431 cm^{-1} , 1641 cm^{-1} (neem stem) due to stretching vibrations of -OH group in hydroxyapatite were well correlated with the available reports.¹¹⁻¹³

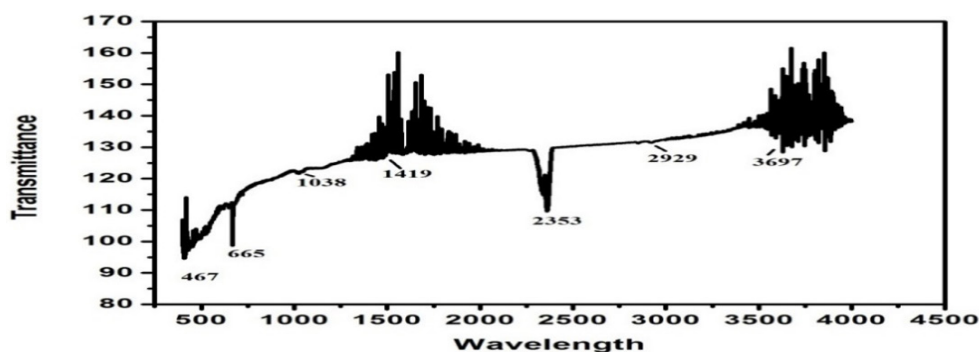


Fig. 1: FT-IR spectrum of *Crassostrea virginica* shell after calcination

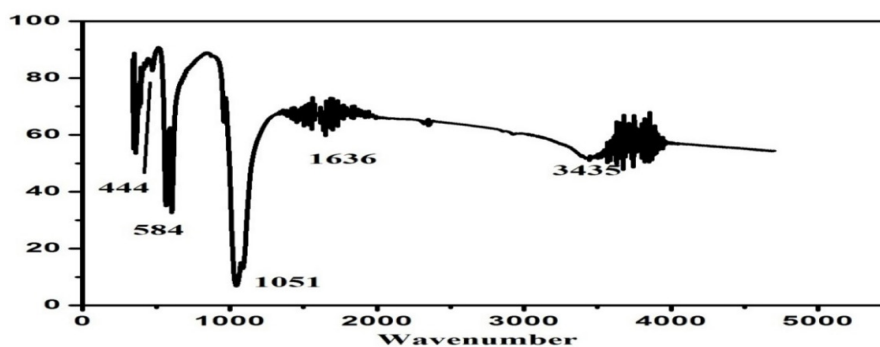
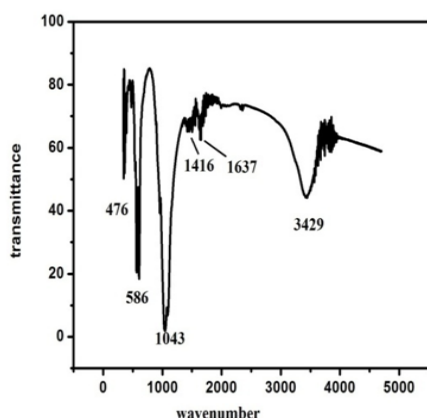
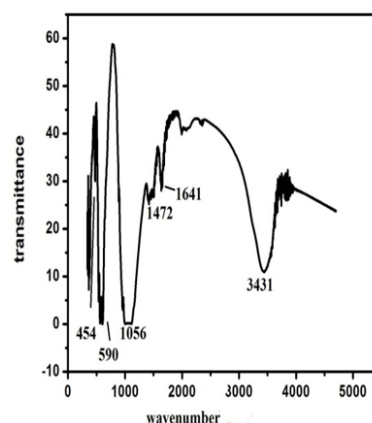


Fig. 2: FT-IR spectrum of HAp obtained from *Crassostrea virginica* seashell



3(a) HAp modified with neem leaf



3(b) HAp modified with neem stem

Fig. 3(3a, 3b) FT-IR spectra of HAp modified with *azadirchata indica* (leaf, stem) extract using *Crassostrea virginica* seashell as precursor

Powdered X ray diffraction (PXRD)

The average crystallite size and crystal phase of calcined oyster shell and Hydroxyapatite obtained from oyster shell were determined using X-ray diffraction analysis. The results are important as it determined the phase purity and phase homogeneity of the major constituent of hydroxyapatite for further exploration of its applications.

PXRD Spectrum of Calcined *Crassostreao virginica* Shell

The PXRD of calcite form of calcium carbonate obtained from calcined oyster shell is given in the figure 4. Sudhaparimala et al.,^{13,14} reported that aragonite and calcite form of calcium carbonate to be present in *Crassostreao virginica* shell which is taken

as a precursor for synthesis of HAp in the present study. The aragonite and calcite form of *Crassostreao virginica* shell was calcined at 709 °C and it is converted in to highly stable form of calcite phase. The presence of calcium carbonate was identified in the calcite phase (JCPDS 86-2334). The presence of less intense unknown peak could not be specified which may be due to other elemental components present in the shell powder. The average crystallite size for the calcined oyster shell was found to be 89.02nm with the rhombohedral(trigonal) structure which correlated with the report of.^{14,15} This clearly indicated that calcined bio waste is in the nano scale and hence can be a very valuable precursor for the synthesis of calcium-based materials.

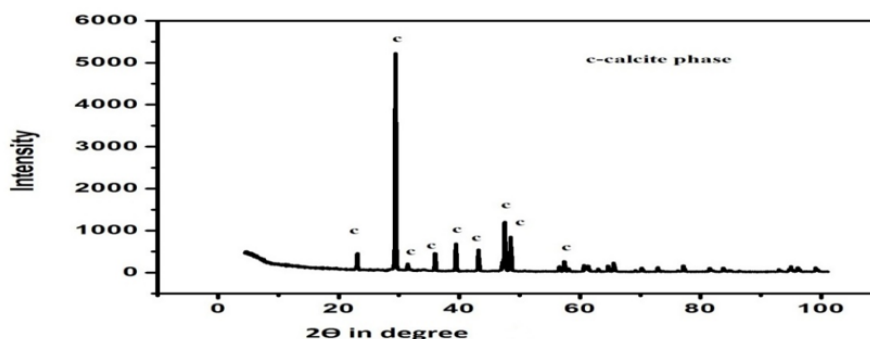


Fig.4: PXRD spectrum of *Crassostreao virginica* seashell after calcination

PXRD Spectrum of Hydroxyapatite Obtained from *Crassostreao virginica* Seashell

The PXRD spectrum of HAp obtained from calcined *Crassostreao virginica* seashell as given in the figure 5, clearly indicated the presence of HAp in the hexagonal phase (JCPDS 09-0432 and 01-09-1243). The presence of single phase of calcium

oxide was also observed in HAp may be due to incomplete conversion of CaCO_3 to Hydroxyapatite at 650°C.^{9,11} The average crystallite size of hydroxyapatite was found to be 38.42 nm. The formation of hexagonal phased HAp in the nano scale is phenomenal since it resembles that of human tooth enamel.^{15,16,17}

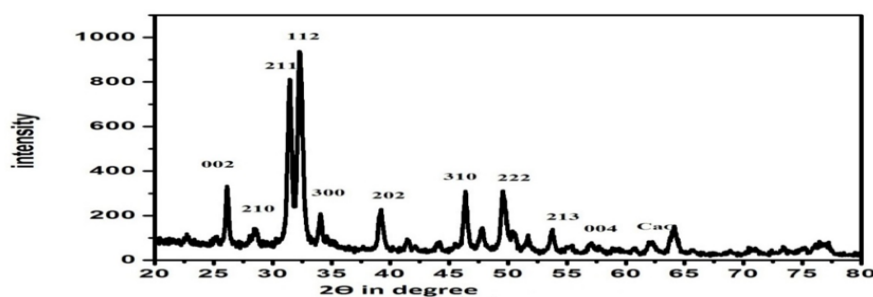


Fig. 5: PXRD spectrum of Hydroxyapatite obtained from *Crassostreao virginica* seashell

PXRD Spectrum of Hydroxyapatite Obtained from *Crassostrea virginica* Seashell Modified with *Azadirchata indica* Extract (Leaf)

The PXRD spectrum of Hydroxyapatite HAp modified with *azadirchata indica* leaf extract as given in the figure 6, indicated the presence of HAp in the hexagonal phase (JCPDS 09-0432 and 01-09-1243). The presence of single phase of calcium oxide ($2\theta = 64.180$) was observed in HAp which may be due to incomplete conversion of CaCO_3 to Hydroxyapatite at 650°C .⁹ The average crystallite size of Hydroxyapatite was found to be 49.53 nm and 52.48 nm after modification with neem leaf and neem stem respectively. This clearly indicated that that Hydroxyapatite obtained from *Crassostrea virginica* shell in the nanoscale and hexagonal phase is like that of human tooth enamel.^{16,17,18}

Scanning Electron Microscopy (SEM)

SEM Image of Hydroxyapatite Obtained from *Crassostrea virginica* Shell

The surface morphology of HAp powder obtained from calcined *Crassostrea virginica* shell figure 7. The near spherical shape on the surface indicating formation of hydroxyapatite. The agglomeration was found to be less which may be due the presence of other elements assisting in the formation of well defined morphology of the sample. Porous morphology of HAp on the surface is attractive for bone regeneration, enhanced biodegradation and good growth property. The presence of small cluster of tidy grains indicates formation of the calcium oxide phase on the surface of hydroxyapatite. This observation also correlates with the report.^{16,17}

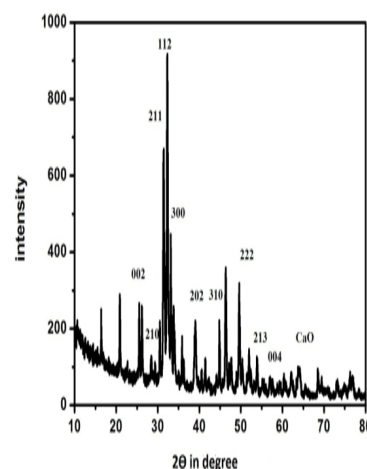
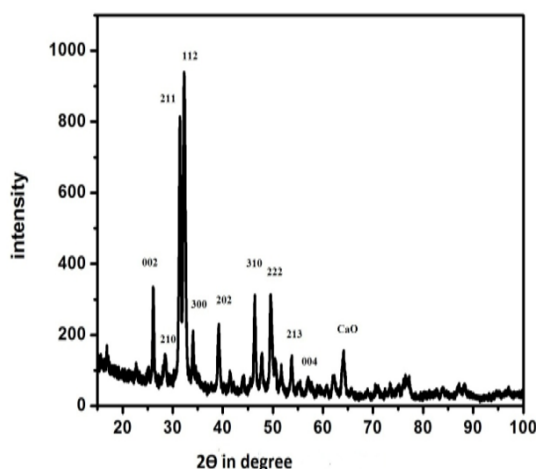


Fig.6(6a, 6b): PXRD spectrum of Hydroxyapatite modified with *azadirchata indica* extract (leaf, stem) obtained from *Crassostrea virginica* shell

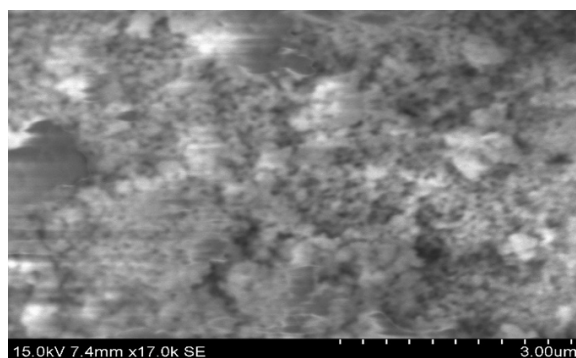


Fig.7: SEM image of hydroxyapatite obtained from *Crassostrea virginica* shell

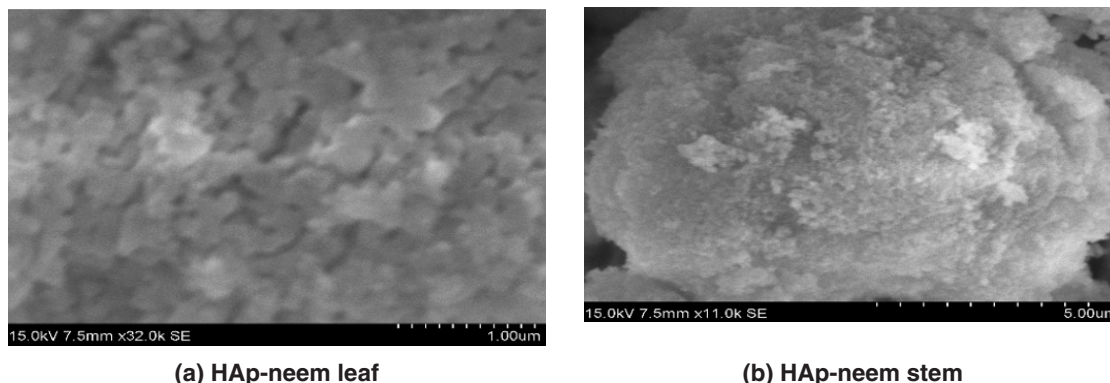


Fig. 8: SEM image of hydroxyapatite modified with azadirchata indica extract [neem leaf (a) and neem stem (b)] using *Crassostrea virginica* seashell

SEM Image of Hydroxyapatite Modified with Azadirchata indica Extract (Leaf, Stem) as Medium using *Crassostrea virginica* Seashell

The surface morphology of hydroxyapatite modified with azadirchata indica [8(a) with leaf extract and 8(b) with stem extract] are shown in the figure 8. The HAp modified with azadirchata indica obtained from *Crassostrea virginica* shell observed to have near spherical shape. The HAp modified with stem extracts having higher crystallinity with large agglomeration than that of leaf extract. The flower like shape obtained from modification HAp with stem extract. The presence of small aggregates indicates formation of the calcium oxide phase on the surface of HAp. This observation also correlates with the report.^{17,18}

Energy Dispersive X Ray Analysis (EDAX)

The results of EDAX analysis done for synthesis of hydroxyapatite obtained from *Crassostrea virginica* seashell and modification of HAp with azadirchata indica extract as medium are tabulated in the Table (1). The Ca/P ratio was intended for 1.67 at the time of preparation, The Ca/P ratio was found to be near for modification HAp with azadirchata indica extract. This Ca/P ratio is like that of HAp present in bone and tooth of human. So that seashells are suitable precursor for synthesis of HAp. This is correlated with the report.^{18,19}

Antimicrobial Activity of HAp and HAp Modified with Azadirchata indica Extract

Nancy.F et al.,^{19,20} reported that common dental infections arise from streptococcus mutans bacteria

and candida albicans fungi. Hence in the present study toxicity of HAp towards those bacteria and fungi were focused. Antibacterial and antifungal studies of hydroxyapatite obtained from seashell and modified HAp are tabulated in the table (2) and table (3).

Antibacterial Activity

Antibacterial activity of HAp obtained from seashell and modification of HAp are tabulated in table 2. There was 50 percent toxicity to streptococcus mutans cells at the concentration level of 500 µg in both the HAp samples with respect to the positive control of tetracycline (500 µg). The enhanced antibacterial activity of hydroxyapatite modified with azadirchata indica than that of HAp obtained from seashell may be attributed to the presence of other anti-microbial elements in neem extract and seashell viz:- zinc, Magnesium added from the precursor of seashell. This correlated with the report H. S. Ragab.^{20,21}

Antifungal Activity

The results of antifungal activity of HAp obtained from seashell and HAp modified with azadirchata indica extract are tabulated as table 3. The antifungal activities are not encouraging. It was observed that there was 50 percent toxicity to candida albicans cells at the concentration level of 1500 µg compared to the control of ketoconazole (positive control 500 µg). The antifungal activity of HAp modified with azadirchata indica extract was found to be nil even at higher concentration of 2000 µg/ml. The toxicity of neem leaf aqueous extract was

Table 1: Comparison of weight percentage of Ca and P and their ratio of HAp and modified HAp samples with *Azadirchata indica* extract using *Crassostreao virginica* seashell as a precursor

Name of the sample	Weight percentage of calcium	Weight percentage of phosphorous	Ca/P ratio of modified HAp	Ca/P ratio of without modification of HAp
HAp-Crassostreao virginica seashell	27.56	16.94	-	1.63
HAp-Crassostreao virginica seashell-neem leaf	32.90	12.90	2.55	-
HAp- Crassostreao virginica virginica seashell-neem stem	23.51	13.97	1.68	-

Table 2: Antibacterial activity of hydroxyapatite and modified hydroxyapatite with *Azadirchata indica* extract

Name of the sample	modification	antibacterial activity	concentration	zone inhibition sample	positive control
HAp-Crassostreao virginica seashell	Without	Name of the bacteria : Streptococcus mutans	2000	22	36
HAp-Crassostreao virginica seashell (leaf)	With	Name of the bacteria : Streptococcus mutans	2000	20	38
HAp-Crassostreao virginica seashell (stem)	With	Name of the bacteria : Streptococcus mutans	2000	20	39

Table 3: Antibacterial activity of hydroxyapatite and modified hydroxyapatite with *Azadirchata indica* extract

Name of the sample	modification	antibacterial activity	concentration	zone inhibition sample	positive control
HAp-Crassostreao virginica seashell	Without	Name of the bacteria : Candida albicans	2000	10	27
HAp-Crassostreao virginica seashell (leaf)	With	Name of the bacteria : Candida albicans	2000	-	27
HAp-Crassostreao virginica seashell (stem)	With	Name of the bacteria : Candida albicans	2000	-	27

reported at a concentration level of $2000\mu\text{g}$.^{21,22} The antimicrobial constituents of neem undergo decomposition at the calcination temperature of 650°C . The residual organic components of the neem after decomposition would have played a role in the solubility of the sample in the solvent taken for fungal study.²³

Discussion

The biowaste of seashell (*Crassostrea virginica* shell) was identified as a rich source of calcium carbonate. Aragonite and calcite form of CaCO_3 was converted into highly stable calcite phase at lower calcination temperature of 709°C . Such potential bio waste into a nanoscale (38.42nm) Hydroxyapatite and modified HAp [49.53nm (neem leaf) and 52.48nm (neem stem)] using azadirachta indica extract by wet chemical precipitation method at lower calcination temperature of 650°C . The average crystallite size was found to be increased during modification. The microstructure, surface morphology and elemental composition (Ca/P ratio) of the as synthesized HAp

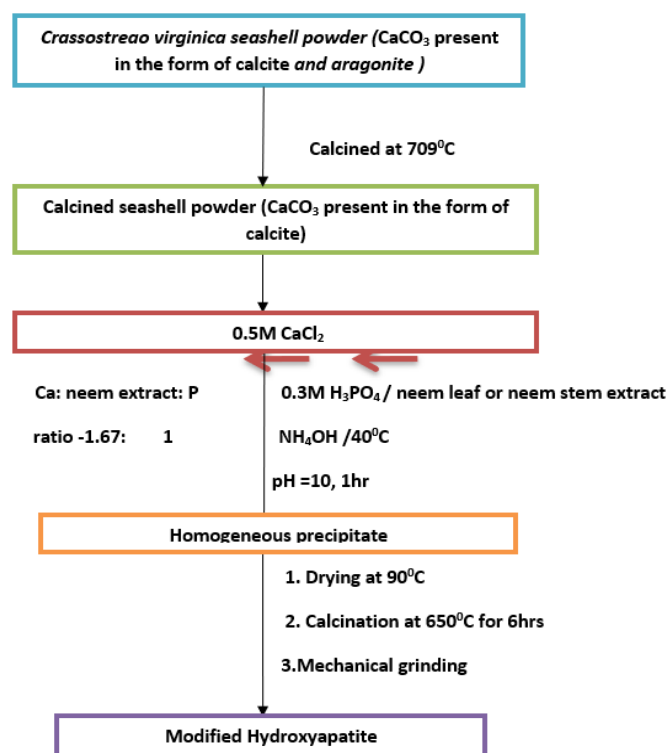
and modified HAp samples matched very well with the HAp found in human bone. Porous morphology of HAp on the surface is attractive for bone regeneration, enhanced biodegradation and good growth property. The antibacterial (*Streptococcus mutans*) and antifungal (*Candida albicans*) activity of the as synthesized HAp was enhanced when seashell was used as the precursor for Ca. Also, the in situ modification of HAp carried out with the leaf and stem extract of *Azadirachta indica* influenced more on the surface morphology but very less on the antimicrobial (bacterial and fungal) activity. Hence the present study ultimately provided an energy efficient, economically cheaper method of converting sea waste into bio mineral with enhanced bio-active and bio compatible properties for wide ranging applications.

Materials and Methods

Materials

The chemicals used for the synthesis of hydroxyapatite were seashell (*Crassostrea*

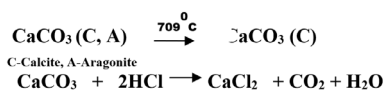
Scheme of in Situ Modification of Hydroxyapatite using Neem Stem and Leaf Extract [*Azadirachta Indica*]



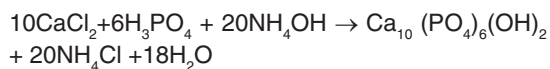
virginica seashell) powder which was used as natural precursor, azadirachta indica extract (leaf and stem), phosphoric acid (Molychem), hydrochloric acid, ammonium hydroxide. These crassostero virginica species are not frequently found in India but particularly available in the seashores of Gulf of Mannar namely, Rameshwaram which were taken up for the present study. They were cleaned with hot water, dil.HCl and bleaching powder and dried.

Wet Precipitation Method

Crassostrea virginica seashell was calcined at 709°C for 4hrs in a muffle furnace. The Aragonite and calcite form of oyster shell converted into calcite form of calcium carbonate during calcination. The calcite form of calcium carbonate obtained from oyster shell was converted into calcium chloride using hydrochloric acid.



Calcium chloride (0.5M) obtained from the above process was treated with 0.3M phosphoric acid and ammonium hydroxide and keeping the pH at 10 and the reaction proceed up to one hour at 40 °C so that Ca/P=1.67.



The obtained precipitate was filtered, dried at 90°C for up to 24hrs. Then the precipitate was calcined at 650°C for about 6hrs in muffle furnace. The similar method was used for modification of HAp using azadirachta indica extract as medium. The assynthesized hydroxyapatite was ground for further characterization.

Analysis of the Samples by Instrumental Methods

The presence of functional groups was identified from FT-IR spectra using FT-IR (SHIMADZU) spectrometer. The spectra were collected in the range frequency range of 400-4000 cm⁻¹ and recorded against the background of KBr. The nature of crystallinity, crystal size, of the HAp samples

were determined from the Powder X-ray Diffraction patterns (PXRD). They were recorded using CuKα radiation with the diffraction angle between 20° and 80°. PXRD analysis was performed using a PANalytical model Xpert pro instrument. The average crystallite size 'D' of the sample was calculated using the Scherrer equation, $D = K\lambda / \beta \cos\theta$, D- crystallite size of the sample, K-Scherrer constant (0.9), β-full width half maximum, θ- diffraction angle. The surface morphology and composition of the seashell samples were studied from the Scanning Microscopic (SEM) images. It was carried out using F E IQUANTA FEG 200. The relative percentage of elements at the surface of the images was determined from the Energy dispersive X-Ray analysis data (EDAX).

Anti Microbial (Anti Bacterial and Anti Fungal Activity of Hydroxyapatites

The synthesized samples were also screened for antibacterial and antifungal activity against human pathogens such as streptococcus mutans and candida albicans using agar well diffusion method.

Samples for the study were prepared by dissolving 10mg of the hydroxyapatites in 500 µl of Ethanol + 500 µl of Acetone + 500 µl of Isopropanol. Nutrient Agar (NA) plates were inoculated with test microorganisms and were evenly spread out. Each well was loaded with 500,1000,1500,2000 µl of corresponding concentration of sample and 10 mg of Tetracycline dissolved in 1 ml of DMSO was used as a Positive control for antibacterial activity. Ketaconazole (100µg/well) was used as a positive control for antifungal activity. The plates were incubated for 24h at 37 °C. The development of inhibition zone around the well was measured (diameter) and recorded.

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References

- 1 Hasan Mahmud Md, KaziAbdus Salam, Gafur M. A, AshequlAlam Rana, Rakibul Qadir Md, Masum Shah Md, Mithun Sarker and Mohammad Mainul Karim. Chemical Characteristics of Hydroxyapatite from Oyster Shell by Thermo-Chemical Process. *International Journal of Innovative Research in Science Engineering and Technology*. 7: 5040-5047 (2015).
- 2 Mehdi ebrahimi, Michael G.botelho, sergey V.Dorozhkin. Biphasic calcium phosphate bioceramic (HA/TCP): Concept, physico chemical properties and the impact of standardisation of study protocols in biomaterials research. *Material science and engineering*. 71,1293-1312 (2017).
- 3 Mythili Prakasam , Janis Locs , Kristine Salma-Ancane , Dagnija Loca, Alain Largeteau, and Liga Berzina-Cimdina. Fabrication, Properties and Applications of Dense Hydroxyapatite: A Review. *Journal of Functionalised Biomaterial*. 6,1099-1140 (2015).
- 4 Linbo Dou, Yancong Zhang and Hanwen Sun , *Advances in Synthesis and Functional Modification of Nanohydroxyapatite*, *Journal of Nanomaterials* Volume 2018, Article ID 3106214, 7 pages.
- 5 Humair A. Siddique, Kim L. Pickering 1 and Michael R. A Review on the Use of Hydroxyapatite Carbonaceous Structure Composites in Bone Replacement Materials for Strengthening Purposes Mucalo,* *Materials*. 11(10), 1813;1-32, (2018)
- 6 Sawittree Rujitanapanich, Piton Kumpapan and PanthongWanjanoi. Synthesis of hydroxyapatite from oyster shell via precipitation. *11th Eco-Energy and Material Science and Engineering*. 56:112-117,(2014)
- 7 Hadeel Alobeedallah, Jeffrey L.Ellis, Ramin Rohanizadeh, Hanscoster and fabrica dehghani. Preparation of nanostructured hydroxyapatite in organic solvent in clinical application. *Materials*. 25,12-19 (2011)
- 8 Dhayani and Sadhna Tripathi, Quercetin-a potential compound of azadirachta indicaa. juss.(neem) leaves exhibiting activity against wood decaying fungi and termiteswati 38 *Wood Preservation Discipline Forest Products Division, Forest Research Institute Dehradun, India*
- 9 Enamulhoque Md, Muhammed shehryar, khandakan and Nurul Islam Md. Processing and characterization of cockle shell calcium carbonate bioceramic for potencial applicationin bone tissue enginnering. *Journal of Material Science and Engineering*. 2: 1-6, (2013)
- 10 Nurul Islam Kh, Zuki Bin Abu Bakar Md, EaqubAli Md, MohdZobir Bin Hussein, Mustapha M. Noordin, Loqman M.Y, Gous Miah, Hanif Wahid and UdaHashim. A novel method for the synthesis of calcium carbonates (aragonite) nanoparticles from cockle shells. *Powder Technology*. 235: 70-75. (2013)
- 11 Dadkhah M, Salavati-Niasari M and Mir N. Synthesis and Characterization of Nano-Size CaCO_3 via Thermal Treatment and Solid State Method. *Journal of Nanostructures*. 2:153-158.(2012)
- 12 Migule galvin ruiz, Mario Enriquez Rodriguez-garica. Characterization of calcium carbonate, calcium oxide and calcium hydroxide as starting point to the improvement of lime for. *Journal of materials in civil engineering*;4: 1-20.(2009)
- 13 Adrian paz, Dainelys geradarama monica, copez, Jesus E. Comparative study of hydroxyapatite nanoparticle synthesized by different routes. *Biomaterial and nanostructures group*. 35: 1-8.(2012)
- 14 Sudhaparimala S* and Usha.R. Quality (Nanoscale) assessments of Calcium carbonate present in shells of *Anadara granosa*, and *Crassostrea virginica* marine species located in the coastal part of South India. *Advances in Natural and Applied science*. 11(9): 205-211.(2017)
- 15 Arunseshan chandrasekar, Suresh sagadevan and Arivuoli Dakshna moorthy. Synthesis and characterization of nano hydroxyapatite using the wet chemical precipitation technique.

- Journal of Material Science and Engineering. 32; 1639-1645.(2013)
- 16 Piergiorgio Gentile, caroline J.wilcock, Miller A, Robert Moorehead and paul V.Hatton. Process optimization of control the physico-chemical characteristics of biomimetic nanoscale hydroxyapatite prepared using wet chemical precipitation. *Materials*.8: 2297-2310.(2015)
 - 17 Mei-sheng Xia, Zhi-tong Yao, Liu-qinGe, Tao Chen and Hai-yan Li. A potential bio-filler: The substitution effect of furfural modified clam shell for carbonate calcium in Poly propylene. *Journal of Composite Materials*. 0(0): 1–10. (2014)
 - 18 Nurhayati, Muhdarina, Amilia Linggawati, Sofiaanita and Tengku Ariful Amri. Preparation and characterization of calcium oxide Heterogeneous Catalyst Derived from *Anadara granosa* shell for Biodiesel synthesis. *Environment and Renewable Energy* 2015; 2016: 1-8.
 - 19 Nurfatirah Nordin, Zainab Hamzah, Othman Hashim, Farizul Hafiz Kasim and Rozaini Abdullah. Effect of temperature in calcination process of seashells. *Malaysian Journal of Analytical Sciences*. 19(1): 65-70.(2015)
 - 20 Nancy F, Crum-cianflone. Bacterial, Fungal, Parasitic, and Viral Myositis. *Clinical Microbiology reviews*. 21(3): 474-494.(2008)
 - 21 Ragab H. S, Ibrahim F. A, Abdallah F, Attieh A, Al-Ghamdi, Farid El-Tantawy, Neyara Radwan, Yakup hanoglu F. Synthesis and In Vitro Antibacterial Properties of Hydroxyapatite Nanoparticles. *IOSR journal of pharmacy and biological science*. 7: 77-85. (2014)
 - 22 Syed sibte asghar abidi, qasim murtaza. Synthesis and characterization of nano hydroxyapatite powder using wet chemical precipitation. *U.P.B. Sci. Bull.,series*. 75: 3-12. (2013)
 - 23 Himanshu khandewal, Satya prakesh. Synthesis and characterization of hydroxyapatite powder by egg shell. *Journal of Minerals and Materials Characterization and Engineering*. 4: 119-126.(2016)