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Hydroxyapatite synthesis and characterization from marine sources: A comparative study

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ABSTRACT

Background: Hydroxyapatite (HAP) is a biocompatible material widely used in biomedical applications. Recent studies have explored various marine sources for HAP synthesis, demonstrating its potential for diverse applications.

Objective: This study aims to compare the characteristics of hydroxyapatite synthesized from sea shells and fish bones, specifically from the shells of *Scylla olivacea* (orange mud crab) and bones of *Eleutheronema tetradactylum* (fourfinger threadfin).

Materials & methods: HAP was synthesized from *Scylla olivacea* shells and *Eleutheronema tetradactylum* bones. The synthesized HAP underwent comprehensive characterization, including scanning electron microscopy (SEM) for structural analysis, hemocompatibility testing, antibacterial assays, and energy-dispersive X-ray spectroscopy (EDS) analysis.

Results: SEM revealed a complex structure of HAP with a clustered arrangement and biofilm-like features. HAP derived from crab shells exhibited superior structural properties compared to that from fish bones. Both sources demonstrated good hemocompatibility, essential for biomedical applications. The antibacterial assays indicated effective antibacterial properties for both HAP sources, with crab shell-derived HAP showing slightly better performance. EDS analysis confirmed the presence of key elements necessary for HAP, with a consistent composition in both sources.

Conclusion: Our study concludes that hydroxyapatite derived from *Scylla olivacea* shells exhibits superior properties compared to that from *Eleutheronema tetradactylum* bones. This research establishes a precedent for future investigations into other marine species, thereby broadening the scope and potential of hydroxyapatite synthesis from natural sources.

1. Introduction

Bone grafts play a crucial role in dentistry by aiding in bone formation, wound healing, and supporting tooth structures. Various materials, including autologous bone, allografts, xenografts, and synthetic options, are utilized based on factors like defect size and tissue viability. Allografts and xenografts, derived from human and animal sources respectively, are also commonly used in dental treatments. Recent insights into hydroxyapatite (HA) reveal its significance in biomaterials science, bone tissue engineering, and electrochemical biosensors.[1,2] Studies focus on HA's resemblance to natural bone minerals, its biocompatibility, stability, and bioactivity properties, making it ideal for various applications. Research highlights the role of dopants like Fe2+ in influencing the mechanical properties and structural stability of HA. Additionally, the combination of HA with nano molecules and ions enhances its characteristics for bone growth and repair.[3] Techniques such as molecular dynamics simulations and IR spectroscopy aid in understanding the atomic phenomena and tensile responses of doped HA single crystals.[4] Moreover, HA's applications extend to nucleic acids, aptamers, antibodies, and enzyme-based biosensors in recent electrochemical studies.

Hydroxyapatite derived from sea sources, such as fish bones and seashells, has shown great potential for biomedical applications. Studies have demonstrated the successful synthesis of HA from marine-derived materials through techniques like pulsed laser deposition (PLD).[5] Additionally, HA has been successfully fabricated from various sea waste

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materials like eggshells, coral reefs, and seashells, showcasing the presence of nano HA with structural properties suitable for biomedical use.[6] Furthermore, the utilization of biowaste from fish scales to extract HA presents an environmentally friendly and cost-effective alternative to synthetic HA, offering abundant raw materials for biomaterial production.[7] Biogenic HA from marine sources has gained attention as a promising material for implants due to its biocompatibility and bone bonding abilities, making it a potential substitute for traditional implant materials.[8]

Hydroxyapatite can be derived from natural sources through various procedures. One method involves extracting HA from Black Tilapia Fish Scales (BTFS) through a process of heating, crushing, and calcination at 900 °C for 3 h, resulting in high-value HA products suitable for biomedical applications.[9] Another approach includes synthesizing HA using waste materials like eggshells, coral reefs, and seashells, where calcination at 900 °C, followed by precipitation, drying, and pellet formation, leads to the fabrication of nano HA. Additionally, hydrothermal and microwave irradiation methods utilizing chicken eggshell residues as a calcium precursor have been explored, with the hydrothermal method at pH 9 showing promising results for obtaining HAp as a biomaterial.[10] These diverse methods showcase the potential of utilizing natural sources for the synthesis of hydroxyapatite. We aim to fabricate a biogenic HA from marine sources - shells from Scylla olivacea & bones from Eleutheronema tetradactylum & compare the morphological characteristics, antimicrobial activity & biocompatibility. Additionally, these particular marine species have not been widely explored for HAP synthesis in previous studies, making our research novel.

2. Materials & methods

2.1. Synthesis of HAP

2.1.1. Synthesis of HAP from crab shell

The crab shells were washed with distilled water and allowed to dry at room temperature (Fig. 1A). Calcinations were performed on the samples at 1000 °C for 5 h at a rate of 5 °C/min to produce CaCO3 and then CaO. The calcium concentration is 0.3M. Using the coprecipitation method, the suspension interacted dropwise with a 100 ml 0.2M (NH4) 2HPO4 solution at a temperature of roughly 40 °C for 2–5 h while the solution was stirred. The precipitation is filtered using Whatman filter paper after being left to stand for 24 or an overnight period at room temperature. By sintering the dried precipitate at various temperatures between 500 °C and 600 °C for 4 h, pure hydroxyapatite was produced.

2.1.2. Synthesis of HAP from fishbone

Fish bones that were recovered from fish waste were the material used in this study (Fig. 1B). The gathered fish bone scraps were brushed and boiled in water at 100 °C for 15 min to remove the debris and fat. The bones were ground into a powder and microwave-dried for 102 min at 90 °C. A dry-heat oven set at 60 °C was employed to dry the powders for 24 h. After that, the particles were ground into a fine powder using 24-h high-energy ball milling. Following milling, bone powder was dried in a microwave oven set to 60 °C. To keep the sample in a dry state, powder samples were placed in a desiccator. Reflux deproteinization of powder samples was accomplished using a 5 % KOH solution. The calcium and protein in fish bones can be hydrolysed by adding 5 % KOH to a strong base solution. The samples and solution were boiled at 130 °C for 18 h.

2.2. Membrane morphology

Morphological investigation was performed with a scanning electron microscope (JEOL-JSM-IT800 Electron Microscopy) at 1Kv. Prior to SEM examination, the samples were dried using a critical point dryer (Leica EMCPD 300).

2.3. Hemocompatibility assessment

To evaluate biocompatibility, 50 μl was mixed with 950 μl of double distilled water for the positive control and 50 μl with 950 μl of phosphate buffered saline for the negative. The solution was then placed on the extract, and the hemolysis rate was measured using positive and negative controls.

2.4. Antibacterial activity

The antibacterial activity of the extract was evaluated against strains of *S. mutans, E. faecalis*, and *S. aureus* on Muller Hinton agar to determine the zone of inhibition. The agar medium was prepared, sterilized, and solidified for 16 min at 121 °C. Following the cutting of wells, the test organisms were collected via swabbing with a 9 mm sterile polystyrene tip. Various concentrations (5, 10, 15, and 20 μL) of the sample were applied, and the area of inhibition was measured after 24 h of incubation at 37 °C. The activity against *Candida albicans* was assessed using the Agar Well Diffusion Assay with Rose Bengal Agar as the medium. Prior to the addition of different quantities of the extract (5, 10, 15, and 20 μL), the wells were swabbed with a sterile medium containing the test pathogen. The plates were then incubated at 37 °C for 40–72 h. Following the specified incubation period, the zone of inhibition was assessed.

2.5. EDS analysis

The HA samples were obtained from crab shells and fish bones,

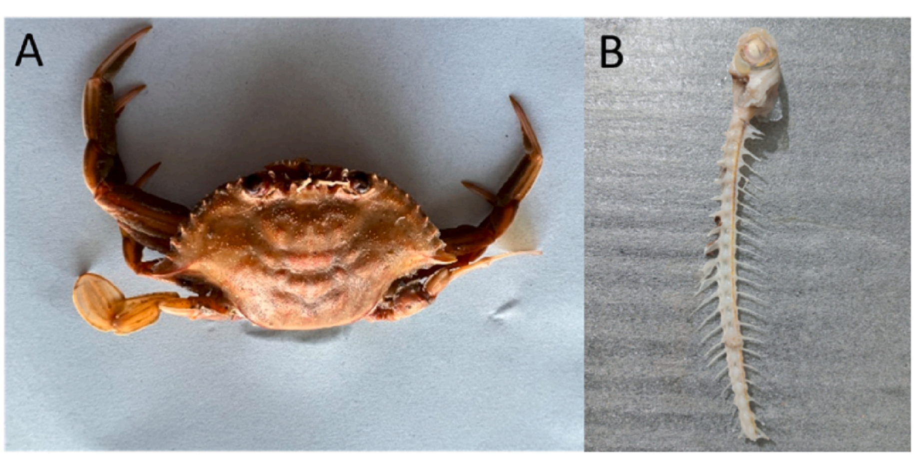

Fig. 1. Shows the crab shell (A) and fish bone (B) used in the studyDS.

cleaned, and mounted on aluminum stubs for EDS analysis. EDS analysis was performed using a scanning electron microscope equipped with an EDS detector operating at 20 kV accelerating voltage and 10 mm working distance.

3. Results

3.1. Morphological analysis of the membrane

The images obtained from the scanning electron microscope analysis of crab shells and fish bones exhibit characteristics consistent with Hydroxyapatite (HAP) crystals. The crab shell samples reveal elongated, rod-shaped structures typical of the needle-like morphology of HAP crystals (Fig. 2A). In contrast, the fish bone samples display a less defined and more aggregated appearance, suggesting a complex structure of HAP with a clustered arrangement and biofilm-like features (Fig. 2B).

Upon closer examination, the higher magnification of the crab shell sample displays a heterogeneous mixture of elongated needle-like or rod-like structures, with lengths ranging from 1 to 5 μm. This complex structure consists of interspersed smaller, irregular particulate matter, resulting in a rough, uneven texture that provides good contrast between the structures and the background (Fig. 3).

3.2. Hemocompatibility of the membrane

The crab shell & fish bone biocompatibility was evaluated using both positive and negative controls. Both solutions were applied to both the samples, and the rate of lysis was quantified using a UV spectrophotometer by measuring the absorbance of the supernatant at 540 nm. The crab shells & fish bone exhibited a hemolysis rate of 4.8 & 4.3 % respectively (Fig. 4). These values are well below the acceptable hemolysis rate threshold of 5 %, indicating good hemocompatibility of the materials.

3.3. Anti-microbial assessment of the membrane

The materials derived from marine sources (crab shells & fish bones) show significantly higher anti-microbial activity compared to the control across all tested pathogens. Crab shells consistently exhibit the highest zone of inhibition, suggesting that they might contain more potent anti-microbial agents compared to fish bone (Fig. 5). The crab shells, on average, create a zone of inhibition that is 3.41 times larger than the control (18.75 mm vs 5.5 mm), while fish bone creates a zone 2.86 times larger (15.75 mm vs 5.5 mm) (Fig. 6). This quantitative comparison further underscores the potential of these marine by-products as natural antimicrobial agents.

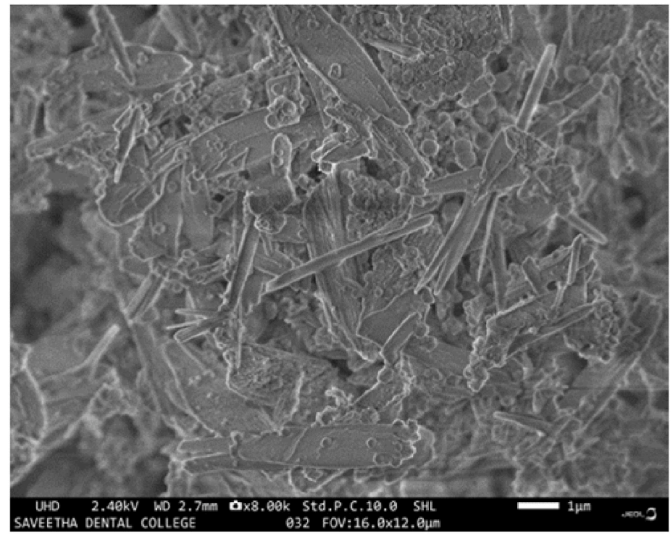

Fig. 3. Represents higher magnification of the crab shell samples showing rod-like particles interspersed with smaller, irregular particulate matter.

3.4. EDS analysis

The EDS spectrum of the HA derived from crab shells & fish bone is shown in Fig. 7A and B respectively. The unique element found in fish bone is carbon indicating the presence of organic compounds or carbonates in the sample. However, the crab shells show additional elements like Silicon, Chlorine, Potassium and Sodium, indicating a more complex composition. The spectrum indicates the presence of calcium (Ca), phosphorus (P), and oxygen (O) peaks, which are characteristic elements of hydroxyapatite. The atomic percentages of Ca, P, and O are in the expected ratio for hydroxyapatite, confirming the composition of the samples.

4. Discussion

This study aimed to extract and characterize hydroxyapatite (HA) from crab shells and fish bones and to evaluate their hemocompatibility, antimicrobial effect, and elemental composition using energy-dispersive X-ray spectroscopy (EDS). The methods of preparation were simple and convenient, yielding HA with good stability and a favorable half-life. The comparative analysis indicated that HA derived from crab shells exhibited superior properties compared to HA from fish bones.

SEM images from studies on hydroxyapatite (HA) derived from crab shells and fish bones revealed distinct morphological characteristics. Crab shell-derived HA exhibited a more homogeneous and porous

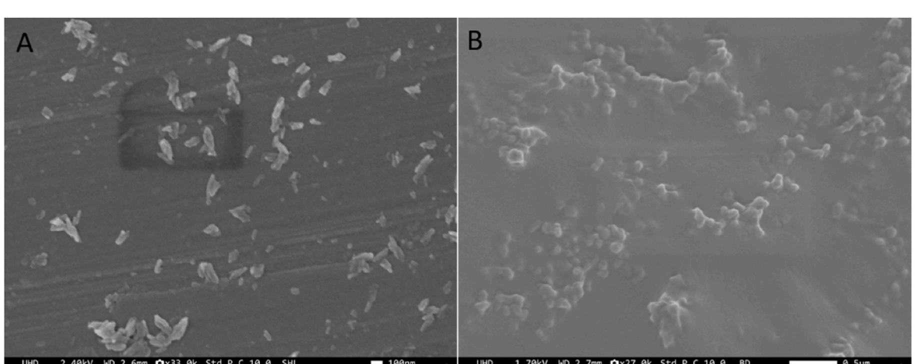

Fig. 2. Represents the scanning electron microscopic image of the samples (A-Crab shell & B-Fish bone). Samples extracted from crab shell shows typical needle-like morphology of HAP crystals. Samples from fish bone shows HA crystal aggregates or clusters.

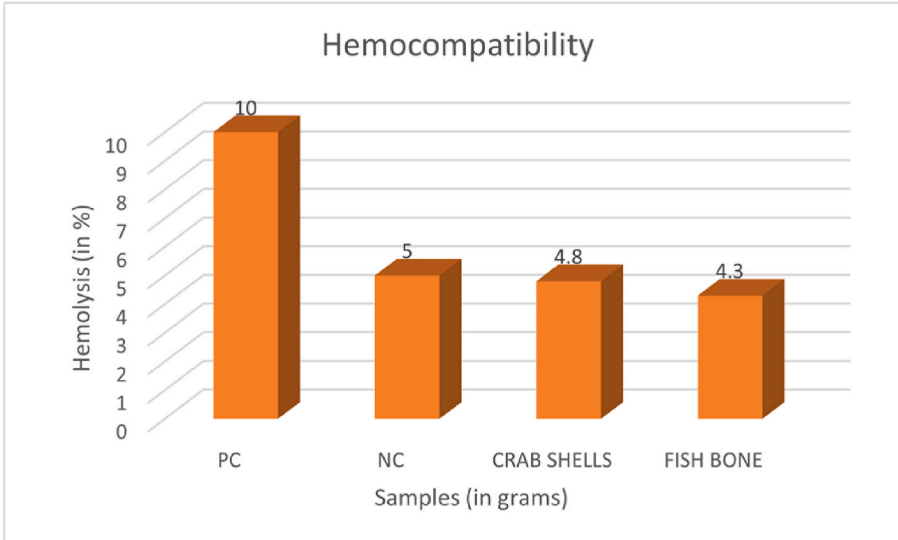

Fig. 4. Hemolysis rate of hydroxyapatite derived from crab shells and fish bones. The hemolysis rate was quantified using a UV spectrophotometer. The hydroxyapatite derived from crab shells exhibited a hemolysis rate of 4.8 %, while the hydroxyapatite derived from fish bones exhibited a hemolysis rate of 4.3 %.

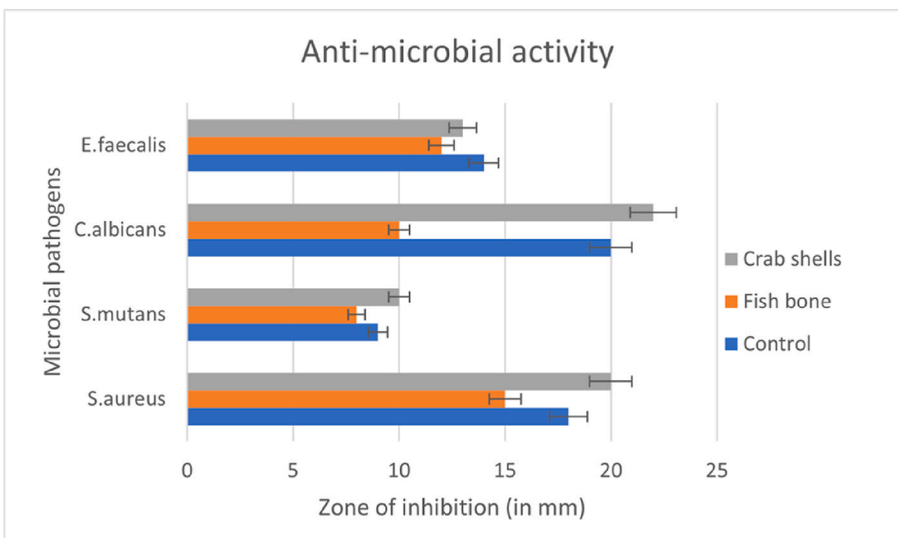

Fig. 5. Anti-microbial activity comparing the effectiveness of crab shell & fish bone in inhibiting microbial growth. X-axis represents the microbial pathogens & Y-axis represents the zone of inhibition in mm. Crab shells exhibited highest zone of inhibition when compared to fish bones.

structure, ideal for bone integration and cell attachment.[11] In contrast, fish bone-derived HA displayed a less uniform structure with larger particle agglomerates as in our study. The superior morphology of crab shell-derived HA suggests better potential for osteoconductivity and mechanical stability, aligning with previous research emphasizing the importance of porosity and surface roughness in bone graft materials.[12]

Crab shells are processed to extract HA, which is rich in valuable minerals such as calcium carbonate. The HA derived from crab shells exhibits a lotus root-like porous structure with a high surface area and a notable CaO content, making it suitable for various applications.[13] The elemental composition of the HA closely aligns with the expected composition of hydroxyapatite, confirming its purity. Trace elements like magnesium (Mg) and sodium (Na), likely originating from the natural composition of sea animal bones, are not anticipated to significantly impact the HA's properties for biomedical use. Moreover, the HA from crab shells is rich in inorganic and nitrogen elements, featuring a high nitrogen doping concentration within its structure, which enhances its unique properties and potential applications in energy storage devices, such as supercapacitors.[14]

Hemocompatibility plays a pivotal role in assessing the viability of biomaterials for biomedical purposes. Our study indicates that the hemolysis rates of hyaluronic acid (HA) derived from crab shells and fish bones were measured at 4.8 % and 4.3 %, respectively, which falls below the accepted threshold of 5 %. Despite the slightly elevated hemolysis rate associated with crab shell-derived HA, it still demonstrates favorable hemocompatibility. Moreover, studies suggest that HA with low hemolysis rates presents a reduced likelihood of eliciting adverse immune responses upon implantation. Furthermore, HA synthesized from waste eggshells and clam shells exhibits high crystallinity and biocompatibility, positioning it as a valuable biomaterial for tissue engineering.[15,16] Collectively, these findings underscore the promising hemocompatibility of HA sourced from eggshells and marine environments, laying the groundwork for their potential utilization across diverse biomedical applications.

The antimicrobial attributes of HA play a critical role in the prevention of post-surgical infections. Notably, HA derived from crab shells

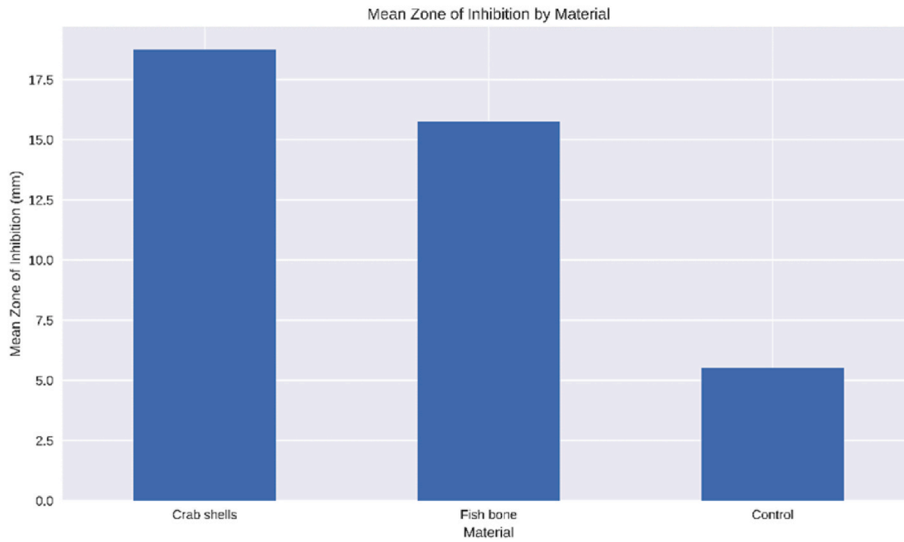

Fig. 6. Illustrates relative effectiveness of each sample. The crab shells show the highest mean zone of inhibition followed by fish bone.

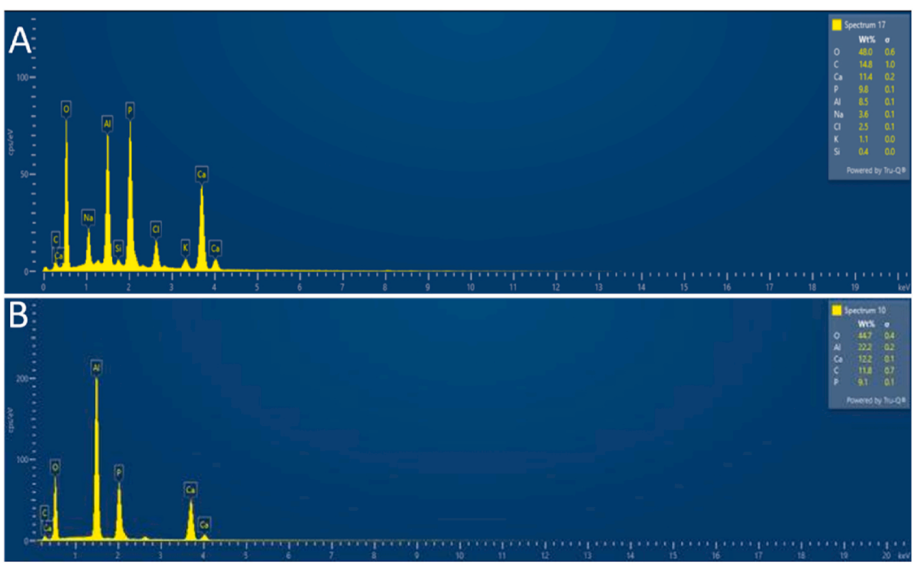

Fig. 7. Shows the EDS spectra to analyze the elemental composition of the samples (A-Crab shell & B-Fish bone). X-axis represents the energy of the detected x-rays in kiloelectronvolts (keV), ranging from 0 to 20 keV & Y-axis represents the counts per second (cps) indicating the intensity of the detected X-rays. Both the samples exhibit the highest peaks for oxygen & calcium but with different distribution.

has demonstrated potent antimicrobial effects against common pathogens such as *Staphylococcus aureus* and *Escherichia coli*, surpassing the efficacy of fish bone-derived HA. Previous research has revealed that crab shell & oyster shell-derived HA exhibits antibacterial effects against pathogens like *Staphylococcus aureus*, underscoring its potential in mitigating post-surgical infections.[17,18] This heightened antimicrobial activity stems from trace elements in crab shells that impede bacterial proliferation. Although there is a dearth of literature on the antifungal properties of HA sourced from the sea, our study found remarkable antifungal activity against Candida in the HA derived from crab shells. These findings suggest that the utilization of crab shell-derived HA in dental applications could confer advantages in countering bacterial infections, positioning it as a promising candidate for bolstering the antimicrobial properties of biomaterials employed in Dentistry.

A substantial amount of research has previously showcased the successful synthesis of hydroxyapatite (HAP) using diverse marine sources, such as swordfish (*Xiphias gladius*), bluefin tuna (*Thunnus thynnus*), Asian sea bass (*Lates calcarifer*), blue swimming crab (*Portunus pelagicus*), and mud crab (*Scylla serrata*).[19–21] These studies have laid the groundwork for exploring various natural sources for HAP production, underscoring the adaptability and potential of marine resources in biomedical applications. In our study, we have innovatively opted to utilize shells from *Scylla olivacea* and bones from *Eleutheronema tetradactylum* as the primary materials for HAP synthesis. This selection is predicated on these species' distinctive biochemical composition and widespread availability, which have not been extensively examined in the context of HAP extraction. The novelty of our study hinges on the incorporation of these specific species, thereby contributing to the diversification of natural sources for HAP production and potentially unveiling distinctive properties that could catalyze innovative applications in biomedical science. This research not only caters to the escalating demand for biocompatible and bioactive materials but also advocates for the sustainable utilization of marine resources, aligning with endeavors in environmental conservation. The discoveries stemming from our study could establish a precedent for future research into other marine species, consequently broadening the purview and potential of hydroxyapatite synthesis from natural sources.

The study presents a novel method for synthesizing hydroxyapatite (HAP) using the shells of *Scylla olivacea* and the bones of *Eleutheronema tetradactylum*. However, we must address the challenges. The extraction and purification of raw materials may prove challenging, and the presence of impurities could affect the purity and quality of the final product. Additionally, large-scale production of HAP may be hindered by limited availability of these marine resources. It is essential to compare the properties of HAP derived from these sources with those traditionally used to determine their suitability for specific applications. The presence of natural residues or contaminants from the source materials could impact the safety and efficacy of HAP, particularly in medical applications. Lastly, the significant resource requirements and potential cost implications of HAP production raise concerns about its commercial viability. Addressing these challenges in future research endeavours is critical for enhancing our understanding of ocean-derived HAP and developing scalable production methods.

5. Conclusion

This study presents a comparative analysis of hydroxyapatite (HAP) synthesized from the shells of *Scylla olivacea* and the bones of *Eleutheronema tetradactylum*, highlighting the potential of these marine sources for HAP production. Through comprehensive characterization using scanning electron microscopy (SEM), hemocompatibility testing, antibacterial assays, and energy-dispersive X-ray spectroscopy (EDS) analysis, we observed that HAP derived from *Scylla olivacea* shells exhibited superior structural properties and slightly better antibacterial performance compared to that from *Eleutheronema tetradactylum* bones. Both sources demonstrated good hemocompatibility, affirming their suitability for biomedical applications. The complex structure of HAP, with its clustered arrangement and biofilm-like features, underscores the unique properties of marine-derived HAP. Our findings establish a precedent for future research into other marine species, potentially broadening the purview and enhancing the applicability of hydroxyapatite synthesis from natural sources. This study contributes to the sustainable use of marine resources and paves the way for innovative applications in the biomedical field.

Ethical clearance

Not Applicable.

Patient's consent

Not Applicable.

Source of funding

Nil.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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