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Extraction of Hydroxyapatites from Fish Scales of *Catla catla* using Alkaline Heat Treatment and Evaluated for their *In Vitro* Antimicrobial Activity

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Abstract: Hydroxyapatites (HAp) were extracted from *Catla catla* fish scales using alkaline heat treatment, and their *in vitro* antimicrobial activity was tested against several different bacterial and fungal strains. The hydroxyapatites were found to be effective against gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*, as well as gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*. In order to quantify the antibacterial activity, the zone of inhibition was measured using the disc diffusion method. Hydroxyapatite concentrations have a linear relationship with antibacterial efficacy. Antibacterial activity was highest against *Escherichia coli,* then against *Staphylococcus aureus, Klebsiella pneumoniae,* and *Bacillus subtilis*. *Candida albicans* is the most fungal species to show considerable antifungal activity, followed by *Candida tropicalis*. In this case, the antibacterial activity was more effective against gram-negative bacteria. Moreover, its antifungal properties were shown. Hydroxapatite's antibacterial properties combined with novel antibiotics or other antimicrobial agents will have important biological uses.

Keywords: *Catla catla,* Hydroxyapatites, Antibacterial, Antifungal, Antimicrobial, Fish

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Introduction

Antibiotics have been crucial in combating microbial illnesses, which in turn has increased the median lifespan. The introduction of antibiotics has had far-reaching benefits, one of which is the decrease in human death and disability brought on by microbial illnesses (Owusu *et al*., 2021). Natural antimicrobials are secondary metabolites present in a wide variety of creatures, including plants, animals, and microbes. Natural products are chemical compounds or substances created by a live organism or found in nature that have pharmacological or biological action. But secondary metabolites could potentially have useful qualities for humans. They have the potential to be employed as medicines against a wide variety of microbial illnesses in humans (Bhatnagar and Kim, 2010). Numerous antibacterial systems have developed in animals as part of host defence mechanisms. Peptide antimicrobials are a common class of endogenous antimicrobials in mammals (protein). Antimicrobial peptides (AMPs) have been discovered in all known forms of life, including bacteria, fungi, plants, and mammals (Hoskin and Ramamoorthy, 2008).

Bioactive synthetic ceramics that closely resemble natural apatite in their properties have attracted increasing research attention. Concerns concerning the appropriate handling and use of by-products have arisen in recent years due to environmental and economic factors. The value-adding potential of fish by-products is enormous but currently underutilised. Use of fish by-products in human food and biochemical products is, however, still a topic of extensive research in the aquaculture sector (Huang *et al*., 2011). Calcium and phosphorus are the primary components of hydroxyapatite (HAp), which has the chemical formula $Ca_{10}(PO_4)6(OH)_2$. HAP may be found in human bone and teeth. Fish bone (Jensen *et al*., 1996; Ozawa and Kanahara, 2005) and fish scale (Mondal *et al*., 2010; Zainon *et al*., 2012) are two examples of natural materials from which HAp is extracted (Sofronia *et al*., 2014). Gómez-Guillén *et al*. (2011) found a correlation between the consumption of bovine and porcine products and negative religious and cultural attitudes. The evolutionary distance between fish and humans suggests that fish are a more safer food source (Venkatesan *et al*., 2015). Hydroxyapatite-based materials (HAp) Fish scales, which make up 30–40% of the overall quantity, are the primary byproduct of the fish-food processing sector, and their management is a source of difficulty for businesses (Gumisiriza *et al*., 2009).

One may save money on trash removal and have a reliable source of raw material by using by-products to create a new biocompatible material. This means that ceramics derived from fish have excellent potential as bioactive medium since they are eco-friendly (Ozawa and Kanahara, 2005). Antibacterial medicines synthesised from bio-waste have been heralded as a unique, environmentally friendly medicinal use. In the present study, HAp was extracted from scales of fish *Catla catla* using alkaline heat treatment and evaluated for their *in vitro* antimicrobial activity against *Escherichia coli* (MTCC 732), *Staphylococcus aureus* (MTCC 3160), *Klebsiella pneumoniae* (MTCC 4031) and *Bacillus subtilis* (MTCC 2423), and antifungal activity against fungal strain *Candida albicans* (MTCC 183) and *Candida tropicalis* (MTCC 184).

Materials and Methods

Fish scale (FS) from *Catla catla* fish (b wt 1.27 kg) were collected from Thanjavur fish market (June 2022). Commercially available hydroxyapatite (CHAp) was bought from Sigma-Aldrich (India).

Hydroxyapatite Isolation from Fish Scales:

Fish scales were cleaned in distilled water to eliminate any remaining organic matter. After that, the scales were allowed to dry naturally. Fish hydroxyapatite (FS-Hap) was manufactured by an alkaline heat treatment process (Kongsri *et al*., 2013). Scales were deproteinized with 0.1 M HCl and rinsed many times with distilled water before drying. Scale proteins that had not been removed were heated and mixed with 5% NaOH (w/v) at 70°C for 5 h. The resulting precipitate was rinsed in pure water and dried at 60 ℃. The powder was mixed with NaOH at a ratio of 50% (w/v), heated to 100°C, and agitated for 1 h. Powdered FS-HAp was washed in deionized water and dried at 60°C. To create HAp ceramics, we calcined treated fish scales at 800° C for 1 hour. To test the antibacterial efficacy of the synthetic ceramics, they were ground into a powder using a mortar and sieve.

The anti-microbial activity was determined using the disc diffusion technique as outlined by NCCLS, (1993) and Awoyinka *et al.* (2007).

Experiment preparations for FS-HAp:

50 mg of FS-HAp was added to 1 ml of water, and the standard solutions included chloramphenicol (25 mg per ml of distilled water) and fluconazole (25 mg per ml of distilled water) to kill bacteria and fungus, respectively. These solutions were stored in the fridge.

Preparing dried filter paper discs:

Four discs, each about 6 mm in diameter, were cut from Whattman filter paper (No. 1) and sterilised with hot air. Discs were loaded with 200 µl, 400 µl, and 600 µl of FS-HAp after they had been sterilised; the test solution was compared to a 30 µl standard solution containing chloramphenicol and fluconazole. Unless they were being utilized in the experiment, they were stored in the fridge.

Detection of antimicrobial activity:

The disc diffusion approach (NCCLS, 1993; Awoyinka *et al*., 2007) was used to create the antibiogram. 30 ml of NA/PDA media were poured onto petri dishes to get them ready. Using a micropipette, the test organism was inoculated onto a plate of solidified agar, where it was disseminated and left to dry for 10 min. Bacteria from a broth culture were used to inoculate the medium surfaces. Standardized microorganisms test suspension is used to inoculate the Nutrient agar /PDA plates, and a sterile cotton swab is used to uniformly inoculate the whole surface of the plates. Spread on plates of Nutrient agar/PDA were inoculums containing various microorganisms. Aseptic techniques were used to place sterile filter sheets (6 mm in diameter) containing 200 µl, 400 µl, and 600 µl of FS-HAp, Standard solution as Chloramphenicol and Fluconazole 30 µl, on the surface of the infected agar plate. Both the bacterial and fungal strains were allowed to grow on plates for 24 and 48 h, respectively, at 37°C. We ran the tests on each sample three times for accuracy. Measurements of the average millimeter-wide zone of inhibition surrounding the disc were used to rank the test chemicals for their antibacterial efficacy. There was a millimetre scale used to quantify the samples' ability to suppress growth.

Results

Hydroxyapatites' antibacterial properties were tested using the disc diffusion method (Table 1, Fig. 1). Hydroxyapatites were tested for their antibacterial activity against a variety of bacteria and fungus at three different concentrations (200 µl, 400 µl, and 600 µl) with results compared to those of the gold standards chloramphenicol and fluconazole. With respect to Escherichia coli, the highest antibacterial activity was recorded as 6.80 mm at 600 µl, while the lowest was recorded as 1.60 mm at 200 µl. The antibacterial activity against Staphylococcus aureus ranged from a maximum of 6.35 mm at 600 µl to a low of 1.45 mm at 200 µl. Klebsiella pneumoniae showed the highest antibacterial activity at 600 µl as 6.70 mm, whereas at 200 µl showed the lowest as 1.50 mm. At 600 µl, the antibacterial activity (Bacillus subtilis) was at its highest (6.20 mm), whereas at 200 µl it was at its lowest (1.30 mm). The highest antifungal activity against Candida albicans at 600 µl was 3.80 mm, whereas the lowest was at 200 µl (0.40 mm). The highest antifungal activity against Candida tropicalis at 600 µl was 4.20 mm, whereas at 200 µl it showed the lowest (0.65 mm). Significant antibacterial activity was seen against Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae, and Bacillus subtilis. Strongest antifungal properties were noticed in Candida albicans followed by Candida tropicalis.

Discussion

Because of its biocompatibility and bioactivity, hydroxyapatite (HA) has garnered a lot of interest in the medical field. Due to its outstanding biocompatibility, tight chemical and crystallographic structure with the mineral phase of natural bone, and its widespread interest as a bone filler and implant material, hydroxyapatite (HAp) $Ca_{10}(PO_4)6(OH)_2$ has gained a great deal of attention (Hench, 1991). Because of its high affinity to biomaterials like proteins, hydroxyapatite is used not only as a structural

Table 1: Anti-microbial activity of FS-HAp

Microbial strains	Zone of inhibition (mm) of FS-HAp			
	200 µl	400 µl	600 µl	Std. (30 µl)
Bacterial strains				
Escherichia coli	1.60±0.11	4.35±0.30	6.80±0.47	11.45±0.80
K. pneumoniae	1.50±0.10	3.70±0.25	6.70±0.46	11.40±0.79
S. aureus	1.45±0.10	3.10±0.21	6.35±0.44	11.25±0.78
Bacillus subtilis	1.30±0.09	3.00±0.21	6.20±0.43	11.10±0.77
Fungal strains				
Candida albicans	0.40±0.02	1.55±0.10	3.80±0.26	6.10±0.42
Candida tropicalis	0.65±0.04	1.60±0.11	4.20±0.29	6.40±0.44

Values are expressed as mm; Bacterial standard: Chloramphenicol; Fungal standard: Fluconazole.

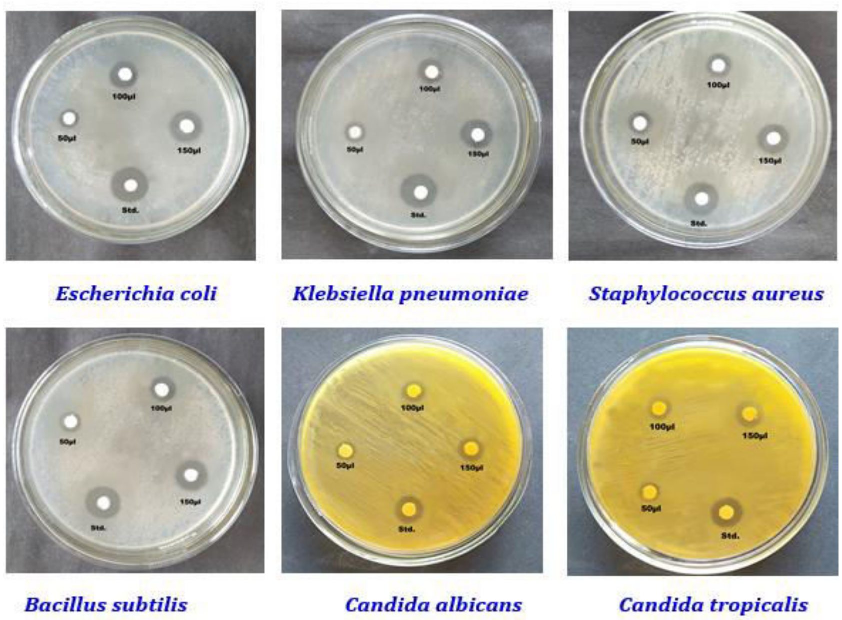

Fig. 1: Anti-microbial activity of FS-HAp.

component of hard tissues like bones and teeth, but also in bioceramics and adsorbents (Kikuchi *et al*., 2004).

By compared to gram-negative bacteria like *Escherichia coli* and *Klebsiella pneumoniae*, gram-positive bacteria like *Staphylococcus aureus* and *Bacillus subtilis* demonstrated the greatest vulnerability to the nanocomposites. Plasmolysis, or the detachment of cytoplasm from the cell wall, may be the most telling indicator of HAp susceptibility in *Staphylococcus aureus* and *Bacillus subtilis*. According to the literature, Gram-positive bacteria have a single membrane surrounded by a thick wall made of multiple layers of peptidoglycan polymer (plasma membrane). Gram-negative bacteria, in contrast, have a more complicated cell wall that consists of an outer membrane and a plasma membrane in addition to a thin coating of peptidoglycan. Many chemicals' permeability is affected by the outer membrane that Gram-negative bacteria cells add. Many chemical agents are less effective against Gram-negative bacteria than they are against Gram-positive organisms, but only under particular circumstances (Tortora *et al*., 2001).

Several processes, including changes to the cell wall and cytoplasm, the membrane, and the suppression of respiratory activity have been proposed as routes by which nanoparticles might elicit their antibacterial effects (Alshemary *et al.*, 2015). The concentration of HAp has a linear relationship with its antimicrobial efficacy. The production of reactive oxygen species (ROS) was sparked and accelerated by HAp, leading to the death of bacteria. There are a number of unique consequences that high amounts of ROS have on bacteria. One of them is lipid peroxidation, which compromises the bacterial membrane and increases ion permeability. The HAp's discharge of positive ions upsets the equilibrium of the cell, leading to its final demise. It is clear that unsaturated oxygen can donate electron to O_2 and activate it through the interaction between oxygen and unsaturated oxygen generated by the dehydration of the hydroxyl groups in the apatite, as evidenced by the simultaneous appearance of ESR centres assigned as unsaturated oxygen and O2 radical oxygen species (Yasuyuki *et al*., 1997).

Conclusion

Hydroxyapatite's inhibitory activity was studied against many bacteria (*Escherichia coli, Staphylococcus, Klebsiella pneumoniae, Bacillus subtilis*) and fungi (*Candida albicans*, *Candida tropicalis*). The antibacterial effect was mostly directed towards Gram-negative bacteria as its major target. In addition to this, evidence of antifungal activity was discovered. The antibacterial characteristics of hydroxyapatite, when coupled with those of newly discovered antibiotics or other antimicrobial agents, will have applications in practical biology.

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