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Green Synthesis and In vitro Applications of Nano Hydroxyapatite for Orthopaedic and Dental Applications

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Abstract

Hydroxyapatite (HAp) is a known Biomaterial applied in Orthopaedic and Dental application as bone implants. Bone can easily be infected by microorganisms. Hydroxyapatite with antibacterial activity can be used for bone and dental treatment defects. In the current study, HAp nanoparticles are synthesized with and without *Moringa oleifera* leaf extract by wet chemical method assisted by Microwave irradiation. Calcium hydroxide and Orthophosphoric acid are used as calcium and phosphorus source respectively. The synthesized samples were characterized by X-ray Diffraction technique (XRD), Fourier Transform Infrared analysis (FTIR), Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray analysis (EDAX). Further in vitro analysis was performed using gram positive bacteria *Staphylococcus aureus,* gram negative bacteria *Klebsiella pneumonia* and fungi *Candida albicans.* X-ray Diffraction (XRD) techniques investigated the crystalline size for both the samples ranges between 6 to 20 nm. FTIR spectrum confirms the presence of hydroxyl (O-H) and phosphate (PO_4^{3-}) groups. SEM and EDAX predict the presence of spherical shaped morphology with proper proportion of 1.67. In vitro analysis reveals the significant zone of inhibition for both bacteria and fungi.

Keywords: HAp, Invitro Analysis, Synthesis

1. Introduction

Hydroxyapatite (HAp) is an excellent biomaterial for bones and dental replacement, repair and reconstruction of tissues as its molecular structure was similar to the human hard tissues. HAp has good compatibility, bioactivity and osteoconductivity[1]. Calcium phosphates are commonly used attractive biomedical materials due to their excellent biocompatibility and the non-toxicity of their element components[2].

The element composition of Hydroxyapatite (HAp) is very close to that of human hard tissues, like bone and teeth and has been widely applied in various biomedical fields[3,4]. The bone is mainly constituent with collagen (20 wt.%), calcium phosphate (69 wt.%) and water (9 wt.%). Bone is an organ that can provide basic mechanical support to the body. But bone can be easily infected by microorganisms. To rectify this problem, the HAp was used as fillers to replace amputated bone and it is also used as coating to promote bone in growth. HAP is used in various fields like drug delivery, hip replacements, dental implants, bone conduction implants, DNA/Protein adsorption and repair tooth enamel the prepared HAp and its composites are used as promising material[5].

HAp can be synthesized using various methods such as wet chemical deposition, sol-gel route, biomimetic deposition. Plant mediated synthesis of nanoparticle that connects nanotechnology with plants[6]. Synthesizing NPs are formed at ambient temperature, low costs and environmentally friendly fashion. Plants and plant extract are the best option for green synthesis. They are cost effective and need low maintenance. Hydroxyapatite (HAp) has been

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prepared by various methods. Such as sol-gel technique, thermo hydrolysis, hydro thermal method, chemical vapour deposition technique and microwave irradiation method. Microwave irradiation method offers outstanding compositional control, molecular level high homogeneity, lower crystallization temperature and possibility of producing very fine routes[7,8].

Moringa oleifera is a traditional medicine for long time in India and it has good antibacterial property[9]. Further this leaves contain excellent source of vitamins and minerals. The chopped leaves (20 grams) contain 2 grams of protein, 19% of vitamin B6, 12% of vitamin C, 11% of iron, 11% of Riboflavin (B2), 9% of vitamin C and 8% of magnesium. It also contains high levels of antinutrients. Further it can be used as capsule form or else powder form to reduce the blood sugar and cholesterol. In this current work we account a plant extract mediated green synthetic method for HAp nanoparticles capped with fresh leaf extract of *Moringa oleifera*. The HAp nanopowders was synthesized by wet chemical assisted microwave irradiation method and characterized by XRD, FTIR, SEM, EDAX and Antimicrobial activity.

2. Materials and Methods

2.1 Green Synthesis of HAp

Calcium hydroxide ($Ca(OH)_2$), Orthophosphoric acid (H_3PO_4) and sodium hydroxide (NaOH) were received from Merck, Mumbai, India. *Moringa oleifera* leaves (Figure 1) was collected and washed using double distilled water to clear dust particles and dried at room temperature. Take two beakers each containing 250 ml of double distilled water with 50 g of chopped leaves and boil at 100°C. The extracts were cooled and filtered. In this present study HAp nanopowders using leaf extracts, 1 M of $Ca(OH)_2$, 0.6 M of H_3PO_4. The pH of without leaf extract was found to be 7 and with leaf extract having the pH of 12 which is higher than the chemical synthesis.

1 M of $Ca(OH)_2$ was mixed with 50 ml of extract and stir the solution for about half an hour at room temperature. After stirring, H_3PO_4 solution was added drop by drop into calcium hydroxide $Ca(OH)_2$ solution and then stirred about 1 h. The gelatinous precipitate produced was placed under room temperature. The precipitate thus obtained was washed out using distilled water twice. The prepared precipitate was dried in domestic microwave oven at 35 W and grinded into fine powder. The synthesized HAp nanopowders using M. oleifera leaf extract were named as MOL:HAp (MHAp). Further, HAp with no leaf extract was synthesized and named as Pure HAp (PHAp) for assessment.

2.2 Characterization Techniques

2.2.1 FTIR

The functional groups of prepared samples were identified using Fourier transform spectroscopy analysis. The spectrum was recorded in the range of 4000-400 cm^{-1} region[10].

2.2.2 XRD

The prepared samples were analyzed using XRD (X-ray Diffraction) technique. This XRD pattern predicts the lattice parameter (a and c), unit cell volume and crystalline size of the sample. The XRD pattern of prepared samples was well matched with JCPDS card no: 09-0432(which is corresponding to hexagonal phase). The lattice parameter of the sample was calculated using the following equation:

$$\mathbf{1/d^2 = (4(h^2+hk+k^2)/3a^2) +(l^2/c^2))}$$

Figure 1. Moringa oleifera leaf.

Where, d is the spacing between the planes, a and c are the lattice parameter. The unit cell Volume (V) of the sample was described using the given equation:

$$V = (\sqrt{3}/2) + a^2+c^2$$

The average crystalline size of the sample was determined by using the scherrer's formula.

$$D = K\lambda/\beta\cos\theta$$

Where D denotes the average crystalline size of the sample, K represents the broadening constant, λ denotes the wavelength of CuKα radiation source(1.54A°), β represents the full width at half maximum, angle of diffraction is denoted by θ[11].

2.2.3 SEM and EDAX

The surface morphologies of synthesised HAp samples were analysed using Scanning Electron Microscopic analysis (SEM). Energy dispersive spectroscopy is used to identify the elemental composition of the sample.

Antimicrobial Activity

Antibacterial activity of the synthesised samples was predicted by Agar well diffusion method. For performing antimicrobial test, 100 mg of the prepared samples were pushed in a well to form a circular disk, with diameter of 14 mm and thickness of 1 mm. Then the microorganisms subjected for study like Escherichia coli and Staphylococcus aureus were spread on the surface of nutrient agar plates. After spreading individual bacteria in each plate, disks of each sample was positioned on the surface of the culture plate and incubated for 24 h at 37 ± 0.5 °C. The zone of inhibition of the microbes including disk was calculated subsequent to the incubation period and its images were documented[12].

3. Results

3.1 XRD Analysis

The resultant XRD pattern of PHAp and MHAp samples are revealed in (Figure 2). XRD patterns of both the samples matched well with JCPDS data card No 09-0432. The diffraction peak at 2θ = 25.9°, 29.6° and 32.3° are clearly distinguishable and could be indexed to the hkl values are (002), (210) and (112). The calculated average crystalline size of PHAp and MHAp are 6.1 and 19.6 nm. The lattice parameter, average crystallite size and unit cell volume were calculated for both the samples and displayed in (Table 1). The crystallite size is attributed by the result of interfacial reaction. The average crystalline size of both the samples decrease with the increases of the unit cell volume and lattice constant as shown in table. No other impurity peaks were detected from the XRD results.

3.2 FTIR Analysis

FTIR spectrum of the prepared samples reveals the elemental composition of the samples (Figure 3). HAp can be identified from the different vibration modes of the phosphate and hydroxyl group. The FTIR spectrum of PHAp

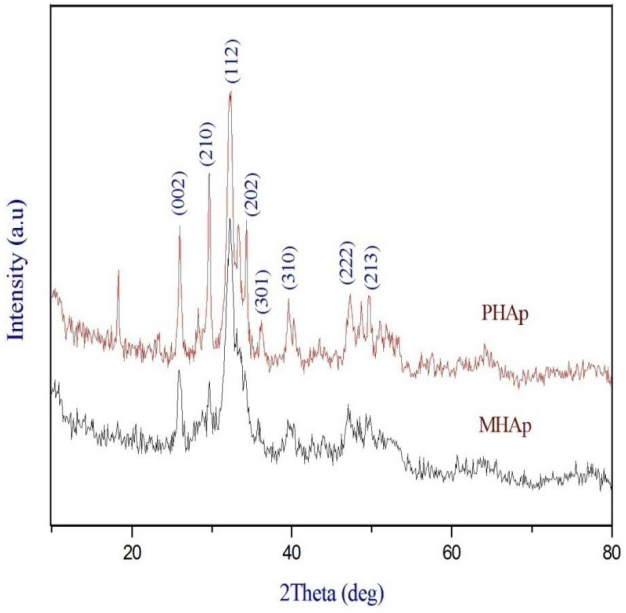

Figure 2. Synthesized sample with XRD pattern for PHAp and MHAp.

Table 1. Lattice constant, crystalline size and unit cell volume of the synthesized samples

S.No	Hkl	D	Lattice constant (Å)		Cryatalline size D (nm)	Unit cell volume (V)
			A = b	C		
PHAp	002 210 112	3.42930 3.01587 2.77520	9.37	6.86	10.0 21.3 27.5	523.10
MHAp	002 210 211	3.43208 3.01551 2.76538	9.6	6.85	6.68 5.6 6.08	548.56

shows the vibration modes at 469.68, 567.41, 603.78, 962.54 and 1039.41 cm^{-1} represents the occurrence of phosphate group and hydroxyl group at 3435 cm^{-1}. The FTIR spectrum of MHAp shows the vibration modes of phosphate at 468.93, 569.75, 604.48 and 1038 cm^{-1}. A broad extending band from 2500 to 3900 cm^{-1} which represents the O-H vibration mode was revealed in both the samples. The peak at 870.14 represents the S-O stretching and peaks at 1414.62 and 1478.91 cm^{-1} which are owed to C=O stretching of carboxylic acid.

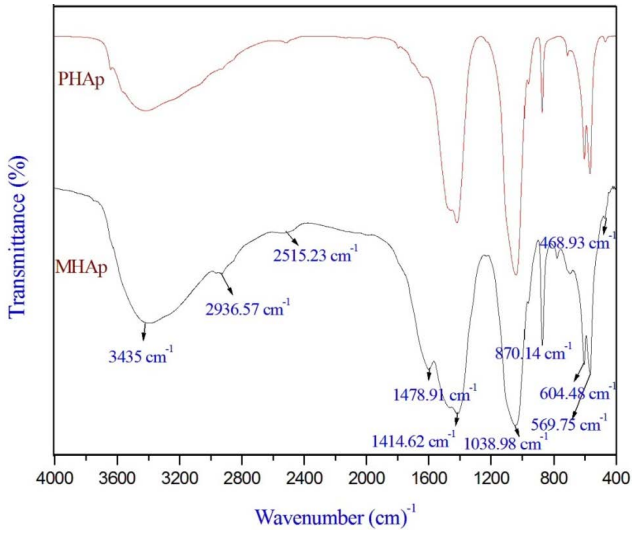

Figure 3. The FTIR spectrum of PHAp and MHAp.

3.3 SEM and EDAX

The SEM micrographs of synthesized PHAp and MHAp shows to influence the size of the nanoparticles. The PHAp nanoparticles appeared with an approximate size of 53 nm in diameter and MHAp appeared with 69 nm in diameter. The synthesized particles exhibit the spherical like nanostructure. It is seen from the both figure there are agglomeration crystallites due to uncontrolled coagulation of tiny crystallites are combined together which forms a great agglomerated structure. At the same time image clarity of MHAp was distinct compared with PHAp. Because the particle size of MHAp was low down due to the vander walls force. Further the EDX analysis reveals the Ca/P ratio for both the samples are approximately 1.69, which demonstrate the purity of the sample showed in (Figure 4).

3.4 Antimicrobial Activity

Photographs of antibacterial activity (Figure 4) of PHAp and MHAp were evaluated against gram positive *Staphylococcus aureus* bacteria, gram negative *Klebsiella pneumoniae* bacteria and *Candida albicans* fungi using agar

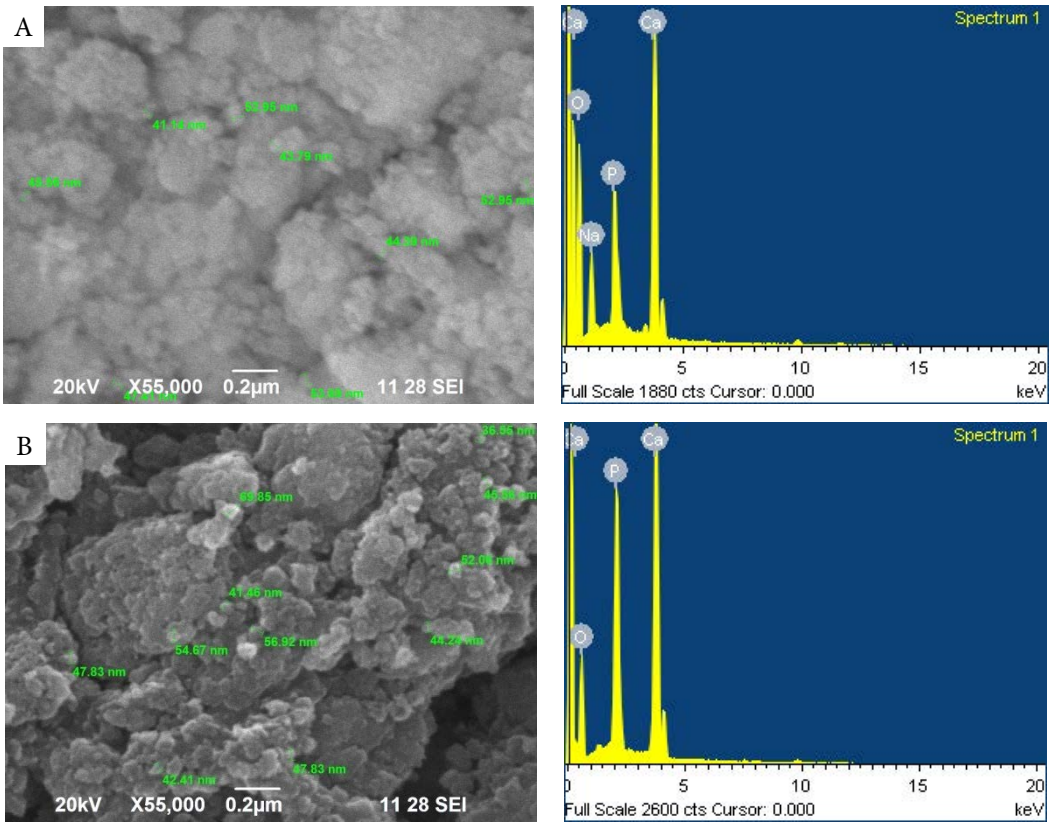

Figure 4. SEM and EDAX analysis of PHAp (A) and MHAp (B).

well diffusion technique. The inhibition zone of microbes for PHAp and MHAp are tabulated in Table 2. These results showed that good zone of inhibition for MHAp compared with PHAp. From the result it reveals that the *Moringa oleifera* leaf extract solution has incorporated into HAp. Ciprofloxacin was the standard used for the bacterial strains. Ciprofloxacin is an antibiotic used to treat a number of bacterial infections[13]. This includes bone and hip joint infections. Nanoparticles are easily move through the tissues because of their size.

4. Discussion

The present research work reports the green synthesis of HAp using *Moringa oleifera* leaf extract. There are several reports on the biological and physiological activities of *Moringa oleifera*. These include hypotensive properties, hypoglycemic and hypocholesterolemic effects, anti inflammiatory and anti hepatotoxic activities, anti-helmic analgesic and in the management of heart diseases and ulcers. In chemical synthesis of nanoparticles substances from chemical source while as in green synthesis of nanoparticle substances from a biological source. Green synthesis of nanoparticles reduces the use of substances which are hazardous to human or environment.

The XRD pattern represents the sharp peaks of the prepared sample. The functional groups are formed from the amide bands and proteins in the leaf extract. The SEM image of synthesized PHAp and MHAp influenced the size nanoparticle range. EDX demonstrate the purity of the sample. *Staphylococcus aureus* and *Klebsiella pneumoniae* are clinically relevant pathogen due to its antimicrobial resistance. *Canadida albicans* is an opportunistic fungus that cause high resistance against both the samples[14]. The capping of MOLE enhanced the bacterial and fungal activity of Hydroxyapatite. The leaf extract which act as a good antimicrobial and chelating agent had a ability to kill microorganisms and stop their growth. The process which comes under the three main mechanisms. Initially antibiotic disrupt bacterial cell envelop. Then block the production of new proteins. And finally retard DNA replication.

5. Conclusion

Hydroxyapatite nanoparticles were synthesized by chemical and green synthesis method. XRD analysis predicts the crystalline size, lattice parameter and unit cell volume of the sample. The morphological structure was revealed by SEM. EDAX analysis confirms the occurrence of calcium and phosphate groups which is in the ratio of 1.67. Antimicrobial activity confirmed the presence of inhibition zone for MHAp than PHAp. From this result, the prepared MHAp reduces the crystalline size of the sample compare than PHAp. Thus we conclude that green synthe-

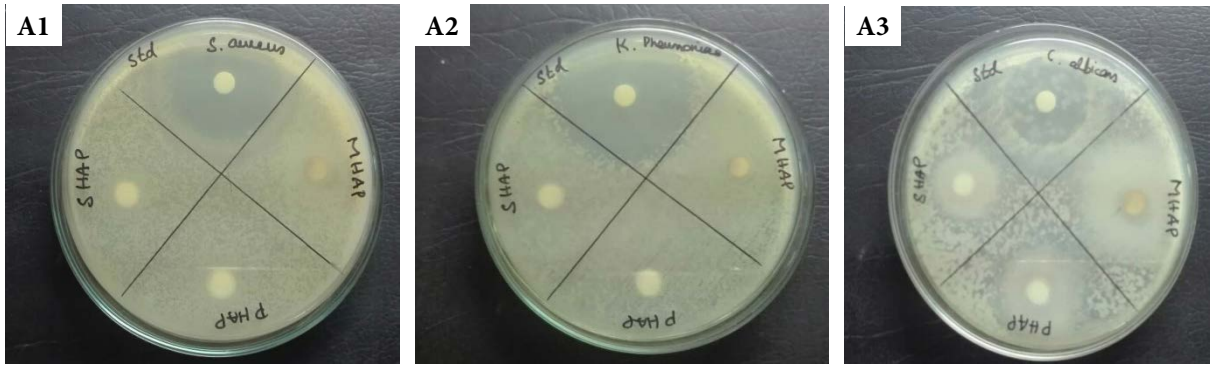

Figure 5. Photographs of antimicrobial results of PHAp and MHAp for Gram positive *Staphylococcus aureus* bacteria (A1), Gram negative *Klebsiella pneumoniae* bacteria (A2) and fungi *Candida albicans* (A3).

Table 2. Summary of the zone growth inhibition related to PHAp and MHAp nanoparticles for Gram positive *S. aureus* bacteria (A1), Gram negative *K. pneumonia* bacteria (A2) and fungi *C. albicans* (A3)

s.no	Sample name	Zone of inhibition (mm)		
		Gram positive	Gram negative	Fungi
		S. aureus	K.pneumonia	C. albicans
1.	**MHAp**	10	10	12
2.	**PHAp**	11	-	10

sized hydroxyapatite nanoparticles can be used as a best candidate for orthopaedic and dental applications.

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