BIOSYNTHESIS OF NANOCOMPOSITE FROM Alpinia Galanga FOR PHYTOCHEMICAL AND CHARACTERISATION ANALYSIS FOR ORTHOPAEDIC REPLACEMENTS

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ABSTRACT

Hydroxyapatite is now widely used as the coating of artificial joint components used in orthopaedics and surgical procedures, and because of its bioactivity, dissolution range, and resorption properties similar to those of natural bones, it has great potential as a biomaterial for bone implantation. Hydroxyapatite (HA) nano powders were synthesised with calcium nitrate and phosphoric acid as calcium and phosphorus precursors using the sol-gel route in this analysis. Phytochemical experiments using standard processes were carried out for the bio-synthesized nanocomposite. In order to get a better viewpoint, synthesised nanocomposite were also subjected to different characterisation tests.

Keywords: Hydroxyapatite, *Alpiniagalanga*, phytochemical analysis, characterisation studies, orthopaedics and dental implants

INTRODUCTION

In recent times, nanomaterials have created immense interest because of their promising applications in different science and technology fields. Among these, due to their properties that depend on their size and morphology, metal nanoparticles are most capable, making them a proper aspirant in multiple applications (1). For nanoparticle synthesis, several methods are available, such as chemistry, photochemistry, electrochemistry, and biology (2). Many of these routes of production include the use of hazardous chemicals which require harsh conditions of reaction. Because of its short reaction time, the chemical method of nanoparticles synthesis is still the most popular. The chemical reagents used as reducing and capping agents, however, are typically toxic in this process and contribute to environmental contamination. With a growing emphasis on green chemistry, due to

its versatility, environmentally friendly nature, and cost-effectiveness, biological synthesis of nanocomposite has recently gained more attention. For nano synthesis, plant extracts and many microorganisms have been used, such as bacteria, fungi, and yeast.

In last years many attempts have been focused on the preparation of synthetic hydroxyapatite (HA), which is quite similar to bone apatite and has excellent bone connectivity. Forming and sintering of apatite crystals at low temperatures were the main contributions of the solgel process in comparison with conventional synthesis methods for HA powder (3). Hydroxyapatite (HA) is used effectively as a material for bio implants that is like bone apatite and has good biocompatibility. A modified sol-gel process to synthesize in less than 16 hours nano crystalline hydroxyapatite (NHA) powder for biomedical applications using calcium nitrate tetra hydrate [$Ca(NO_3)2x4H_2O$] and phosphorus pentoxide [P_2O_5] as precursors, is previously presented and discussed. The results showed that there was an increase in the size of nanocrystals HA (NHA), ranging from 30 nm for the sample treated at 600 °C and up to 500 nm for the sample heat-treated at 1200°C.

A significant Ayurvedic medicinal herb belonging to the Zingiberaceae family is Alpinia galangal Wild. It is a perennial herb that is widely found in India's Western Ghats. Different therapeutic processes, such as anti-inflammatory, *antiallergic, antifungal, antibacterial and antidiabetic*, have been found to be present in the plant. In different Ayurvedic preparations, such as *Dasamoolarishtam, RasnadiChoornam, Aswagandharishtam, Balarishtam*, and so on, it is used as a constituent (4). In addition, rhizomes are used to treat rheumatism, bad breath, ulcers, infections of the throat, digestive disorders and skin diseases. Tannins and flavonoids such *as* kaempferol, galangin, and alpinine are present in the rhizome. In addition, chavicol acetate, acetyl euginol acetate, p-coumaryl alcohol, methyl cinnamate, cineole, pinene, and camphor are also found to contain *acetoxy* to some degree. Medicinal plants have medicinal properties and hence have positive pharmacological impacts on the body of the animal. Plants are the source of about 25 percent of the world's prescription medicines. Around 80 percent of people in developing countries rely on conventional plant-based drugs for their primary health care needs. There is an abundance of medicinal plants, but few of them are being studied for their biological and pharmacological activities. The broad range of medicinal plant parts such as flowers, leaves, barks, stem, fruits, root extracts are used as effective

raw drugs with a variety of pharmacological activities. *Alpiniagalanga* is the selected plant material for this study based on comprehensive literature review (5).

The sol gel method was used for synthesising nanohydroxyapatite in this study. The plant material chosen for the study was *Alpiniagalanga*. For the bio-synthesized nanocomposite, phytochemical experiments using standard processes were performed. Synthesized nanocomposite was also subjected to numerous characterization experiments in order to get a better point of view.

MATERIALS AND METHODS

Collection of plant sample

Preparation of Hydroethanolic extract from *Alpiniagalanga*

Dried *Alpiniagalanga* rhizomes were collected and powdered. 50grams of fine powder was soaked with 250ml of hydroethanol (50%) at room temperature and kept in shaker for 3-4 days. Filtered and evaporates by evaporation in the hot air oven at 25-30°C. The final part of extract was taken and stored at 5°C for further study.

Synthesis of Hydroxyapatite:

The analytical grade available from commercial sources was used for all the chemicals. 99% sigma Aldrich calcium hydroxide, 98% sigma Aldrich orthophosphoric acid. The host precursors for calcium and phosphate are calcium hydroxide and Orthophosphoric acid. Using double distilled water, 1 M of calcium hydroxide and 0.6 M of orthophosphoric acid were prepared and agitated separately for 30 minutes. To the calcium hydroxide solution, the orthophosphoric acid solution was applied in drops. The precipitated solution was vigorously stirred for an hour after inserting it. With the addition of ammonia solution drop wise, the pH of 11 was retained. The solution had been aged for 24 hrs. The precipitate was dried less than 200°C in the oven for 6 h. The product thus developed was put in the furnace for 3 hours at various temperatures ranging from 400°C to 700°C.

Phytochemical analysis

In order to detect the existence of different forms of phytoconstituents such as alkaloids , carbohydrates, flavonoids, glycosides, diterpenes, proteins, aminoacids, phenolic compounds, phytosterols, saponins and tannins, preliminary phytochemical research was carried out with sample

extracts (6). The extracts, with their respective solvents, were diluted and used for photochemical analysis.

DETECTION OF ALKALOIDS:

Test for Alkaloids were performed with three different methods **(7)**.

a)Wagner's test:

0.1ml of sample extract was treated with Wagner's reagent Iodine in potassium iodide

b)Hager's test:

0.1ml of sample extract was treated with Hager's reagent (saturated sulphuric acid solution).

c) Mayer's test:

0.1ml of sample extract was treated with few drops of 2% Hydrochloric acid.

DETECTION OF CARBOHYDRATES:

Fehling's test:

0.1ml of the extract were hydrolysed with 0.5ml of dilute HCl, neutralised with 0.5ml of alkali and added 4-5 drops of Fehling's solutions and heated in a boiling water bath for 5minutes. **(7)**

DETECTION OF FLAVONOIDS:

Alkaline reagent Test:

The 1.0 ml of extracts treated with 10% of the sodium hydroxide solution resulted in an improvement in yellow colour strength. The colour was subsequently reduced by adding a few drops of dilute Hydrochloric acid.

DETECTION OF GLYCOSIDES:

Modified Borntrager's test:

2.0ml of 10 percent ferric chloride solution was applied to 2.0ml of extracts and heated in a boiling water bath for around 5 minutes. The mixture was cooled with 4.0ml of benzene and treated with it. The ammonia solution was isolated and diluted with the benzene layer**.**

DETECTION OF DITERPENES:

Salkowski Test:

1.0ml of chloroform and 1.0ml of concentrated sulphuric acid were treated with 0.5ml of extracts, **(8)**.

DETECTION OF PHENOLS:

Lead Acetate Test:

3.0 ml of 10 percent lead acetate solution was applied to 2.0 ml of the extracts**(8).**

DETECTION OF PROTEINS:

Xanthoproteic test:

A few drops of concentrated nitric acid were used to treat 3.0 ml of the extracts.

DETECTION OF AMINOACIDS:

Ninhydrin test:

Three drops of 0.25 percent ninhydrin reagent were added to 3ml of the extracts and boiled for a few minutes.

DETECTION OF PHYTOSTEROLS:

LibermannBurchard's test:

2.0 ml of the extracts is treated with, and purified with, 2.0 ml of chloroform. The filtrate was treated, distilled, and cooled with 1.0 ml of acetic anhydride. Added 2 drops of concentrated sulphuric acid.

DETECTION OF SAPONINS:

Froth Test:

The extracts were diluted with 5.0 ml of distilled water and shaken for 15 minutes in the graduated cylinder**.**

DETECTION OF TANNINS:

Ferric Chloride Test:

1.0 ml of neutral 5 percent ferric chloride solution was applied to 2.0 ml of the extracts**(9).**

Characterization techniques

1.Fourier Transform Infrared Spectroscopy (FTIR)

Infrared Spectroscopy is a standard method of analytical pharmacy and chemistry which provides the images of vibration of atoms of compound. Therefore it is also referred to as vibrational spectroscopy. IR spectrum is obtained by passing infra-red radiation through the sample and determining what fraction of the incident radiation is absorbed at a particular frequency. Identification

and characterization of homo polymers, copolymers and polymer bends, monitoring polymerization reactions, characterization of the structural properties of polymers, including tactility, branching, crystallinity, hydrogen bonding and orientation, investigation of the surface properties of polymers and understand polymer degradation. Estimation of the secondary structure of proteins by IR is common **(10).**

2. EDX analysis

The morphology of the samples was carried out on an ultra-high vacuum and high resolution MAIA3 TESCAN scanning electron microscopic—energy dispersive X-ray analysis (SEM-EDX) operated at 5 kV. The samples were prepared by gold sputtering the surface of the samples using a low deposition rate.

3.Field Emission Scanning Electron Microscope (FESEM)

The field emission scanning electron microscope **(FE-SEM)** images a sample surface by raster scanning over it with a high energy beam of electrons. The electrons interact with the atoms comprising the sample to produce signals that contain information about surface topography, morphology, composition and other properties, such as electrical conductivity. The function of the electron gun is to provide a large and stable current in a small beam.

4. TEM analysis

The TEM images were obtained using a JEOL JEM-1200EX II electron microscope. A small amount of sample (<10 mg) was dispersed in neat methanol and then ultrasonicated for 2 min to yield a very dilute suspension. A few drops of the resulting suspension were then deposited on a carbon-coated copper grid, which was used as the TEM specimen. The grid was dried prior to use in the double-tilt holder of the TEM. Image J software (version 5.0) was used to estimate the particle size.

5.X-ray diffractometery (XRD)

Powder X-ray Diffraction **(PXRD)** techniques uses this principle to elucidate the crystalline nature of materials. The scattering of X-rays from atoms produce a diffraction pattern that contains information about the atomic arrangement in crystal. Amorphous materials like glass do not have periodic array with long-range order so; they do not produce any significant peak in diffraction pattern **(10).**

6.DLS and Zeta Potential Analysis

Zeta Potential analysis is a technique for determining the surface charge of nanoparticles in solution (colloids).

Nanoparticles have a surface charge that attracts a thin layer of ions of opposite charge to the nanoparticles surface. The electric potential at the boundary of the double layer is known as the Zeta potential of the particles and has values that typically range from +100 mV to -100 mV. The magnitude of the zeta potential is predictive of the colloidal stability. Nanoparticles with Zeta Potential values greater +25mV or less than -25 mV typically have high degrees of stability. Dispersions with a low zeta potential value will eventually aggregate due to Van Der Waal inter-particle attractions. Zeta Potential is an important tool for understanding the state of the nanoparticles surface and predicting the long term stability of the nanoparticles.

Dynamic light scattering (DLS) measurements were performed using a Malvern Instruments Zetasizer operated in backscatter (173°) mode. The sample slurry was produced with a solid content ~1% by volume and diluted with methanol. To disperse the sample, this suspension was kept in an ultrasonic bath for 10 min. Square cuvettes with a path length of nominally 10 mm were used for measurements.

RESULTS AND INTERPRETATION

Table 1: Phytochemical analysis

S.No	Phytochemicals	*Alpiniagalanga*
1	Alkaloids	+
2	Flavonoids	+
3	Terpenoids given diterpenes in methods	+
4	Tannins	+
5	Total sugars given as carbohydrate in methodology	+
6	Protein	+
7	Phenols	+
8	Saponins	+
9	Steroids given as phytosterols in methods	+
10	Glycosides	_
11	Aminoacid is given in methods not included here	

1.FOURIER TRANSFORM INFRARED SPECTROSCOPY

FTIR study The FTIR experiments were carried out to classify the different HAp-related functional groups. In the FTIR spectrum, all HAp NPs displayed the characteristic signature HAp bands. The signature HAp peaks reported at ~565cm-1 are due to the phosphate group bending modes of P-O bonds, with a contribution of ~605cm-1 from the -OH of the apatite group. The ~1031cm-1 peaks noted are due to the PO4 stretching modes. -OH bonds are due to the bands in the 3400cm-1 region and those detected close ~3571cm-1 are correlated with -OH stretching HAp vibration (11)

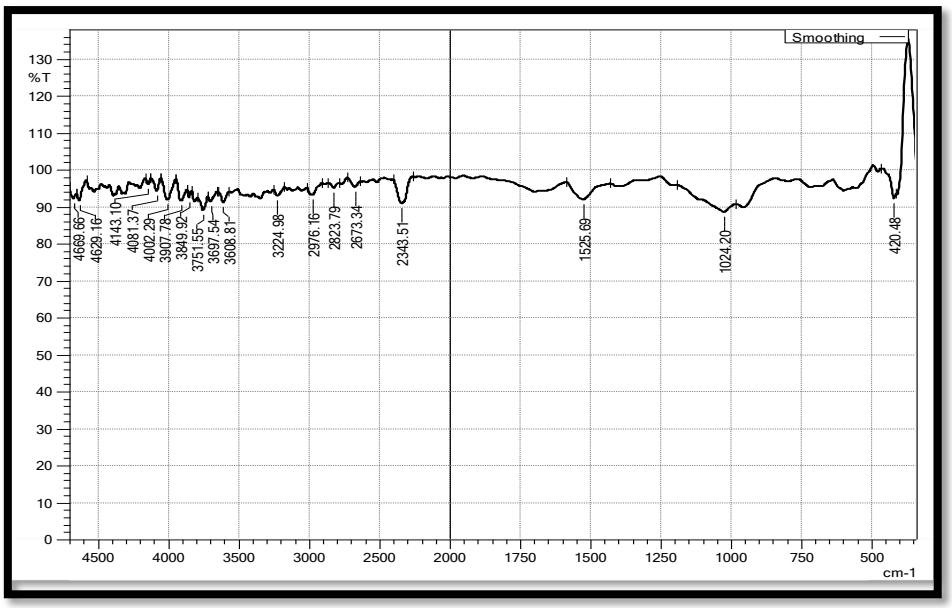

Fig 1: Fourier Transform Infrared Spectroscopy

2.EDX analysis

The X-ray energy dispersive study of all synthesised HAp is shown in (Figure 2). The elementary composition of materials is defined by EDAX analysis. SEM instruments are connected to EDAX systems. The EDAX spectrum showed a peak corresponding to calcium (Ca), phosphorous (P) and oxygen (O) for all synthesised HAp.

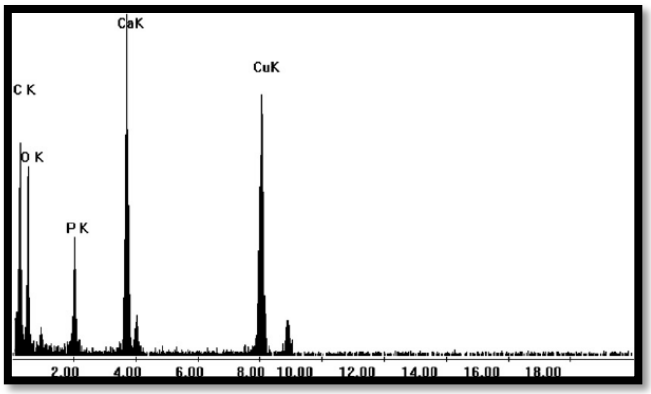

Fig 2: EDX Analysis

3.FESEM

The FE scanning electron microscope (SEM) was used for the morphological study of nanoparticles of hydroxyapatite. Figure 3 shows the FESEM images of the as-prepared hydroxyapatite nanoparticles. The hydroxyapatite nanoparticles formed were highly agglomerated. The agglomeration of the nanoparticles might be because of Ostwald ripening. The spherical shaped

particles with clumped distributions are visible from the FESEM analysis. The FESEM images show the spherical shaped particles as confirmed by Ferraz et al. (2004) for reported results of hydroxyapatite nanoparticles.

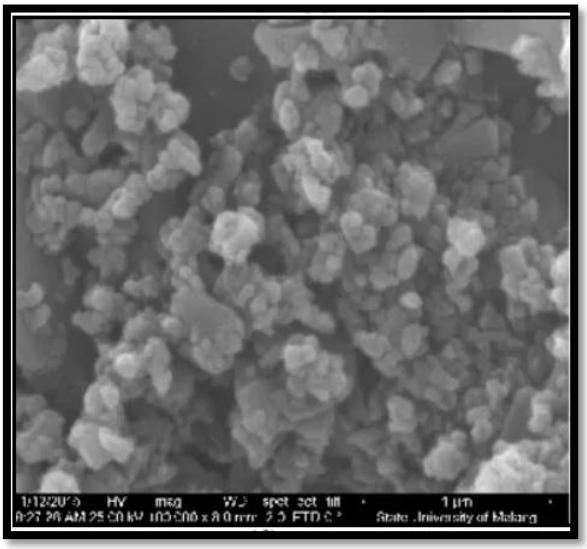

Fig 3: EDX Analysis

4.TEM analysis

Morphologies of samples were confirmed using TEM analysis. Depending on the conditions used in the reaction mixture, the newly formed particles exhibited rod morphology with various aspect ratios. The shape changed to short rods with an average length 35nm and an average diameter 7nm when DMSO was used as a solvent keeping TEA as the stabilizing agent (Figure 4). Thin rods of average length 62.5nm and diameter 6.2nm were obtained when the stabilizing agent was changed to ACA while maintaining DMSO as the solvent. When water was used as the solvent and ACA as the stabilizing agent, the particles obtained were irregular shaped with mixed morphologies

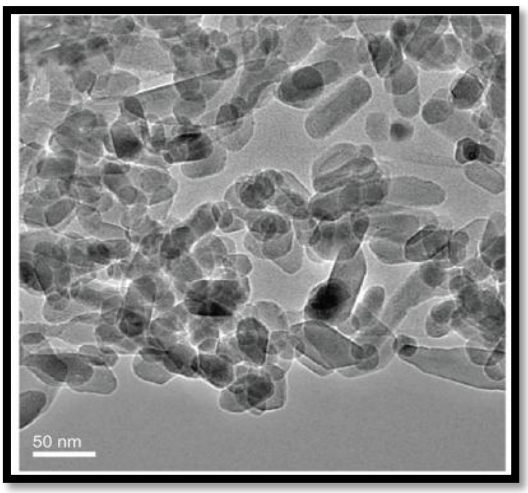

Fig 4: TEM Analysis

5.XRD analysis

Using XRD analysis, the phase purity and crystallinity of samples were determined. Of both the synthesised HAp and commercially purchased sigma control HAp, the XRD spectra corresponded well to the hexagonal HAp crystal (ICDD 09-0432). All the HAp characteristic peaks corresponding to the [hkl] lattice of [002], [211] and [300] at 25.8°, 31.34° and 33.97° were observed in the spectra, respectively. As well as ACA as a stabilising agent and water as a solvent, the sample prepared without a stabilising agent showed ßTCP peaks along with HAp peaks. In each spectrum, the relative intensities of characteristic HAp peaks ([002], [211] and [300]) vary, suggesting the possibility of difference in growth orientation under different conditions of synthesis. Similar results were found by few researchers where differences in peak intensities were due to variations in the conditions of synthesis such as pH, reaction time, and temperatures and time of calcination (12). Table 2 presents the crystallite size measured for [002] and [300] lattice, crystallinity and cell unit parameters of commercially available HAp and synthesised HAp. HAp synthesised with ACA and DMSO was lower, while HAp synthesised with water and TEA showed greater crystallinity and crystallite size.

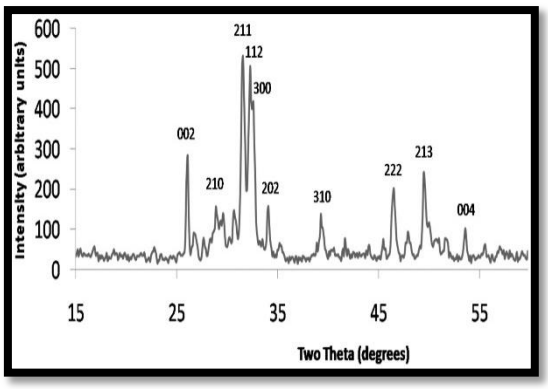

Fig 5: XRD Analysis

Table 2: Crystallite size, crystalline and unit cell parameters of fabricated cells

HAp samples synthesized with various conditions		Crystalline Size Xp (nm)		Crystallinity Xs	Unit cell parameters	
Solvent	**Stabilizing**	**[002]**	**[300]**		**a(A)**	**C(A)**
Water	TEA	37.9	25.22	0.890	9.97	6.93
DMSO	TEA	32.0	19.70	0.753	9.67	6.84
DMSO	ACA	28.8	14.00	0.774	9.58	6.80
Water	ACA	35.0	24.00	0.915	9.72	6.95
Water	NA	31.1	18.60	0.732	9.83	6.88
Commercially available HAp		30.3	17.03	0.872	9.72	6.90

6.DLS and Zeta potential measurement

To assess surface charge, Zeta potential analysis of all the synthesised HAp was performed. For thin rod, short rod, rod, mixed and irregular morphology and microrod-shaped HAps, Zeta potential values were respectively 13.25, 3.85, 2.12 6.92 and 4.37 mv. As ACA was used as a stabilising agent, the Zeta potential value of HAp NPs displayed greater positive value, and this could be due to the acidic nature of the positive surface charge contributing to ACA. Similar findings were recorded by Berg et al, in which the effect of pH on zeta potential was tested using TiO2, Fe2O3, Al2O3, ZnO, and CeO2 NPs, and the positive zeta potential increased with a decrease in pH.

CONCLUSION

The present study concluded that the extract of *Alpiniagalanga* Hydroxyapatite nanocomposites can be synthesised rapidly using the extract of *Alpiniagalanga* green. These nanocomposites were cheap, non-toxic and eco-friendly, displaying rod structures with an average size of 41 nm. The results of this study have demonstrated a wide range of notable future applications of nanocomposites in medical fields, especially in orthopaedics.

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