Current Trends in Biotechnology and Pharmacy
Vol. 18 (Supplementry Issue 4A) 77 - 87, November 2024, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2024.4s.7

Synthesis of phyto-hydroxyapatite using *Ocimum sanctum* and its characterization

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Abstract

Hydroxyapatite is a mineral naturally occurring in the form of calcium apatite which is important to make biocompatible bone grafts, dental implants and dental fillings. The objective of the study is to synthesize a herbal hydroxyapatite using *Ocimum sanctum* (Basil) leaf aqueous extract ofto impart the anti-inflammatory, anti-oxidant and anti-microbial properties to hydroxyapatite to improve its applications. The formed phyto-hydroxyapatite (phyto-HAP) was subjected to various characterization using Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and X-ray diffraction (XRD) studies. The aqueous leaf extract of *Ocimum sanctum* was subjected to anti-microbial, anti-oxidant and anti-inflammatory activity. The results showed that the synthesized phyto-hydroxyapatite has the crystalline structure similar to natural hydroxyapatite with the bioactive compounds of *Ocimum sanctum* which could make HAP a more effective material to be used in dentistry as filling materials, as herbal bone grafts, as composite herbal dental grafts, as coating material for dental implants, in orthopaedics and in other biomedical applications.

Keywords: *Ocimum sanctum*, Phyto hydroxyapatite, Hydroxyapatite, Herbal Bone graft, Dental implant, Basil leaf, Dental filling material.

Introduction

Hydroxyapatite (HAP) is a versatile biomaterial explored by the researchers worldwide in making bone implants. Because of its ability to differentiate osteoblast, being biocompatible, ability to bond with osseous tissues, and natural occurrence in the human body, HAP has been widely applied in areas of dentistry and tissue engineering.This property of HAP promotes its application in various biomedical fields(1, 2).

When HAP is incorporated in scaffolds, shows excellent osteoconduction by integrating with bone without eliciting an immune response (3).Although synthetic HAP shows great promise in bone tissue engineering, its low fracture toughness, brittleness, and weak mechanical strength make it unsuitable for load-carrying applications (4-7). Moreover, there is a possibility of infection occurring in the implant site as HAP doesn't exhibit antimicrobial property by its own (8). To overcome this, researchers showed great interest in designing and developing biomaterials with the infusion of indigenous herbs which indeed stabilizes the composition and property of the biomaterials through its phytochemicals without inducing any side effects to the host and also imparting antimicrobial and anti-inflammatory properties to HAP. Herbal biomaterials have gained momentum in orthopedic requirements, biomedical applications and also in dentistry. Dental implants are at the

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risk of failure due to deposits of oral pathogens. Attempts are made by the researchers to develop coating materials for preventing biofilm formation on dental implants by using zinc oxide coated hydroxyapatite (9). This gave the idea of synthesizing hydroxyapatite with herbs having antimicrobial property which on coating with dental implants or prepared as dental or bone grafts can prevent infection in the implant site.

Indigenous herbs with various therapeutic purposes are abundant in India which can be used as medication sources in a conjugated form (10). Phenols, Tannins, Saponins, and Flavonoids are the significant bioactive compounds found in medicinal plants imparting the therapeutic value to the plant (11). Among these, *Ocimum sanctum* (Holy basil), a well-known traditional herb for many years in India belongs to the family Labiateae, used in treating headaches, common colds, inflammation, heart disease, stomach disorders, various forms of poisoning and malaria (12,13). Holy basil or Tulasi possess a strong aroma with an astringent taste and also considered as the queen of herbs (14). Several research articles showed that *Ocimum sanctum L* is a promising herb containing antioxidant, antiarthritic, antimicrobial (15, 16), anti-inflammatory, anticancer and antistress properties. Kalaiselvi et al used *Moringa oliefera* flower extract and capped with hydroxyapatite to form nano rods. The herbal hydroxyapatite nano rod was characterized and the results supported that, the inclusion of the plant extract favours in stabilizing the hydroxyapatite (17). Based on this, the objective of this study is to synthesize hydroxyapatite with the phytochemicals of *Ocimum sanctum* an insitu method to incorporate the bioactive compounds of *Ocimum* into hydroxyapatite and to characterize theirstructural, morphological and other biological activities namely antimicrobial, antioxidant and anti-inflammatory activities. This novel phyto-HAP with the biological activities of *Ocimum sanctum* can be applied in the field of dentistry, dental implant coating, bone grafts and in orthopedic applications.

Materials and Methods

Chemicals

Ethanol of 95% purity, calcium nitrate tetra hydrate, Di-ammonium Hydrogen Phosphate, and ammonia were purchased from Sigma Aldrich.

Preparation of *Ocimum sanctum* leaf aqueous Extract

2.0 g of *Ocimum sanctum* leaf was collected and rinsed with deionized water. Then it was subjected to boiling in deionized water for 10 min and filtered. The obtained *Ocimum sanctum* aqueous filtrate was used for the synthesis of Phyto-HAP.

In situ synthesis of phyto hydroxyapatite

On modifying the approach employed by Nayar et al (18), 30 ml of the *Ocimum sanctum* aqueous filtrate was taken and mixed with350 ml of 0.4 M alkaline calcium nitrate tetra hydrate. The mixture was kept for incubation at 30°C for 24 h. The resulting solution was added to 400 ml of 0.156 M alkaline diammonium hydrogen phosphate salt solution. The reaction mixture was stirred for 7 days at an ambient temperature of 30 ±0.5 °C. After ageing, the precipitate was collected, rinsed with de-ionized water and dried at 80°C.

The obtained phyto-HAP was subjected to Fourier transform infrared (FT-IR) spectra were performed by pelleting the phyto-HAP with KBr (Nicolet 400) and the peaks were obtained in the region 400– 4000 cm^{-1}. X-ray diffraction (XRD) studies for phyto-HAP was done usingThermofisher ARL Equinox 3000 with Cu Kα radiation running at 45 kV and 40 mA with an angular 2θ range of 20–80° and a sampling interval of 0.002°. Scanning electron microscopy (SEM) studies were performed using JOEL by mounting the sample on a copper disc and images were recorded at 20 kV.

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Antimicrobial activity

Media and inoculum

To perform antimicrobial activity, bacterial strains namely *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 67120 and for fungus *Candida albicans* (ATCC 10231) were used in this study. Müller-Hinton agar (MHA) was used in cultivating the bacteria at 35°C for 24 h. For fungi cultivation, Sabouraud dextrose agar (SDA) was used.

Preparation of Sabouraud Dextrose Agar (SDA)

6.5 g of the medium should be dissolved in 100 ml of purified water. For the medium to fully dissolve, heat it while stirring often and bring it to a boil for one minute. The content should be 15 minutes autoclaved and cooled. Place petri plates with the sterile agar in them.

Preparation of Mueller Hinton Agar (MHA)

15.2 g of the medium should be dissolved in 400 ml of purified water. For the medium to fully dissolve, heat it while stirring often and bring it to a boil for one minute. Autoclave the contents for 15 minutes at 121 °C, then let them cool to room temperature. Mueller Hinton Agar should be poured onto sterilized petri plates on a level, horizontal surface at a constant depth. *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Klebsiella pneumoniae*, and *Candida albicans* are the chosen test microorganisms.

Well Diffusion Method

The antibacterial activity was assessed using the agar well-diffusion method. On Sabouraud Dextrose Agar (SDA) and Mueller Hinton Agar (MHA) plates, 8-hour-old broth cultures of the appropriate fungi and bacteria were swabbed respectively using sterile cotton swabs. Using a sterile cork borer, wells (10 mm in diameter and about 2 cm apart) were drilled into each of these plates. *Ocimum sanctum* extract stock solution was created at a concentration of 1 mg/ml. The wells received around 100 µl of the extract, which was added, and was left to diffuse at room temperature for two hours. They set up control trials using inoculums devoid of plant extract. For bacterial pathogens, the plates were maintained at 37°C for 18–24 h, and for fungal pathogens, the temperature was maintained at 28°C for 48 h. The inhibitory zone's diameter (in mm) and activity index were both determined (19).

***In vitro* Anti Oxidant Activity**

DPPH radical scavenging activity

According to the methodology of Braca et al, the scavenging activity for DPPH free radicals was measured (20). A mixture of 0.1 ml of plant extract/ascorbic acid in various doses (50 – 500 µg) and 3 ml of 0.004% DPPH solution in ethanol was made. Here, different concentrations of ascorbic acid (50 – 500 µg) was taken as a positive control standard. The mixture was well mixed and given 30 minutes to stabilize at room temperature. The absorbance at 517 nm was measured in order to quantify the decolorization of DPPH. The plant extract was substituted with 0.1 ml of ascorbic acid so as to make positive control. By contrasting the absorbance values of the experimental and control tubes, it was possible to calculate the proportion of DPPH radicals that the extract/compound successfully inhibited (21).

$$\text{DPPH Scavenged (\%)} = \frac{\text{(A cont - A test)}}{\text{A cont}} \times 100$$

Where A test is the absorbance for the presence of the sample in the extracts and A cont is the absorbance of the control reaction. The extract's antioxidant activity was quantified as IC_{50}. The amount of extract (measured in µg/ml) needed to inhibit the production of DPPH radicals by 50% is known as the IC_{50} value. The inhibition curve was reported as a percentage of mean inhibition divided by the standard deviation and plotted for repeated experiments. SPSS 2022 One Way Anova was used to plot the different concentrations of DPPH radical-

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scavenging activity (%) against *Ocimum sanctum* extract concentration (µg/ml) which will determine the IC_{50} value of each extract.

Anti-inflammatory activity

Protein Denaturation method

The protein denaturation approach was used to demonstrate anti-arthritic and anti-inflammatory efficacy ***In vitro***. Using this procedure, 0.05 ml of *Ocimum extract* (50–250 µg/ml) was combined with 0.45 ml of 5% bovine serum albumin in distilled water. The reaction mixture's pH was brought down to 6.3 by adding 1N hydrochloric acid. The mixture was heated to 57˚C for three minutes after being incubated for twenty minutes at 37 °C. After cooling the mixture, 2.5 milliliters of phosphate buffer were added. 0.05 ml of distilled water was taken asnegative control by replacing plant extract, and for product control bovine serum albumin was substituted with distilled water (22-24). At 600 nm, the reaction mixture's turbidity was measured.200 µg of diclofenac sodium was taken as standard. Protein denaturation inhibition percentage was calculated as follows,

Percentage Inhibition = 100 – (Test OD – Product Control OD/ Control OD) x 100

100% protein denaturation is considered as negative control. 200 µg/mlof Diclofenac sodium is taken as a positive control and the results were calculated.

Statistical Analysis

The antimicrobial, anti-inflammatory, and DPPH activity findings were shown as the mean±standard deviation of three independent tests (n = 3). Using the statistical software program SPSS, version 22.0, a one-way analysis of variance ($p<0.05$) was performed by comparing the means and then Duncan's multiple range analysis.

Results and Discussion

The present study aims to synthesize phyto-hydroxyapatite using *Ocimum sanctum* and characterize it using FTIR, XRD, SEM EDX, Antioxidant, Anti-inflammatory and anti microbial properties. While researchers have made various attempts in making different biomaterial, infusion of medicinal herbs in biomaterial makes it more versatile in its applications. With substantial scientific evidence, the herbal biomaterials can be applied not only in basic or applied research, instead it can be taken to clinical research.

Herbs possessing both antioxidant, anti microbial and anti-inflammatory properties like *Ocimum sanctum* benefits the biomaterials to overcome the inflammatory response and infection in the implant site. This was demonstrated by Santin et al in his study by using soy bean extract in bone fillers and showed that the extract with antioxidant and anti-inflammatory properties reduces the inflammatory response in the implant site and promotes the osteoblast differentiation of the bone fillers in invivo models (25).

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of Phyto-HAP was shown in Figure 1. The phosphate bandsv3 PO_4^{3-} were observed at 670 cm^{-1} and874.1 cm^{-1}. v1 phosphate band was observed at 963.5 cm^{-1}, 1019 $cm^{-1}$and 1088.4 cm^{-1}which attributes the anti-symmetric bending motion in Phyto-HAP. The peak at 872.2 cm^{-1}, also indicates the CO_3^{2-} stretching band in Phyto-HAP. 3379 $cm^{-1}$and 1638 cm^{-1} peak values corresponds to the phenolic group and the amide group of *Ocimum sanctum* which is due to the presence of euginolin *Ocimum sanctum* (26). The peak at 1422 cm^{-1} shows the presence of aliphatic amines of *Ocimum sanctum* (27). The bands reveal that, the prepared Phyto-HAP contains both the chemical compounds of *Ocimum sanctum* and Hydroxyapatite. Kumar et al prepared a bone graft using hydroxyapatite with the herb *Cassia occidentalis* and characterized it invitro using FTIR, in which the peak values obtained for hydroxyapatite were similar to our findings (28). Moreover, the phenols and amines of *Ocimum sanctum* imparts antimicrobial and anti-inflammatory property

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to Phyto-HAP and which may support thephyto-HAP to overcome the inflammation and infection in the implant site, when it is introduced in a bone graft or a dental implant.

X ray Diffraction Analysis

Figure 2 shows the X-ray diffraction (XRD) pattern of phyto-HAP which shows the presence of peaks at 25.92º, 32.13º, 39.67º, 46.76º, 49.53º, 53.12, 64.07º, 77.17º, and 87.80º indicates the reflection from 002, 210, 300, 130, 222, 321, 511, 513, and 244 crystal planes, respectively, which were compared with JCPDS (740566) data and it clearly indicates the presence of phyto-HAP. The results of XRD analysis obtained in the present investigation are in good agreement with the reported results (29). The results

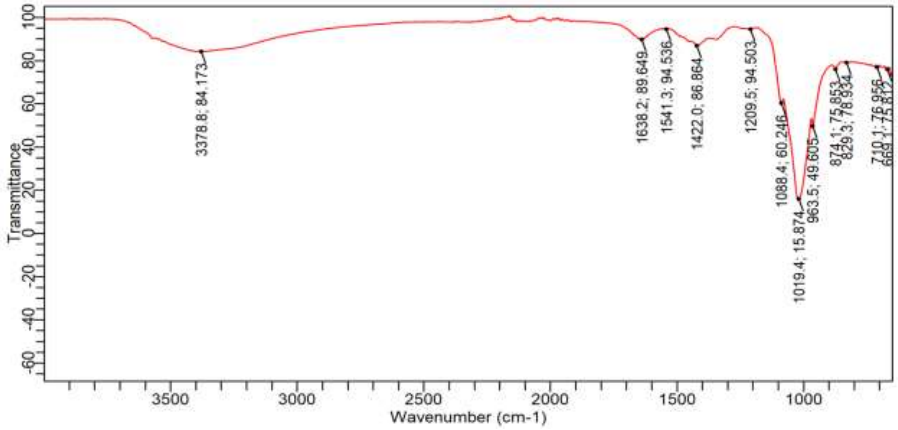

Figure 1: Shows the FTIR pattern of Phyto-hydroxyapatite (Phyto-HAP)

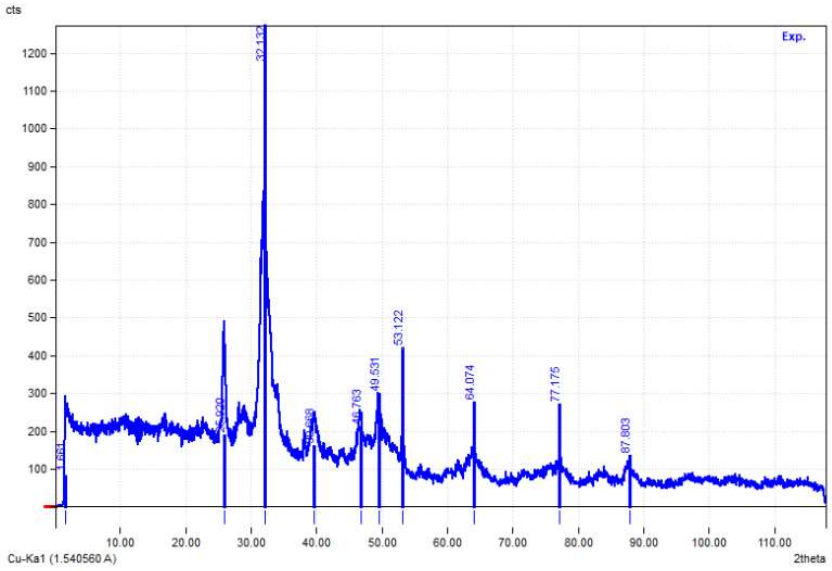

Figure 2: Shows the XRD pattern of Phyto-hydroxyapatite

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obtained in our study are in accordance with the study conducted by Rabiei et al who did a comparative analysis on the crystal structure of hydroxyapatite through XRD analysis and showed the reflections obtained for hydroxyapatite (30). These results supports that the prepared phyto-HAP is similar to natural hydroxyapatite and could be used in coatings of dental implants or can be incorporated in bone grafts.

Scanning Electron Microscopy

The Figure 3 shows the SEM image of phyto hydroxyapatite. The image shows white crystalline deposits of hydroxyapatite along with the phyto chemicals of *Ocimum sanctum*. The standard EDX spectra reveals the calcium and phosphate presence with a ratio of 1.66 which corresponds to the living bone ratio of 1.67(31). Our previous study on the bone graft prepared with the incorporation of *Ormocarpum sennoides*, exhibited a stoichiometric ratio of 1.66 for hydroxyapatite which showed an effective ossification property with the anti-inflammatory properties of the incorporated herb *Ormocarpum sennoides* (32). Similarly, Chandrasekar et al also demonstrated that the synthesized nano hydroxyapatite showed a Ca/P ratio of 1.68 which was so close to our study findings (33).

Anti microbial activity

The current study assessed the antibacterial activity of an aqueous extract of *Ocimum sanctum* leaves against a variety of Gram positive and Gram negative bacteria as well as a fungus that was thought to be a human pathogenic microbe. Using the agar well diffusion method, the plant extract's susceptibility was evaluated. According to Table 1, our initial research revealed that *Ocimum sanctum* aqueous leaf extract with a concentration of 100 mg/l was effective against human pathogens that were isolated locally, including *E. Coli and Staphylococcus aureus* each with a zone of inhibition 19mmand for, *Klebsiella species*the resistance zone was 16 mm, and for *Pseudomonas aeruginosa*

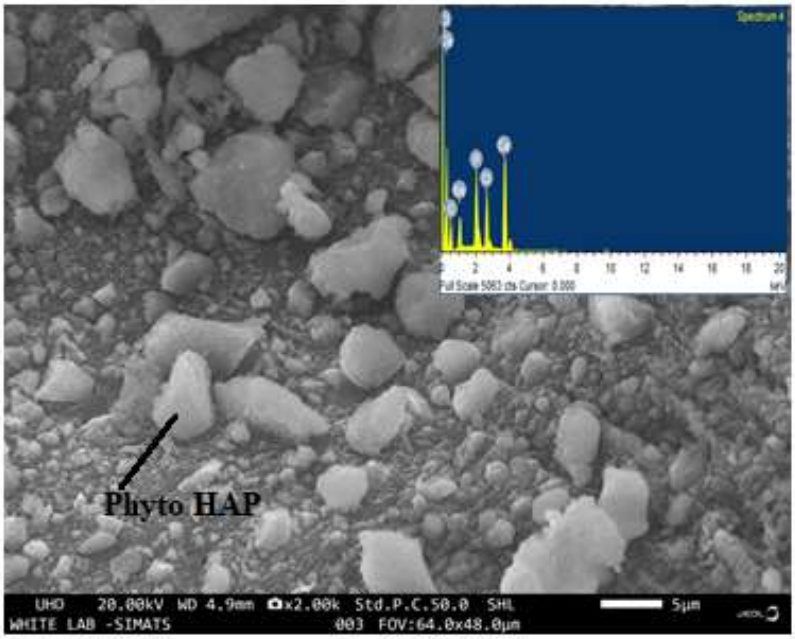

Figure 3: Shows the SEM image of Phyto-hydroxyapatite with EDX

Phyto-Hydroxyapatite Using *Ocimum Sanctum*

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21 mm was exhibited. There were no zone formation for *Candida albicans* which was in accordance with the study conducted by Khan et al who reported that the aqueous extract of *Ocimum sanctum* showed no inhibition against candida albicans (34). The findings of this study are in accordance with the study performed by Ashish Ranjan Singh et al who has reported anti-microbial activity for aqueous, methanolic and ethanolic extracts of *Ocimum sanctum* (35). Hence, the antimicrobial property of *Ocimum sanctum* may be imparted to hydroxyapatite which help to overcome the infections at the implant site or can prevent any biofilm formation on dental implants or bone grafts.

Antioxidant Assay

DPPH Radical Scavenging Activity

At varying doses, *Ocimum sanctum* demonstrated efficient scavenging of the free radicals in a dose-dependent manner. The DPPH free radical scavenging activity is a widely recognized model for mitigating lipid oxidation (36). It was previously believed that antioxidants' capacity to donate hydrogen was the reason behind their impact on DPPH radical scavenging (37). The aqueous extract of *Ocimum sanctum* at varying concentration 50, 100, 200, 300, 400 and 500 µg/ml exhibited 71.15%, 75.59%, 81.11%, 83.57%, 85.19% and 91.76% inhibition respectively. The standard drug ascorbic acid at the same dosage showed 80.9%, 89.64%, 92.73%, 95.58%, 101.62% and 102.81% inhibition respectively. The IC_{50} value for *Ocimum sanctum* was found to be 35.13 µg/ml and for Standard ascorbic acid was 30.9 µg/ml Table 2. The aqueous extract of *Ocimum sanctum* was able to scavenge the free radicals significantly when compared with standard ascorbic acid.Gupta et al studied the antioxidant activity of ethanolic-water extract of *Ocimum sanctum*

Table 1: Shows the antimicrobial activity of *Ocimum sanctum*

S. No	Selected Pathogens	Zone of Inhibition in millimeter (mm)
1	*Escherichia coli*	19 ± 0.5
2	*Candida albicans*	No Zone
3	*Klebsiella sp*	16 ± 0.4
4	*Staphylococus aureus*	19 ± 0.5
5	*Pseudomonas aeruginosa*	21± 0.4
The experiments were performed in triplicates and the values are expressed as Mean ± SEM		

Table 2: Shows the antioxidant activity of *Ocimum sanctum*

Concentration (µg/ml)	DPPH inhibition % of Positive control-Ascorbic acid	DPPH inhibition % of*Ocimum sanctum*
50	80.9 ±2.38	71.15±1.24
100	89.64±2.21	75.59±1.21
200	92.73±0.35	81.11±1.35
300	95.58±1.65	83.57±1.36
400	101.62±2.69	85.19±1.45
500	102.81±2.41	91.76±1.58
IC_{50} (µg/ml)	35.13 µg/ml	30.9 µg/ml

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Table 3: Shows the anti-inflammatory activity of *Ocimum sanctum*

S. No	Conc. (µg/ml)	*Ocimum sanctum* Extract (% inhibition of protein denaturation) (%)	Diclofenac Sodium (% inhibition of protein denaturation) (%)
1	50	23.82 ± 2.06	
2	100	37.62 ± 2.17	
3	200	49.93 ± 1.77	87.69 ± 1.0
4	300	57.62 ±2.34	
5	400	62.71 ±2.21	
6	500	70.37 ± 1.63	
7	1000	77.12 ± 1.54	

The experiments were performed in triplicates and the values are expressed as Mean ± SEM. Diclofenac sodium was used as positive control.

and reported an IC_{50} value of the extract as 34.21 µg/ml. the results are matching with our study (38)

Anti-inflammatory Activity

Denaturation of protein is usually caused by inflammation. Non-steroidal Anti-inflammatory drugs like Diclofenac sodium are considered as good anti-inflammatory drug but in long term it induces side effects in the body (39). As an alternative, traditional herbs like Tulsi can be a suitable substitute for Diclofenac sodium as many researchers have studied about its anti-inflammatory property. Surender singh et al studied about the anti-inflammatory and anti-arthritic activity of *Ocimum sanctum* and found that it significantly reduced the inflammatory edema in rats (40). In this study, *Ocimum sanctum* extract was tested for anti-inflammatory efficacy *In vitro* at different doses inhibiting bovine serum albumin. A dose-dependent rise in percentage inhibition was observed in *Ocimum sanctum* extract at concentrations between 50 and 1000 µg/ml, indicating the extract's potential to prevent denaturation of albumin. These results were comparable to those of diclofenac sodium (200 µg/ml) as mentioned in Table 3. At 50 µg/ml, *Ocimum sanctum* extract was able to prevent denaturation by 23.82%. *Ocimum* extract progressively raised its percentage of denaturation inhibition in a dose-dependent way. The extract was preventing denaturation by 77.12% at 1000 µg/ml, which was almost identical to the 200 µg/ml denaturation inhibition caused by diclofenac sodium. *Ocimum sanctum* extract's anti-inflammatory properties may be attributable to the extract's bioactive compound octadecenoic acid and hexadecenoic acid (24)

Conclusion

The infusion of native herbs in biomaterial makes it a versatile composite for its utilization in various biomedical applications. In this study, the bioactive compounds of *Ocimum sanctum* (Holy basil) leaves were incorporated in the synthesis of hydroxyapatite by an insitu method. The various characterization of the phyto-HAP reveals the inclusion of bioactive compounds of basil leaves and the chemical composition of Hydroxyapatite. Moreover, this combination can help to achieve osteoconduction with the anti-inflammatory, anti-microbial and antioxidant properties of the basil leaves.

Acknowledgement

I would like to acknowledge the management of Sathyabama Dental College & Hospital and SRM Dental College and

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10.5530/ctbp.2024.4s.7

Hospital, Ramapuram campus for providing great support in completing this research work.

Conflicts of Interest

The authors declare no conflict of interest.

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