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FUNCTIONALIZATION OF NATURAL HYDROXYAPATITE BY CLOVE OIL: AN ECONOMIC APPROACH FOR PREPARATION OF INNOVATIVE DENTAL IMPLANT

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ABSTRACT

This research investigation has been designed to synthesize novel clove oil coated natural hydroxyapatite composite particles using residual bones. The bone waste was consumed for extraction of natural bone precursor hydroxyapatite. This work was executed to investigate the benefits of using essential clove oil coated hydroxyapatite composite in dentistry, especially for its preventive, restorative and regenerative applications**.** The clove oil coated natural hydroxyapatite composite particles were characterized using standard physicochemical characterization viz. XRD, SEM, FTIR, TEM and EDX. The thermal stability of clove oil coated natural hydroxyapatite composite particles was ascertained by TGA-DTA analysis. Moreover, the clove oil coated natural hydroxyapatite composite particles were tested for the antimicrobial activities using preferred Gram positive (*S. aureus* ATCC 29213 and *S. epidermidis* ATCC 12228) and Gram negative (*E. coli* ATCC 35218 and *K. pneumoniae* ATCC 700603) bacteria. The antifungal activity was tested using *C. albicans* ATCC 10231 as model organism. The clove oil functionalized hydroxyapatite composite particles material depicted broad spectrum antimicrobial potential which strongly recommends their use as material of choice for potentialdental implants.

Keywords: Cow bones, Hydroxyapatite, Clove oil, Composite, antimicrobial, antifungal

1. INTRODUCTION

Apatite is the main mineral component in bones and teeth; hydroxyapatite (HAP) is most dominant component among apatite in human tissues. HAP is one of the most extensively used materials as bone substitute [1]. Hydroxyapatite preferred as better material than other calcium phosphates due to the reason that its mineral composition matches with the teeth and bone [2]. Hydroxyapatite is considered as one of the best implant materials because it is easy to synthesize, compatible with humane cell and has best stability at biophysical temperature among salts of calcium phosphate. Hydroxyapatite exhibit good bonding with muscles, skin and bone tissues [3, 4].

Consequently, hydroxyapatite has frequently been employed as a bioactive substance to replace the synthetic bone since it is biocompatible, bear chemical and generic likeness with bony tissue. When hydroxyapatite get in touch with physiological fluids, apatite formation occurs, this ultimately creates fresh bones [5]. In human teeth, mainly the enamel is the mineralized tissue which possesses approximately 96 wt% inorganic materials; 4 wt% organic materials and water. Furthermore, in dentin inorganic material correspond to 70 wt% and the inorganic materials are mostly comprised of calcium phosphate linked to hexagonal hydroxyapatite [chemical formula is $Ca_{10}(PO_4)_6{\cdot}2(OH)$] with Ca/P molar ratio of 1.67. In other words, the, hydroxyapatite is key constituent of enamel, form bright white surface and abolishes the diffuse reflectivity of light by means of pores closure at exterior part of enamel [3, 6]. It plays vital role for the re-mineralization of de-mineralized enamel region. Considering the astounding properties of hydroxyapatite, the objective of this study was to isolate hydroxyapatite from the waste bones of cows and to prepare organic-inorganic composite using easily available economical precursors. In recent times, composites, consisting of calciumphosphate encompass a great deal of consideration; for instance, hydroxyapatite is over and over again employed as a bone implant and is also utilized to build up different composites for sophisticated biomedical equipment [7]. This extracted hydroxyapatite was further used to synthesize clove oil functionalized hydroxyapatite composite particles. The essential oil of dried flower buds of clove has extensively been utilized as antimicrobial, antioxidant, antifungal and antiviral material. In addition, clove oil is loaded with anti-inflammatory, cytotoxic, insect repellent and anesthetic activities

(mainly due to presence of Eugenol) also [5]. Customarily, it has been incorporated in dental care system as an antiseptic and analgesic material; in order to cure toothache. Furthermore eugenol is also being exploited as local anesthetic material in dentistry and as additional supplement in dental cement for temporary fillings [5]. Keeping in view the fascinating properties of clove oil and hydroxyapatite; the present work made the use of bone waste hydroxyapatite and clove oil to fabricate clove oil functionalized hydroxyapatite composite particles as innovative, inexpensive dental care material.

2. Experimental

2.1 Synthesis of natural HAP by simple thermal decomposition method

Natural bones were collected from cows, diced in small parts and washed enough to eliminate any impurity. The pristine bones were immersed in acetone and ethanol for 30 minutes to remove the collagens, fats and superfluous contaminations. The samples were transferred to dry oven for 24 at 160 ºC. Furthermore, the crushed samples was kept in a crucible and shifted to the muffle furnace (Barnstead Thermolyne 47900) at different heating temperature such as 200, 400, 500, 600, 700 ^{o}C for 5 h at scan rate of 10 ºC/min. The obtained calcined samples were once again compressed into fine powder using mortar and pestle.

2.2 Synthesis of Hap-clove oil composite particles by simple dip coating method

The functionalization of the synthesized HAP was done by dip coating method .In brief; physical mixing of hydroxyapatite powder with natural clove oil was done. First, 1 g of HAP powder was homogeneously mixed in 1 ml of ethanol. Then 0.25 ml of essential clove oil was added to the HAP powder and mixed well with mortar and pestle for homogeneous mixing.

2.3 Characterization techniques

The pristine and composite materials were characterized using modern physico-chemical techniques. The samples were characterized for crystallinity and phase content by X-Ray Diffraction (XRD, Rigaku, Japan) with Cu Kα radiation over Bragg angle ranging from 15 to 70º.Surface morphology of hydroxyapatite was examined by Scanning Electron Microscopy (SEM, Topcon ABT-32). For the elemental analysis the SEM was equipped with an energy dispersive X-ray attachment (EDX). The elemental composition was identified by EDX.

The analysis of different functional groups present in the pure and composite samples was analyzed by Fourier

Transform Infrared Spectroscopy (FTIR, Bruker Alpha-Atunated Spectrophotometer) from the wavenumber 400 to 4000 cm^{-1}. The thermal behavior of the dried cow bone was examined using thermal gravimetric analysis (TGA) and differential thermal analysis (DTA) with Netzsch STA 449F3 analyzer from an ambient temperature to 1000 ºC in air environment. Initial weight of the sample taken was 15 mg and calcined at scan rate of 20 $^oC\ min^{-1}$.Detailed morphology of the synthesized samples was also analyzed by Tunneling Electron Microscopy (TEM,H-9500, 300 kV). It has stable high voltage power supply and allows ultra-high resolution imaging.

Antimicrobial activity

The antibacterial activity of clove oil coated hydroxyapatite composite particles has been tested in liquid broth following *Amna* et al (2011) method [8]. The bacterial strains were cultured (10^6 CFU) with different concentration of the samples. Double dilution method has been applied to test out the minimum inhibitory concentration (MIC) of the clove oil coated natural hydroxyapatite composite particles. The concentrations used in this study were 100 mg, 50 mg, 25 mg and 12.5mg and 6.25 mg/tube respectively. The growth kinetics was monitored at every 4 h by checking the OD with spectrophotometer in order to verify the MIC. A constant incubation temperature of 37 °C has been kept and the rpm of 150 was maintained in a rotary shaker. The entire incubation period to monitor the growth inhibition in presence of clove oil coated natural hydroxyapatite composite particles was 16 h. The change in absorbance was calculated at 600 nm by Ultra violet (UV) spectrophotometer. The Gram positive and Gram negative strains were procured from American Type Culture Collection (ATCC). The representative Gram positive bacteria were *Staphylococcus aureus* ATCC 29213 and *Staphylococcu epidermidis* ATCC 12228. Likewise the clove oil coated hydroxyapatite composite particles were also tested against representative Gram negative strains such as *Escherichia coli* ATCC 35218 and *Klebsiella pneumoniae* ATCC 700603.

To test the antifungal activity of clove oil coated hydroxyapatite composite particles, *Candida albicans* ATCC 10231 was used in the present research. The *Candida albicans* ATCC 10231 fungus was cultured and maintained in the Sabouraud's dextrose agar. Briefly, after spreading the plates with representative fungus, the inoculated plates were kept in incubator for 30 minutes for incubation. For the fungal culture the growth was monitored after 72 h

incubation period. The experiments were executed in set of three.

3. RESULTS AND DISCUSSION

We used XRD to define the crystal structure of synthesized sample. Figure 1demonstrates the XRD spectra of synthesized pure HAP and clove oil coated HAP particles. All the observed XRD peaks of the pure HAP could be indexed to single phase hexagonal structure of hydroxyapatite (JCPDS No. 82-1943) [9]. Sharp peaks were observed for calcined samples (at 700 ºC for 5 h) suggesting higher degree of crystallinity due to the decomposition of organic component of cow bones at elevated temperature (Fig. 1a). No impurities like hydroxide and phosphates of calcium were noticed, which indicates the formation of pure hydroxyapatite. Whereas, in XRD spectra of composite, the result demonstrated that the addition of clove oil has no distinct influence in crystal structure of clove oil coated hydroxyapatite composite particles, except the peak intensity of composite samples decreased (Fig. 1b). This could be attributed to the presence of clove oil in composite, resulting in increased carbon and amorphous phase of the composite.

Figure 2 illustrated micro-structural study of clove oil coated hydroxyapatite composite particles, which are spherical in shape, highly dispersed and size is less than 1 µm (Fig. 2). To further confirm the size of the particles, we performed TEM characterization. Figure 3 shows a typical TEM image of the clove oil coated hydroxyapatite composite particles. It again confirmed that the particles are having spherical morphology with average size of the particles between 500 nm to 1 µm. Generally, at high temperature the particles size increases and agglomeration occurs [10]. Our results are in consistent with previous reports. The chemical composition of pristine hydroxyapatite and clove oil coated hydroxyapatite particles were analyzed by EDX spectra (fig. 4). As shown in spectra (Fig. 4a), pure HAP powder comprised of calcium (Ca), phosphorous (P) and oxygen only. On other side, the HAP-clove oil composite was found to be comprised of Ca, P, O and a prominent peak of carbon (C) as shown in Fig. 4b. There were no other elements noticed in the spectra, it again confirms the formation of pure material. A quantitative examination of the pure HAP powder was carried out and the atomic percentage of Ca: P ratio was found to be around 1.61. This is consistent with the formula and stoichiometry of hydroxyapatite [11]. Whereas, in HAP-clove oil composite sample, the ratio of Ca/P remains same but the percentage composition of carbon

increased significantly due to the coating of clove oil in hydroxyapatite.

Figure 5 displays the TGA and DTA curve of raw bones dried at 160 ºC. From the TGA graph, the total weight losses calculated were around 37.0 % for the raw bones until 900 °C. A comparatively prominent mass loss happened from 25 and 300 ºC, also confirmed by an endothermic peak because of the loss of water molecules. The major weight loss was noticed from 300 to 800 ºC temperature range, which can be attributed to the decomposition of organic molecules, carbonate into CO_2 evaporation. This prominent weight loss indicates the complete synthesis of hydroxyapatite. DTA plot shows a major exothermic peak in the same temperature range due to the crystallization of HAP. Beyond 600 ºC to 1000 ºC, insignificant weight loss was noticed that confirms the stability of the hydroxyapatite material at high temperature.

FT-IR studies were done to know the molecular structure or detect the functional groups present in HAP particles. Figure 6 depicts the FTIR spectra of the HAP samples synthesized at different temperature. The absorption band at 3570.20 cm^{-1} was assign to the characteristic hydroxyl groups (-OH) stretching vibration in crystal lattice of HAP. Furthermore, the weak peaks at about 1405 and 1460 cm^{-1} was assigned to the absorption bands of CO_3^{2-}, indicating the carbonate ions formation due to the reaction atmosphere. The major peaks at 559, 605, 962.0 and 1027 cm^{-1} could be assigned to the (PO_4 group) PO_4^{3-} [12, 13]. The band intensity seems to increase with temperature.

Figure 7 depicts the FT-IR spectra of pure clove oil, HAP and clove oil coated HAP composite. The pure clove oil displays distinctive peaks at 3500, 1610 and 1434 cm^{-1}, that could be assigned to the peaks of -OH in the benzene and C=C and $=CH_2$ in the vinyl groups, respectively [14]. The peak at 1513 cm^{-1} attributed to the ν(C=C) in benzene ring [15]. Peak at 1269 cm^{-1} attribute to the ν(C-O) bond in eugenol compound (Fig. 7a). Eugenol is a principal compound in clove oil which is responsible for giving cloves their distinctive aroma and taste. In the figure 6b, all the peaks confirmed the formation of HAP particles. In case of clove oil coated HAP composite, all the characteristic peaks of HAP and clove oil are present in the composite (Fig. 7c). It suggested that the identity of HAP and clove oil is maintained and they coexist in the composite.

The *in vitro* antimicrobial action of clove oil coated HAP composite against four bacterial strains (two Gram-positive

and two Gram-negative bacteria) was examined by means of MIC technique as shown in the Figure 8a. In order to screen the antibacterial activity, four varying concentrations (50 mg, 25 mg 12.5 and 6.25 mg/tube) of the clove oil coated HAP have been used. The observed MIC of clove oil coated HAP particles was found to be 6.25 mg for Gram positive strains (*S. epidermidis* and *S. aureus*). Whilst for Gram negative strains (*E. coli* and *K. pneumonia*) it was found to be 12.5 mg. The clove oil coated HAP composite particles were found more effective against S. *epidermidis* and *S. aureus* strains. Furthermore, regarding all selected pathogenic strains, it has been observed that with the amplification in clove oil coated HAP composite; the bacteriostatic effect was also improved. Perceptible changes in growth kinetics have been observed in case of all the strains between 4–8 h of incubation time. The uppermost value of clove oil coated HAP particles (50 mg) has established excellent reduction in bacterial cells of selected pathogens. The logarithmic stages were established between 4 to 8 h time point and 15 h or more in control samples (Fig. 8a). Clove oil coated HAP has shown inhibition against both Gram-positive and Gram-negative bacterial strains. The obtained results indicate that the inhibitory efficacy is very much dependent on selected concentration of clove oil coated HAP composite and also the doping of clove oil in HAP which is comparable to former studies [16]. The sensitivity of the Gram positive strains towards clove oil coated HAP might be due to the difference in the structure of cell membrane [8].

It has been documented that the dental caries result due to many infections, which are usually portrayed by localized deterioration of teeth. The most common microorganisms which are found in dental caries mainly include yeast and bacteria [17]. Several studies accounted that clove essential oil possess antimicrobial, antifungal, and anti-carcinogenic activities. Nevertheless; numerous compounds which possess antimicrobial activity towards mouth bacteria especially with dental caries have been extracted from *Eugenia caryophyllata*. The compounds have recognized to be competent against diverse bacteria such as: *E. coli, L. monocytogenes, S. enterica, C. jejuni, S. enteritidis,* and *S. aureus* [18]. Besides, bacteriostatic activity, the purpose of this study was also to examine *in vitro* antifungal activity of clove essential oil to unearth a substitute to existing synthetic antibacterial and antifungal medicines. The representative fungal strain used was *C. albicans.* Herein; it was found that highest concentration (100

mg) of clove oil coated HAP possesses admirable antifungal activity (Fig. 8b). The excellent antifungal and antibacterial activity of clove oil coated HAP is possibly because of functionalization of HAP with clove oil. More to the point; the synergistic effect of clove oil and HAP resulted in the excellent antimicrobial action. Additionally; it has already been reported in the literature too that the clove essential oil is incredibly efficient even at very small quantities against numerous mouth pathogens such as *Streptococcus constellatus, Streptococcus mutans, Streptococcus oralis, and Streptococcus mitis* etc. The outcomes of our study verify earlier studies which have specified that clove essential oil possess antibacterial activity against some periodontal microbes. Our data is in admirable harmony with the earlier research [19].

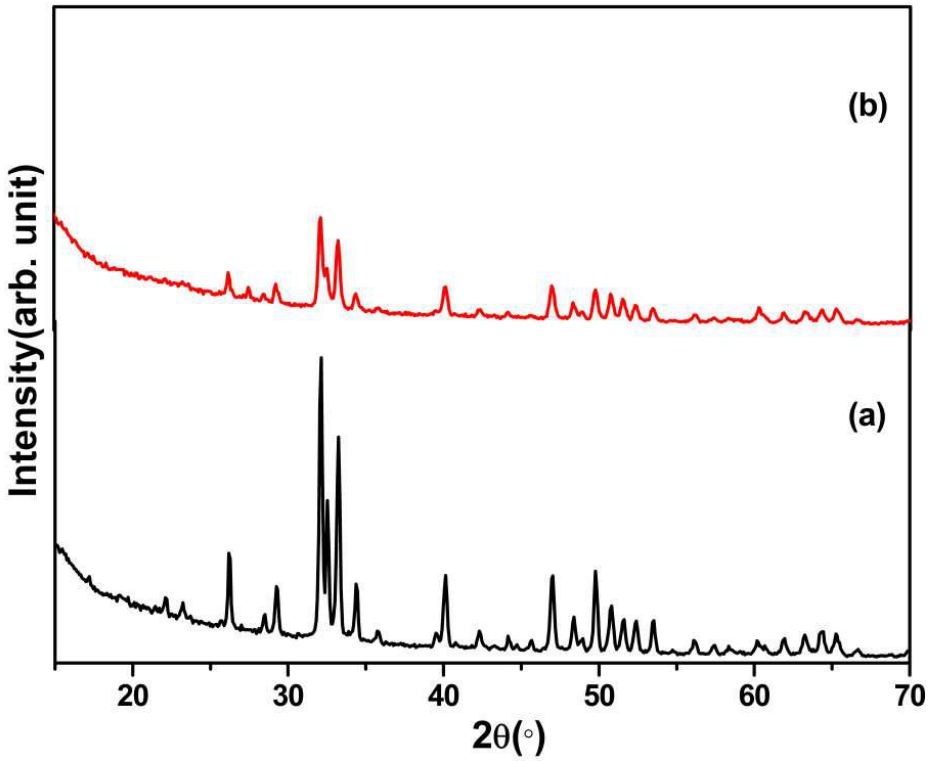

Figure 1: XRD spectra of synthesized pure HAP and clove oil coated HAP particles calcined at 700 ºC

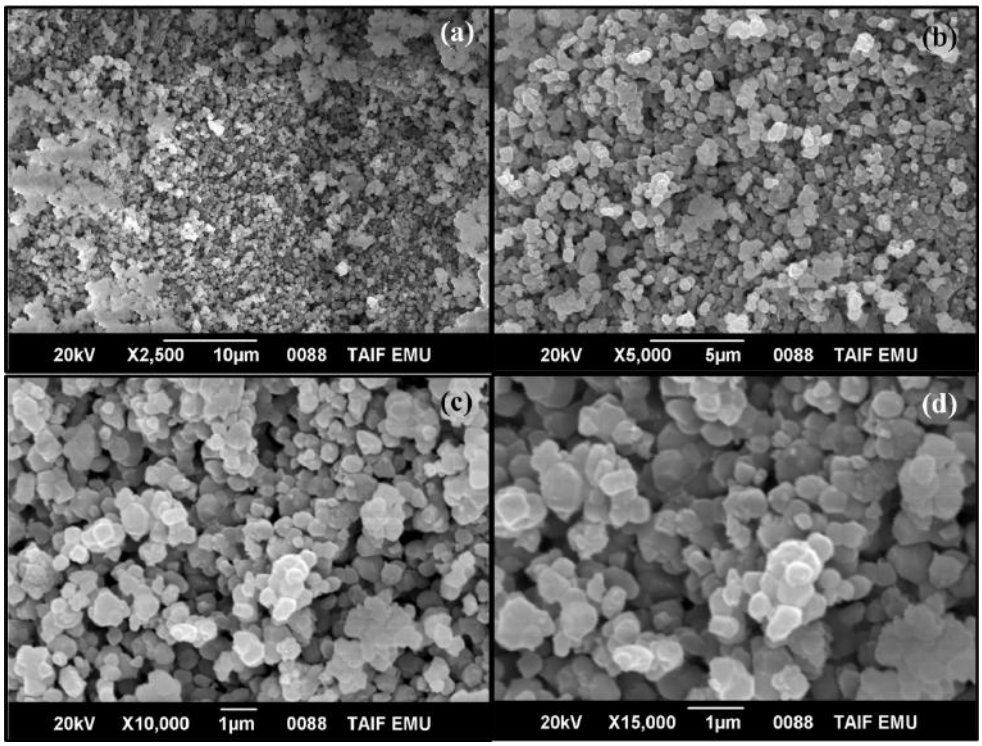

Figure 2: SEM images of synthesized pure HAP particles at different magnifications calcined at 700 ºC

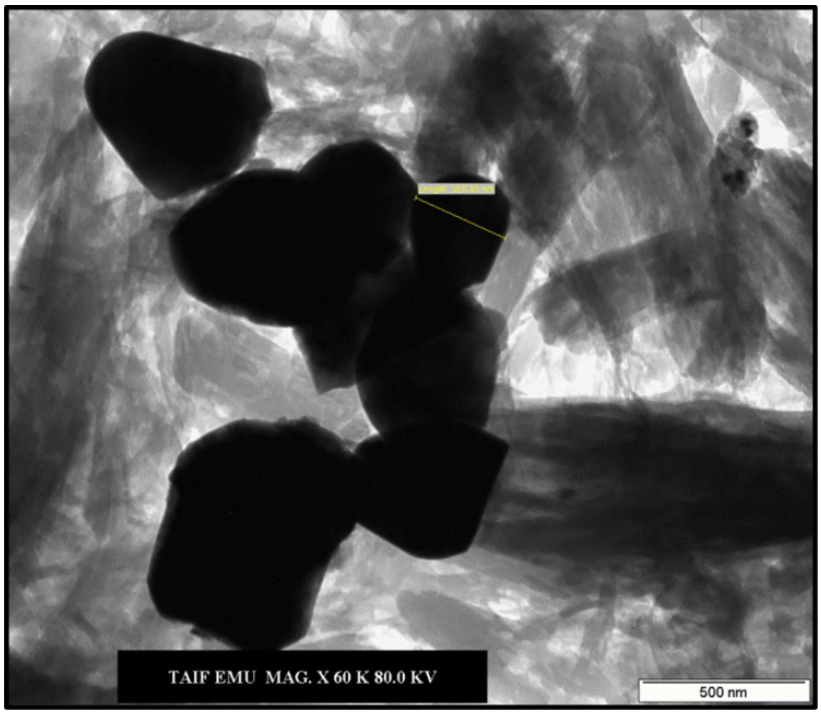

Figure 3: TEM images of synthesized pure HAP particles synthesized at 700 ºC

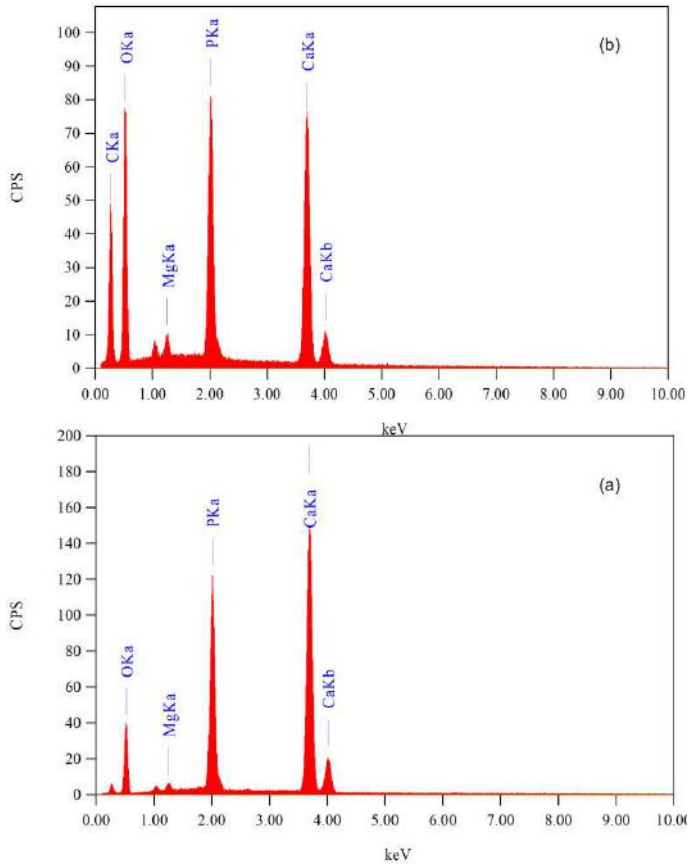

Figure 4: EDX spectra of synthesized pure HAP and clove oil coated HAP particles calcined at 700 ºC

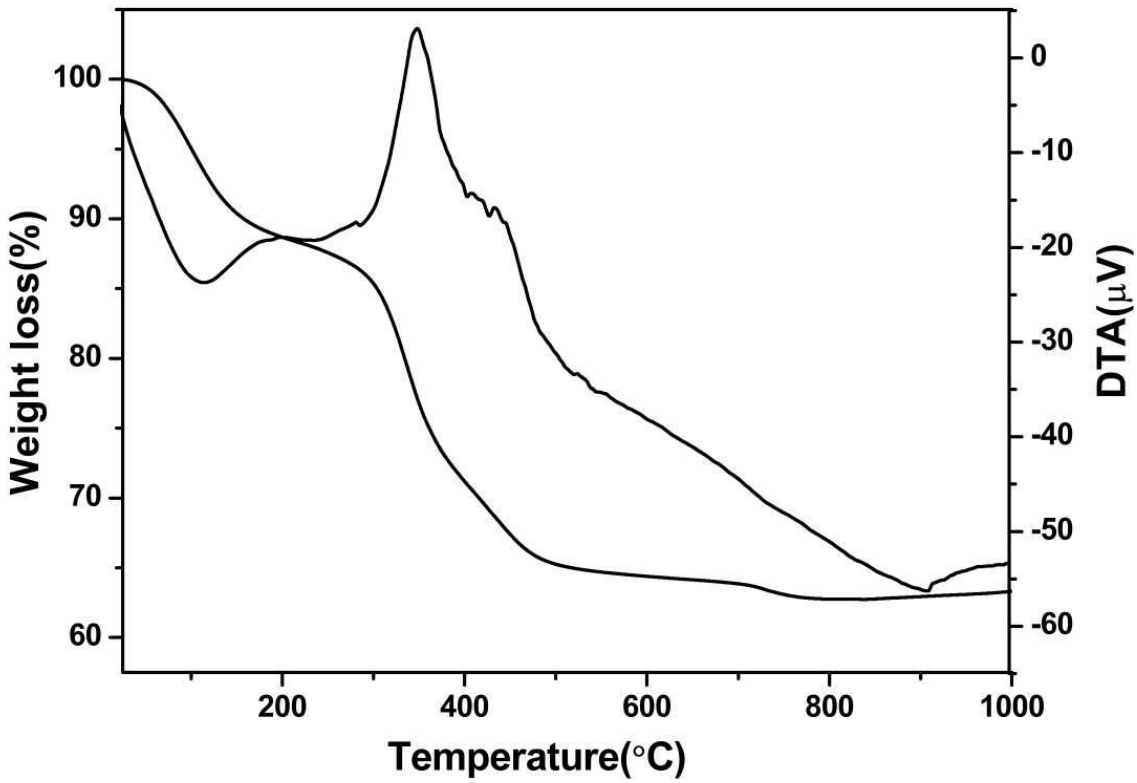

Figure 5: TGA and DTA curve of the raw bones dried at 160 ºC

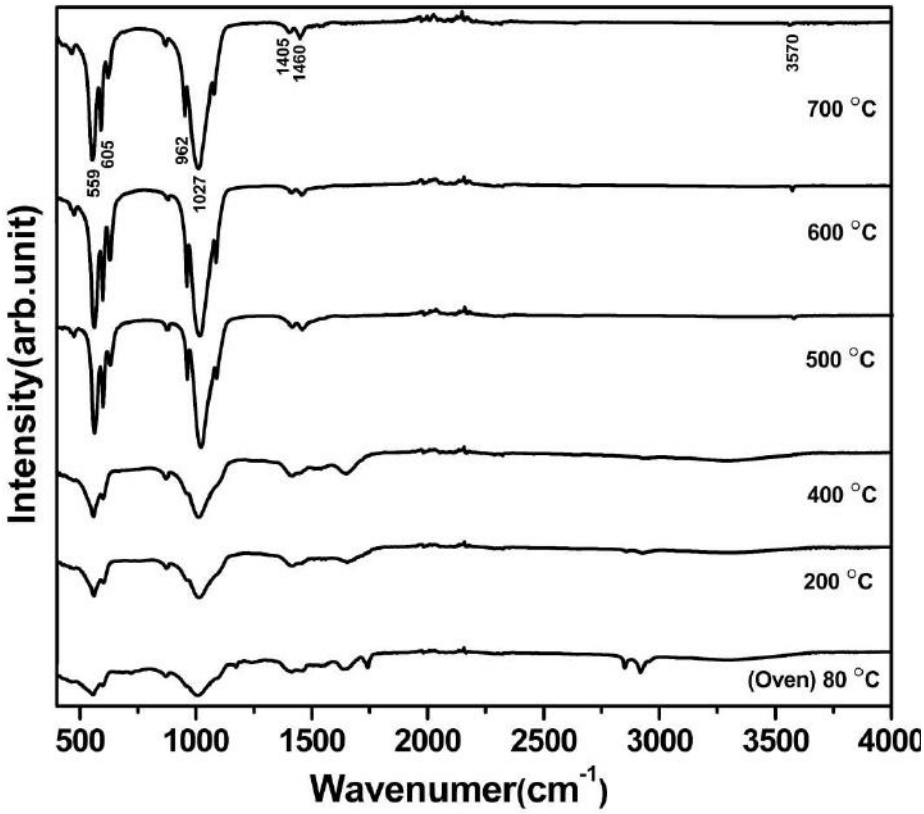

Figure 6: FT-IR spectra of the pure HAP samples synthesized at different temperatures

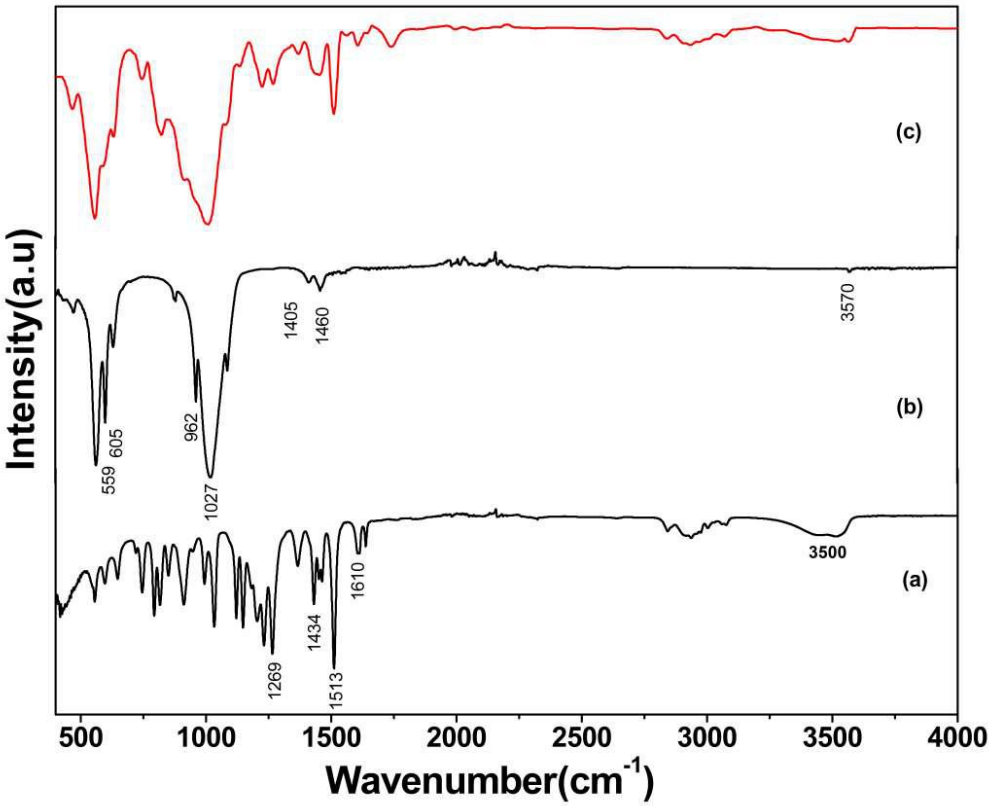

Figure 7: FT-IR spectra of (a) pure clove oil (b) HAP and (c) clove oil coated HAP composite particles

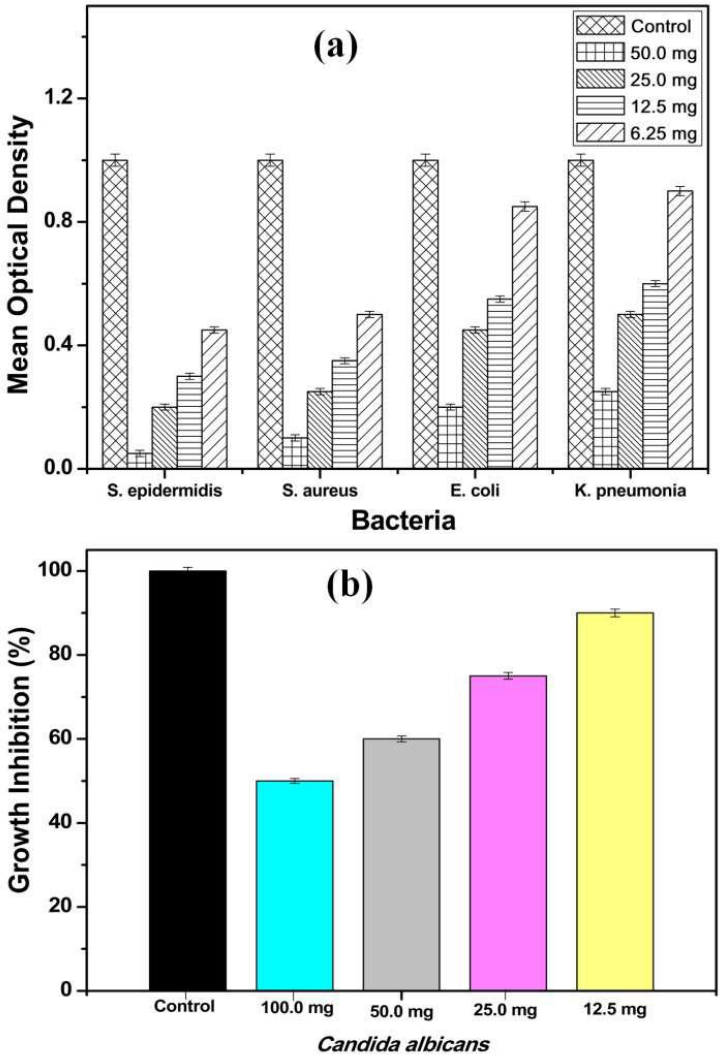

Figure 8: A bar graph representation showing the optical density (600 nm) of supernatants from *S. epidermidis*, *S. aureus*, *E. coli* and *K. pneumonia* after treatment with different concentrations of clove oil coated HAP composite particles (a). *In vitro* antifungal activity of clove oil coated HAP compositeparticles against *C. albicans.* Absorbance (OD at 595 nm) for control cultures in absence of clove oil coated HAP composite and in presence of different concentrations of clove oil coated HAP composite was determined. Fungal growth was expressed as percent growth of control cultures (100% growth represents fungal growth in Sabouraud dextrose medium without clove oil-HAP composite)(b)

CONCLUSION

In conclusion, the study described highly crystalline pure HAP and clove oil coated HAP composite particles which were synthesized using natural bone precursor. The synthesized samples were characterized using XRD, SEM, TEM, EDX and FT-IR. The vigorous utilization of natural products enriched with bioactive components has attracted huge attention of the pharmaceutical, food, and cosmetic companies. A lot of investigations have clearly accounted that clove essential oil possess excellent antimicrobial and anti-carcinogenic activities. In particular; loads of antimicrobial constituents which are very effective in controlling oral bacteria allied with dental caries have been isolated from *Eugenia caryophyllata*. Nevertheless, in this study we have used the clove

essential oil for coating of HAP to prepare a novel dental implant. This composite has revealed superb antimicrobial action against *S. epidermidis*, *S.* aureus, *E. coli* and *K. pneumonia* bacteria and *C. albicans* fungus, respectively. On the other hand, HAP has long been amongst the utmost studied biomaterials due to its biocompatibility and for being the key component of the mineral portion of bone and teeth. Therefore, the combination of HAP and clove essential will be the best suited material to be used in near future for the oral microflora management and dental caries treatment.

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REFERENCE:

[1] A. Tampieri, S. Sprio, A. Ruffini, G. Celotti, I.G. Lesci, N. Roveri, From wood to bone: multi-step process to convert wood hierarchical structures into biomimetic hydroxyapatite scaffolds for bone tissue engineering, Journal of Materials Chemistry, 19 (2009) 4973-4980.

[2] N. Ramesh, S.C. Moratti, G.J. Dias, Hydroxyapatite–polymer biocomposites for bone regeneration: A review of current trends, Journal of Biomedical Materials Research Part B: Applied Biomaterials, 106 (2018) 2046-2057.

[3] S. Kweh, K. Khor, P. Cheang, The production and characterization of hydroxyapatite (HA) powders, Journal of Materials Processing Technology, 89 (1999) 373-377.

[4] V. Orlovskii, V. Komlev, S. Barinov, Hydroxyapatite and hydroxyapatite-based ceramics, Inorganic Materials, 38 (2002) 973-984.

[5] M. Vallet-Regí, Ceramics for medical applications, Journal of the Chemical Society, Dalton Transactions, (2001) 97-108.

[6] R. Young, D. Holcomb, Variability of hydroxyapatite preparations, Calcified tissue international, 34 (1982) S17-32.

[7] K. Azzaoui, A. Lamhamdi, E.M. Mejdoubi, M. Berrabah, B. Hammouti, A. Elidrissi, M.M. Fouda, S.S. Al-Deyab, Synthesis and characterization of composite based on cellulose acetate and hydroxyapatite application to the

absorption of harmful substances, Carbohydrate polymers, 111 (2014) 41-46.

[8] T. Amna, M.S. Hassan, N.A. Barakat, D.R. Pandeya, S.T. Hong, M.-S. Khil, H.Y. Kim, Antibacterial activity and interaction mechanism of electrospun zinc-doped titania nanofibers, Applied microbiology and biotechnology, 93 (2012) 743-751.

[9] D. Núñez, E. Elgueta, K. Varaprasad, P. Oyarzún, Hydroxyapatite nanocrystals synthesized from calcium rich bio-wastes, Materials Letters, 230 (2018) 64-68.

[10] Y. Han, S. Li, X. Wang, L. Jia, J. He, Preparation of hydroxyapatite rod-like crystals by protein precursor method, Materials research bulletin, 42 (2007) 1169-1177.

[11] N.Y. Mostafa, Characterization, thermal stability and sintering of hydroxyapatite powders prepared by different routes, Materials chemistry and physics, 94 (2005) 333-341.

[12] J. Rocha, A. Lemos, S. Kannan, S. Agathopoulos, J. Ferreira, Hydroxyapatite scaffolds hydrothermally grown from aragonitic cuttlefish bones, Journal of Materials Chemistry, 15 (2005) 5007-5011.

[13] H. Varma, S.S. Babu, Synthesis of calcium phosphate bioceramics by citrate gel pyrolysis method, Ceramics International, 31 (2005) 109-114.

[14] B.O. Jung, S.J. Chung, S.B. Lee, Preparation and characterization of eugenol-grafted chitosan hydrogels and their antioxidant activities, Journal of Applied Polymer Science, 99 (2006) 3500-3506.

[15] A. Kadarohman, Hernani, F. Khoerunisa, R.M. Astuti, A potential study on clove oil, eugenol and eugenyl acetate as diesel fuel bio-additives and their performance on one cylinder engine, Transport, 25 (2010) 66-76.

[16] A. Puškárová, M. Bučková, L. Kraková, D. Pangallo, K. Kozics, The antibacterial and antifungal activity of six essential oils and their cyto/genotoxicity to human HEL 12469 cells, Scientific reports, 7 (2017) 8211.

[17] M. Zaremba, W. Stokowska, A. Klimiuk, T. Daniluk, D. Rozkiewicz, D. Cylwik-Rokicka,

D. Waszkiel, G. Tokajuk, A. Kierklo, S. Abdelrazek, Microorganisms in root carious lesions in adults, Advances in medical sciences, 51 (2006) 237-240.

[18] J.P. Ouhayoun, Penetrating the plaque biofilm: impact of essential oil mouthwash, Journal of Clinical Periodontology, 30 (2003) 10-12.

[19] B. Kouidhi, T. Zmantar, A. Bakhrouf, Anticariogenic and cytotoxic activity of clove essential oil (Eugenia caryophyllata) against a large number of oral pathogens, Annals of microbiology, 60 (2010) 599-604.