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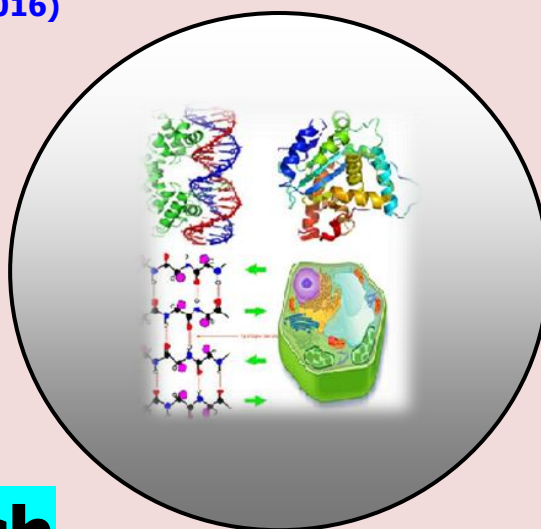
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Synthesis and Spectroscopic Investigation of Nanohydroxyapatite for Orthopedic Applications

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ABSTRACT

Due to its similarities with the inorganic component of bone and tooth, hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA) has been extensively studied for biomedical applications. Our present study is aimed at investigating the contribution of two biologically important cations, Mg^{2+} and Zn^{2+} and in addition to the polymer like PEG (polyethylene glycol), when substituted into the structure of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA), on biocompatibility, bioactivity and osteo conductive properties. The substituted samples were synthesized by an aqueous precipitation method. The structural composition and the morphology of the synthesized samples were analyzed by FTIR, XRD, SEM and EDAX. The bioactivities such as cell viability, anticancer and antimicrobial activities have been studied. The as-synthesized nanocomposite material can act as a potential candidate for orthopedic applications.

Keywords: Hydroxyapatite, Nanoparticles, Mineral, polymer, Bioactivity and SEM.

INTRODUCTION

Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA) is the important biomaterial for bone [Laurencin et al., 2011] since it shows an good biological compatibility and osteo conductivity property [Thian et al., 2013, Song et al., 2013, Liao et al., 1999]. Due to its excellent properties like bone bonding ability and structural composition of the mineralized human bone, HA is widely used as a bioceramic material for hard tissue replacement [Sanosh et al., 2009, Agrawal et al., 2011]. Different ionic substitutions are easy to incorporate in HA structure such as mono, di, tri and tetra valent ions. Most of the divalent ions are substituted to develop the properties of HA such as bioactivity, mechanical strength and solubility by controlling its composition, morphology and particle size [Ciobanu et al., 2015]. Previous authors reported that HA having the compositions by stoichiometric C/P is ratio of 1.67 [Zhang et al., 2012].

The surface morphological properties of HA can be adjusted by different methods like chemical precipitation, sol-gel, wet chemical precipitation, sonochemical method, ultra sonication method, hydrothermal techniques and mechano-chemical methods [Senthilarasan and Sakthivel 2012, Senthilarasan et al., 2014]. For a

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time consuming and expensive facilities chemical precipitation method was used. Compare those methods it was an efficient technique for the synthesis of HA. It exhibit good dispersivity and explain the article dimension [Felsen et al., 2015, Hazar Yoruc and Koca 2009].

For considering osteoporosis in humans Magnesium and zinc was a basic element [Geng et al., 2016, Suresh Kumar et al., 2012]. These most probable elements are found in bone and bone tissues. It was a significant role on the synthesis of substituted Mg/Zn/HA [Lijuan et al., 2013, Zhang et al., 2013]. In order to improve the binding property of HA some of the minerals are used. Here Mg^{2+} and Zn^{2+} ions are used, which are found in bone and teeth. Generally these ions have osteogenic effects on osteoblastic bone formation and selective effect on bone resorption by stimulating cell proliferation synthesis in osteoblastic cells both in vitro and in vivo [Zhang et al., 2013]. These minerals will influence the biological activity of calcium phosphates by changing their material properties.

When the bioactive metallic materials of Zn^{2+} and Mg^{2+} ions exhibit good antibacterial potential, but its antibacterial competence is simply affected by the microstructure of HA [Stanica et al., 2010]. Presently several considerations have been made to produce nanocrystalline HA with controlled morphology by incorporating organic polymers. For producing HA formation poly-(ethylene glycol) (PEG) is used [Zhou et al., 2013, Azzaoui et al., 2013]. Because of better binding ability and to improve their mechanical strength poly ethylene glycol (PEG) is added to HA [Dhanalakshmi et al., 2012]. Hence, the morphological changes of HA crystals in the presence of PEG was a good candidate to investigate by the chemical precipitation method. The present work explains synthesis of nano structured minerals Mg^{2+} , Zn^{2+} substituted HA incorporate with polymer (PEG) by chemical precipitation method.

MATERIALS AND METHODS

CHEMICALS

Analytical reagent grade calcium nitrate ($Ca(NO_3)_2$) and di potassium hydrogen phosphate (K_2HPO_4), ammonia solution (99% pure NH_3), ethanol and polyethylene glycol (PEG) solutions were purchased from Sigma Aldrich (India). Double distilled water was used to prepare solutions and solvent. To maintain the p^H 10% ammonia solution was used. Using chemical precipitation method pure and composite HA powders were prepared as follows 0.5 M calcium nitrate solution was first put into a 250 ml beaker, the solution was kept at 25 °C in a heating mantle with a magnetic stirrer, and its pH of the solution was maintained to 9 - 10 by the addition of ammonia solution (NH_3). 0.3 M di potassium hydrogen phosphate solution was added drop wise to the calcium nitrate solution at the rate of 10 ml/min. The mixture was stirred continuously in the magnetic stirrer. Then the suspension was stirred at ambient condition for 6 h, and then aged in the mother solution for 24 h at room temperature. Then solution was washed 4 times with deionized water or ethanol to remove excess of impurities, and finally dried at 120 °C in a vacuum oven overnight. Then the dried hap was calcined at 600-800°C for 2 h finally it is powdered by mortar and pestle for getting a pure HA powder. In the same way mineralized (Mg/ Zn/ HA) hydroxyapatite was prepared by using 0.5M $Ca(NO_3)_2$, 0.3M K_2HPO_4 and equimolar mixture of 0.1M (Mg and Zn). Mineral and calcium solutions were taken in a beaker, the solution was kept in a heating mantle with a magnetic stirrer, and pH of the solution was maintained at 9 - 10 by the addition of ammonia solution (NH_3). Phosphate solution was added drop wise to the Ca/Mg/Zn at 10 ml/min at 25 °C, maintaining a pH of 9- 10. The solution was stirred well for 6 h, heated in an oven and calcined at temperature to get a mineralised HA.

On the addition of polymer to the composite, the heating rate will depend on polymer temperature. The boiling point of polymer is 140°C so it was heated in an oven only below 100°C. Finally we got the polymer doped composite minerals substituted HA.

CHARACTERISATION TECHNIQUES

FTIR, XRD ANALYSIS

FTIR was a powerful tool for the characterization of biomedical materials. Fourier transform infrared spectroscopy was used to identify the molecular structure of the sample, chemical composition and details about type of bonds. Powdered samples were recorded using KBR matrix. Using a JASCO 6100 FTIR spectrometer it exhibits the spectral range of 4000–400 cm^{-1} . X-ray diffraction was used to analyses the crystalline phases of the sample. The calcined powdered sample were characterized by using (XRD; D8 Advance, Bruker) analyser. It was used to study the structural phase composition of the powder sample.

This diffractometer were recorded using CuK α radiation at 35 kV and 30 mA. Incident radiation allows passing radiation in the range of 20-60 $^{\circ}$. Because of pure HA and composite HA, intensity data were collected from 2 θ range of 35–70 $^{\circ}$ at a scan rate of 0.3 $^{\circ}$ 2 θ /min. For considering the XRD patterns HA was a unique crystalline phase. It was exhibit well defined and sharp peak in high degree of cristanality.

SEM AND EDAX ANALYSIS

Scanning electron microscope (SEM) Philips XL-305 FEG was used to resolute particle size and morphology of the powdered HA sample. EDAX is attached to SEM was used for the elemental analysis of the HA composites.

ANTIMICROBIAL ACTIVITY

The antimicrobial efficacy of the mineral substituted PEG doped HA samples were tested against clinically isolated bacterial pathogens i.e., *Staphylococcus epidermidis* are collected from clinical laboratories, in and around Salem District, Tamil Nadu, India. The given samples were dissolved in 1 ml of DMSO separately and mixed well which was used to perform antimicrobial screening tests. The working standard for bacterial cultures of each test organism was prepared by inoculating a loop-full of mother culture in Müller Hinton broth (5 ml) and incubated at 37 $^{\circ}$ C (in a shaker) for 16 h. The culture turbidity was adjusted to 0.5 McFarland equivalents (1.5×10^8 CFU).

The antibacterial activity of the mineral substituted PEG doped HA samples were determined using agar well diffusion method. A suspension of test culture (50 μ l) was swabbed on the Müller Hinton Agar (MHA) using sterile cotton swab. In each seeded plate, four wells were made using sterile cork borer (5 mm diameter). Then, different concentrations (25, 50, 75 μ l/ μ g) of each sample was separately loaded into wells and allowed to diffuse at room temperature. 25 μ l of ciprofloxacin (1 μ g/ μ l) for bacteria was served as positive control. The bacterial plates were incubated at 37 $^{\circ}$ C for 24 h. After appropriate incubation period, the diameter of zone of growth inhibition was measured (in mm).

ANTICANCER ANALYSIS

The prepared nanocomposite samples were performed by HeLa cell line. An *in vitro* cytotoxicity test method was performed for the substituted HA sample as per ISO 10993:5. The culture medium from the L929 monolayer was replaced with fresh medium. Test sample in duplicates were added on the cells. After incubatssion at 37 $\pm$ 1 $^{\circ}$ C for 18 h, MTT assay were added in all the wells and incubated for 4 h. After incubation, DMSO was added in the wells and read at 570 nm using visible spectrophotometer. Cytotoxicity was calculated by the following formula.

Cytotoxicity = [(Abs Control – Abs Treated) / Abs Control] * 100

Cell viability= (Abs Treated / Abs Control) * 100

CELL VIABILITY ANALYSIS

A cell viability study was determined by the cancerous cell line of HeLa. The culture was maintained by using DMSO solvent, supplemented with foetal bovine serum. For the cell line Trypan blue dye was used.

RESULTS AND DISCUSSION

X- RAY DIFFRACTION ANALYSIS

X-ray diffraction analysis was used to study the crystalline nature of the sample. When the intense peaks are explain about the cristanality of the sample, as well as the particle size of the given composites. HA powdered sample was calcinated at 600 $^{\circ}$ C and the prepared pure sample was used for XRD analysis. The calcined samples show vital role on the formation of Hap. Increase in temperature increases the crystalline structure. While temperature is increased from 400 $^{\circ}$ C to 600 $^{\circ}$ C several peaks of XRD pattern gives distinct HA powder and the widths of the peaks become narrower. From figure1 the powdered samples such as pure HA, Mg/ Zn/ HA, PEG/HA and Mg/ Zn/ PEG/ HA are analyzed. These are exhibiting the 2 θ values of 37.48, 35.00, 34.92, 34.84, 32.16, 31.64, 25.88, 30.00, 30.44, 30.12 and 29.8. Broad and intense peaks are obtained from the XRD pattern. 29.8 and 30.12 are explain the sharp intense peak for pure HA. Where the value of 37.48 describe the crystalline peak of PEG/HA. When consider for the Mg/Zn/PEG/HA the high intense crystalline peak was obtained in range of 35 and 34.92.

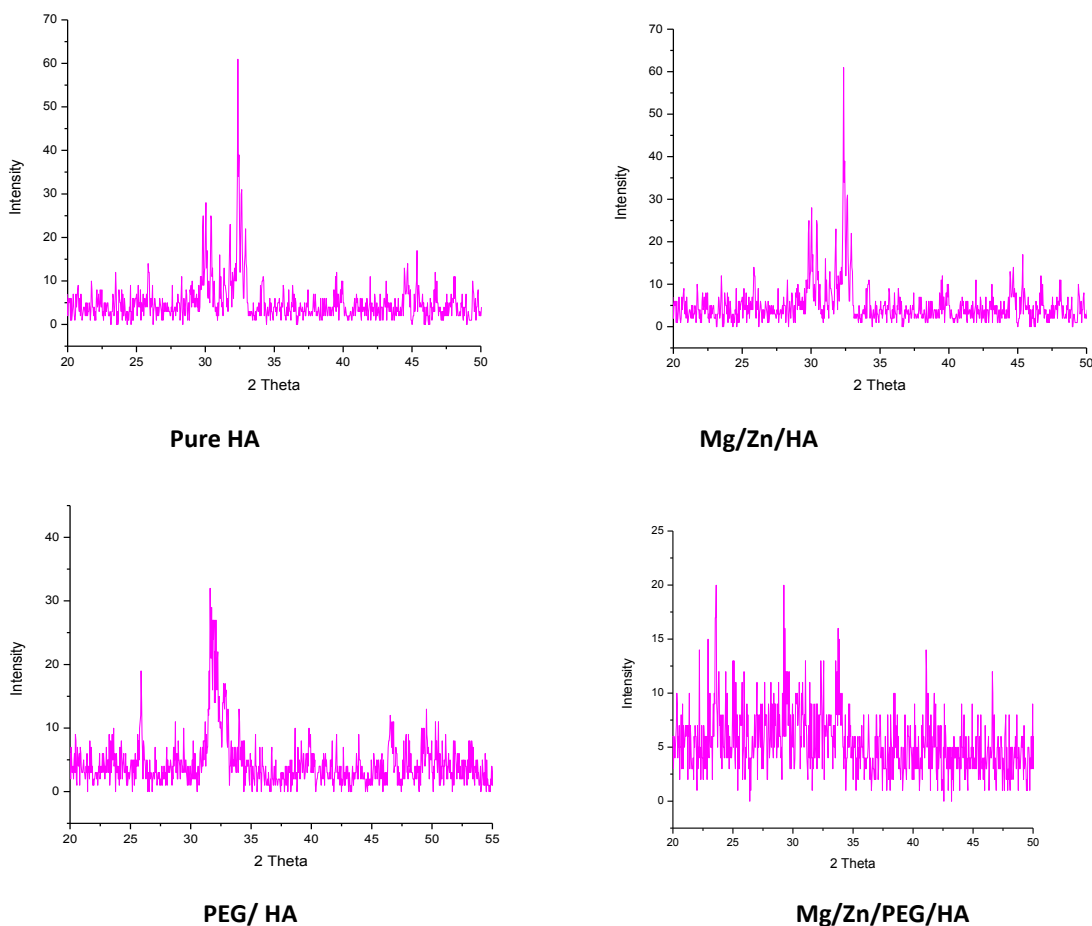


Figure 1. X-ray diffraction analysis (XRD) images for pure HA (a), Mg/Zn/HA/ (b), PEG/HA (c), Mg/Zn/PEG/HA (d) respectively.

FT-IR ANALYSIS

The fig 2(a) represents FTIR spectra of pure Hap sample which exhibit three bands in the range of 3415, 1114, 1037 cm^{-1} . Those are assigned to phosphate, O H, COO and CH bonds respectively. Fig 2(b) represents three bands at 3416, 1114, 1036 cm^{-1} corresponding to stretching vibration of COO (symmetric), COO (asymmetric), CH and O H, respectively. The fig 2(c) represents FTIR spectra of mineral substituted HA sample which exhibit in the peaks range of 3643, 1433, 1384, 1039 cm^{-1} , due to C–OH stretching vibration in the polymer. The fig 2(d) represents FTIR spectra of mineral/polymer HA sample which explained that the bands exhibit in the range of 3396, 2879, 2398, 1619, 1354, 1070 cm^{-1} are assigned to C–OH, COO, CH stretching vibration in the polymer. The peak at 976.89 cm^{-1} corresponds to symmetric stretching mode of PO_4 . The peaks at 623.02 cm^{-1} and 560 cm^{-1} for sample calcined at 400°C and 589.7 cm^{-1} for sample calcined at 700°C indicate the bending mode of PO_4 . The large separation of these bands indicates the presence of crystalline phase.

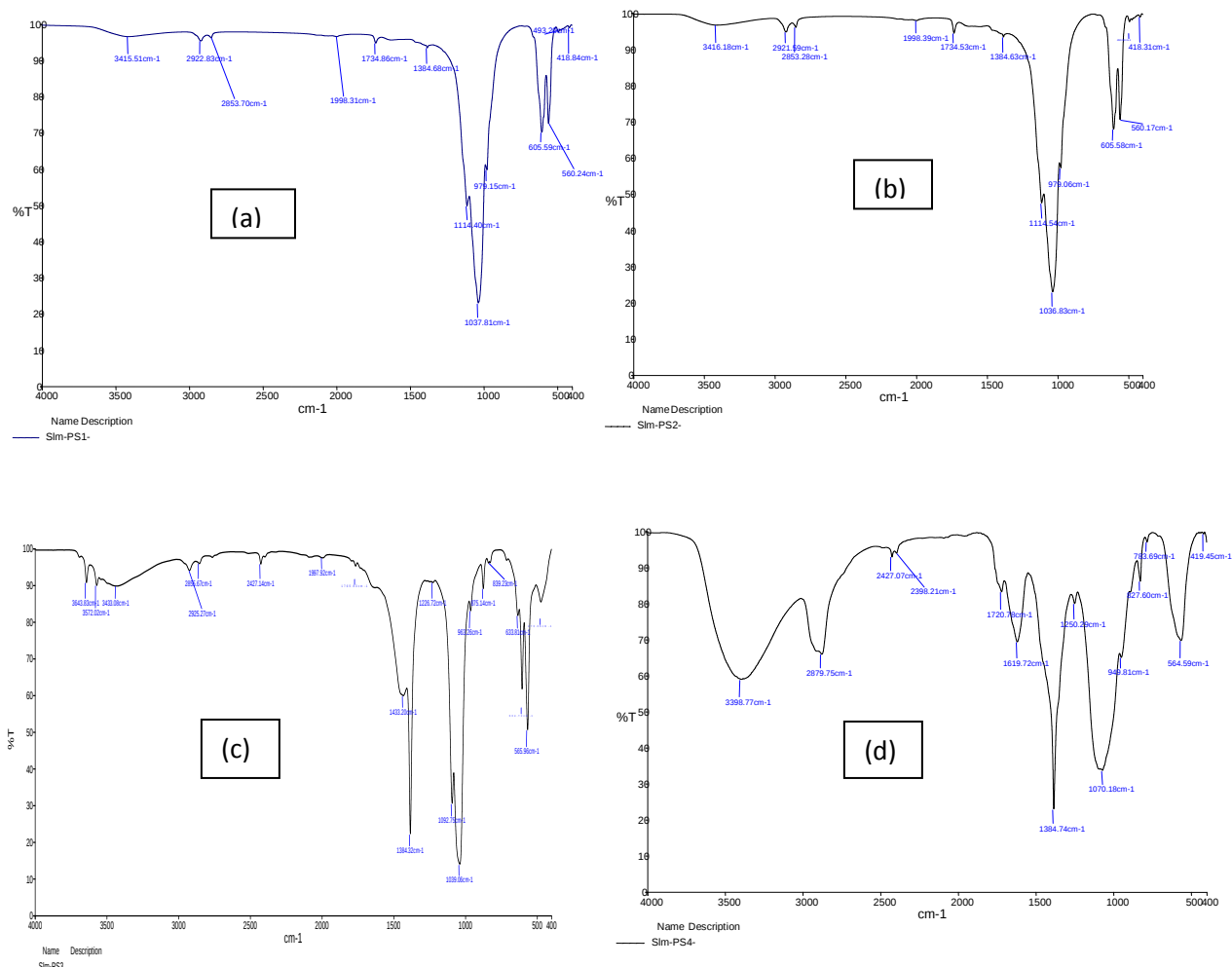


Figure 2. Fourier transform infrared spectroscopy (FTIR) images for pure HA (a), Mg/Zn/HA/ (b), PEG/HA (c), Mg/Zn/PEG/HA (d) respectively.

SEM, EDAX ANALYSIS

From scanning electron microscope (SEM) surface morphology, nanostructure, size and shape of the substituted element can be analyzed. Fig 3 shows that SEM images of various HA samples like pure HA (a), HA/Mg/Zn (b), HA/PEG(c), HA/Mg/Zn/PEG (d) respectively.

From the SEM images HA powder explains the surface treatment of the mineral and composites. When consider polymer HA was increased the agglomeration size. It was observed that Mg, Zinc and polymer composite consist of small particles, porous nature area. This revealed that the polymer and composite material posses high porous nature and high surface area. When comparing with pure HA and composite material, HA posses less porosity and low surface area. From that we conclude that Mg and Zn having good biological adsorption properties.

EDAX ANALYSIS

Edax analysis is to determine the elements present in the powdered sample. Fig 4(a-d) shows the EDAX spectra of the HA samples. It can easily explain about the elemental composition of Hap/Mg/Zn/PEG composites. This EDAX patterns shows the presence of Calcium (Ca), Phosphorous (P), Magnesium (Mg), Zinc (Zn), Oxygen (O) in the structure of synthesized nanoparticles. Which confirm the formation of mineralized PEG doped HA.

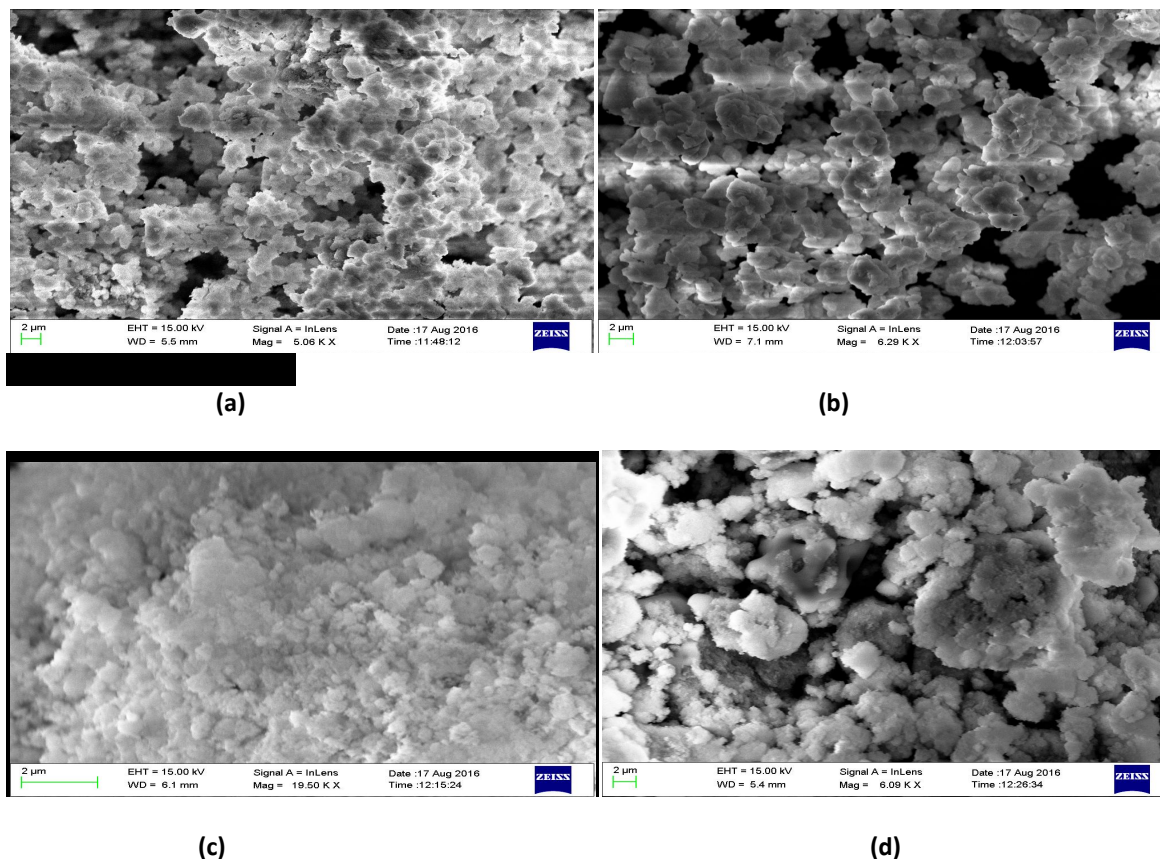


Figure 3. Scanning electron microscope (SEM) images for pure HA (a), Mg/Zn/HA (b), PEG/HA (c), Mg/Zn/PEG/HA (d) respectively.

ANTIMICROBIAL ACTIVITY

Antibacterial activity of the Hap/Mg/Zn/PEG substituted nano powders were performed through *Staphylococcus epidermidis* bacteria. As revealed in fig 5, the level of microbial growth of bacteria increased with increase of substitutions. When consider pure Hap, Mg/Zn, Mg/Zn/PEG the growth of antimicrobial activity increase with composite sample. From fig5 describe that the Hap, Hap/Mg/Zn, Hap/PEG and Hap/Mg/Zn/PEG are having antimicrobial activity. When compare with Hap and Hap/Mg/Zn/PEG, composite having good antimicrobial activity.

ANTICANCER ACTIVITY

The cytotoxicity levels of pure Hap, Hap/Mg/Zn/PEG nanohybrids, were evaluated against HeLa cell lines using MTT assay. The method is based on the reduction of MTT assay. Minimum essential medium is supplemented with foetal bovine serum. The results of in vitro cytotoxicities of pure Hap, Hap/Mg/Zn/PEG composite against HeLa cells for 24h, were demonstrated in Fig 6(a). The pure Hap showed none of toxicity, indicates that it did not induce any effects upon cellular viability and proliferation at 24 h against the Hela cell lines. It was observed that the incubation time (ie 24h) increased the cytotoxicities of Hap/Mg/Zn/PEG against the selected cell lines. The cell lines showed a dose-dependent cytotoxicity toward Hap/Mg/Zn/PEG Fig 6 (b). On the other hand, Hap/Mg/Zn/PEG shows moderate cytotoxicity against HeLa cells at 24 h than pure HA.

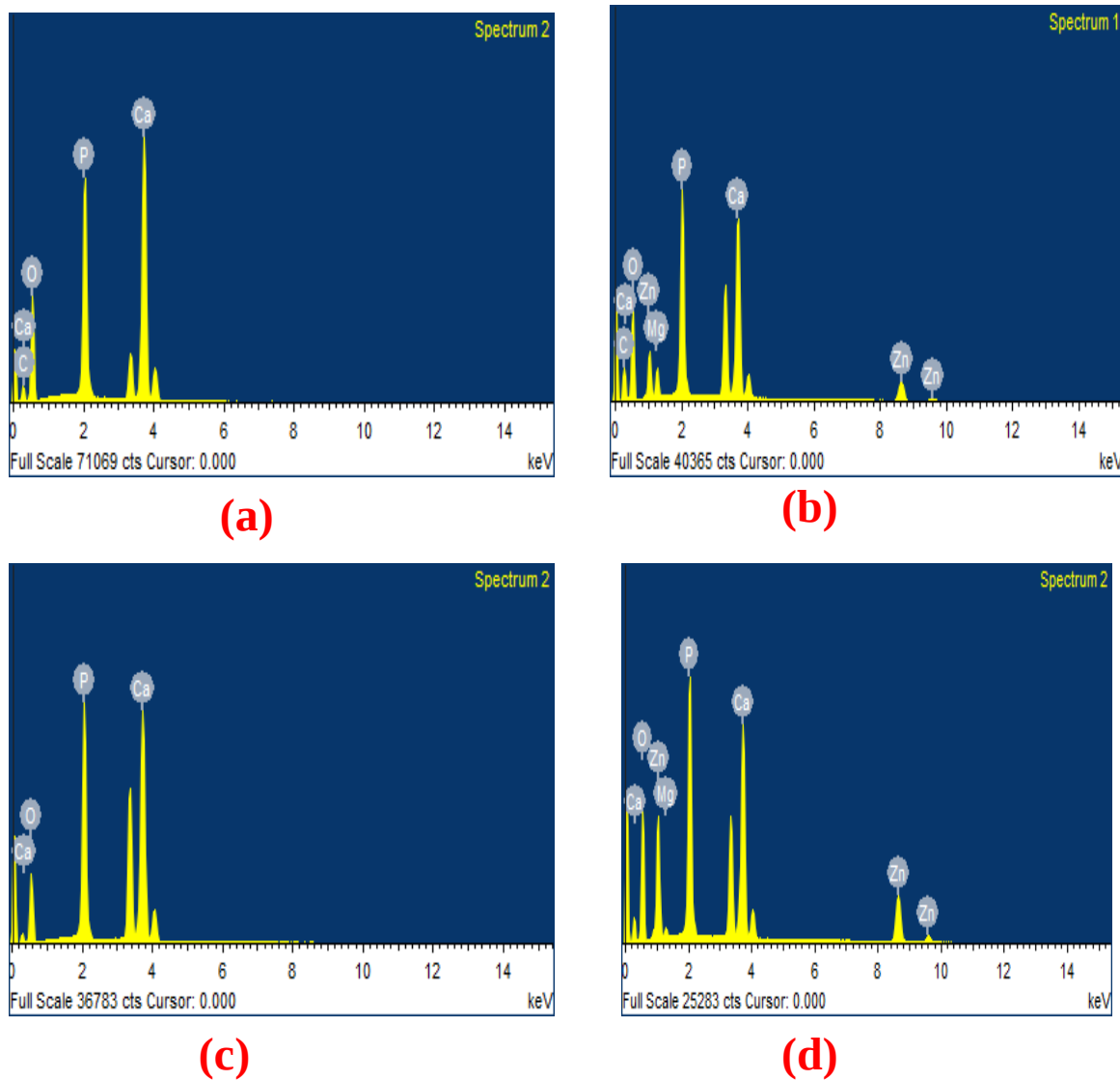


Figure 4. Elemental analysis (EDAX) images for pure HA (a), Mg/Zn/HAp (b), PEG/HAp (c), Mg/Zn/PEG/HAp (d) respectively.

CELL VIABILITY

Cell culture studies were conducted by the source line of NCCS with HeLa cell line. HeLa was an established and well characterized cell line that has demonstrated reproducible results. Minimum essential supplemented with foetal bovine serum. For the cell line trypan blue dye was used. The solution was dissolved by using DMSO medium. When the samples were tested at different concentrations are 50 μ l, 75 μ l 100 μ l. It should exhibit toxic through HeLa cell line and explain the cell suppression of the sample. Fig.7 was explain the cell viability of pure HA and Hap/Mg/Zn/PEG composite, from that we can easily identify the cell suppressed with increase of concentration.

CONCLUSION

The spectroscopic investigation of XRD, FTIR and SEM showed that particles of all samples are of nano size and homogenous in composition, and also exhibit their morphology and biocompatible nature. The above study revealed that the composite Hap/Mg/Zn/PEG powder exhibits a good biocompatible nature. The antibacterial results reveal that the as-synthesized HA/Mg/Zn/PEG powder exhibits a strong antibacterial activity against the *S. epidermidis* bacteria. Biological study evidence that Mg, Zn ions are improved the biological activity of hydroxyapatite. Anticancer and cell viability study explain the toxicity of samples against HeLa cells.

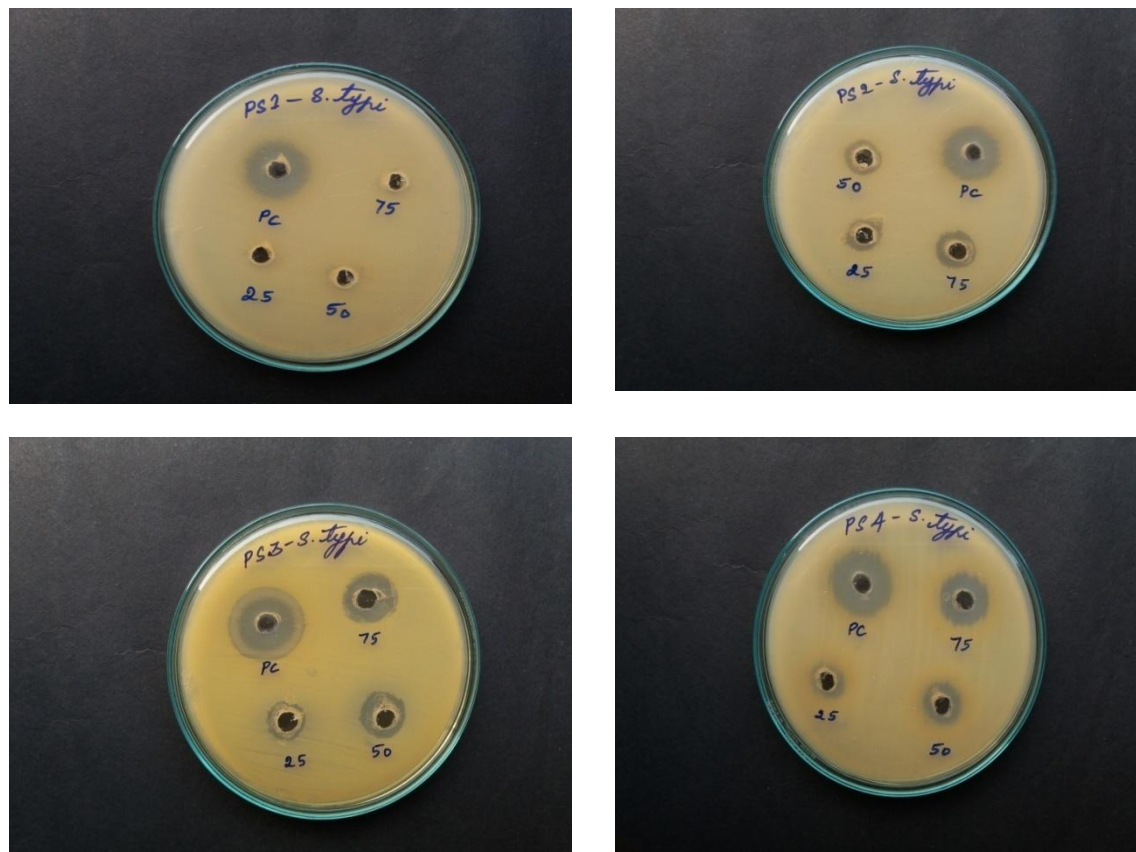
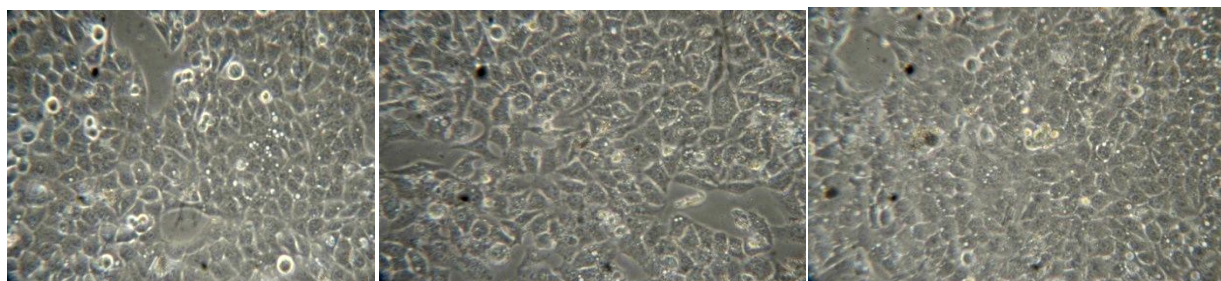


Figure 5. Antimicrobial images for pure HA (a), Mg/Zn/HA (b), PEG/HA (c), Mg/Zn/PEG/HA (d) respectively.

50 μ l

75 μ l

100 μ l



(a) Pure Hap

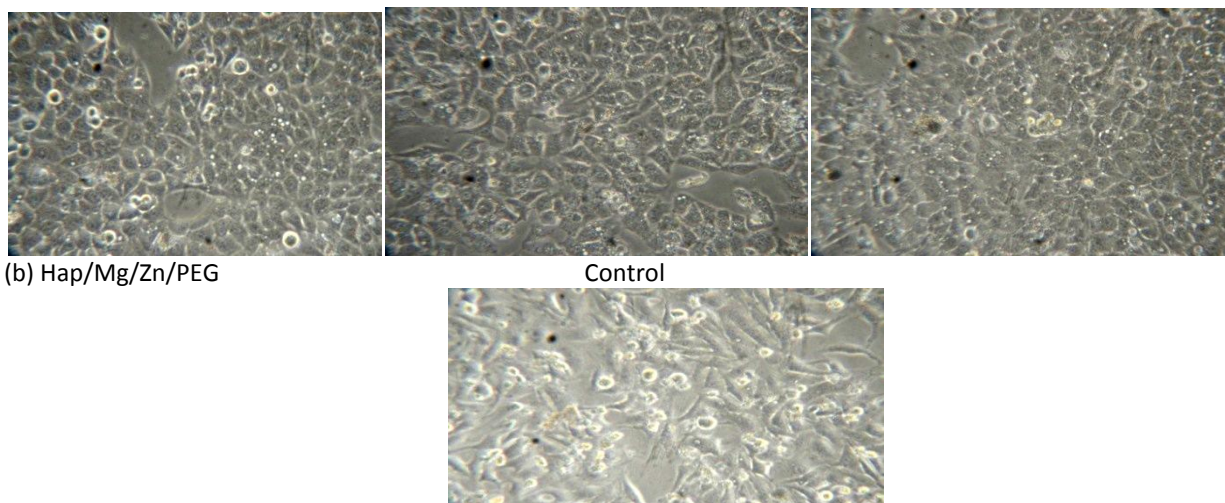


Figure 6. Anticancer images for pure HA (a), Mg/Zn/PEG/HA (b) respectively.

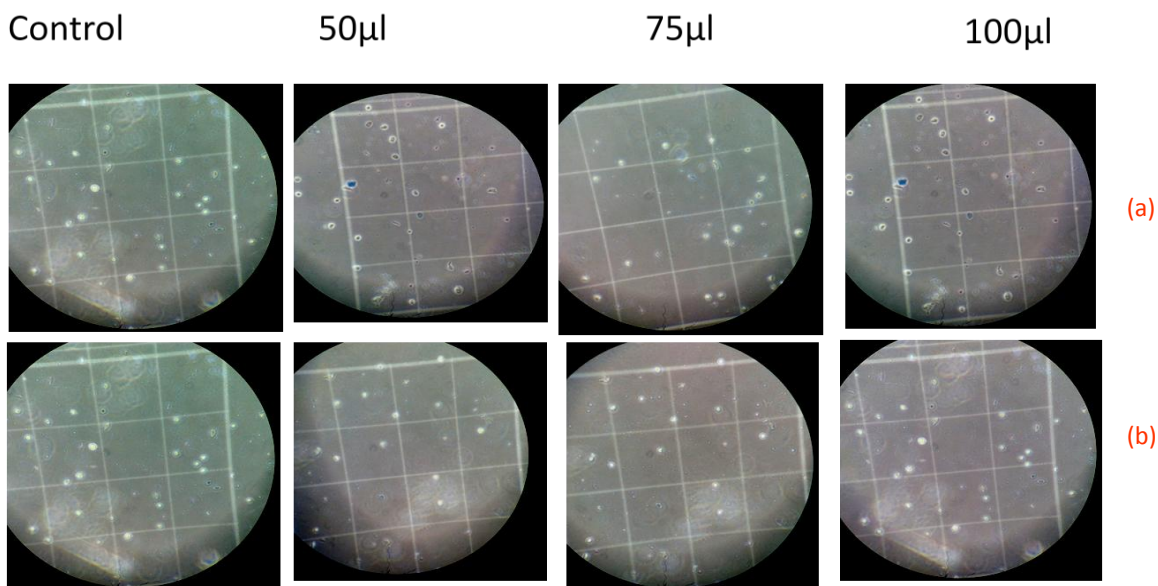


Figure 7. Cell viability images for pure HA (a), Mg/Zn/PEG/HA (b) respectively.

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