Synthesis and Characterization of Polymer Composite Doping on Hydroxyapatite For Biomedical Application

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Abstract : Dual ionic substitutions in addition to the polymer have been anticipated as a tool to improve the mechanical and biological properties of hydroxyapatite (HA). Substitution of trace elements, such as Sr, Mg, Zn ions into the structure of calcium phosphates is the subject of widespread investigation. In the present work Ca/Cu/Zn/PEG–HA nanoparticles were prepared by precipitation method, which enhances biocompatibility, bioactivity and mechanical properties. The synthesized polymer composite material was characterized by FTIR, XRD, SEM EDAX. Also, cell viability, anticancer and antimicrobial studies were carried out. The as-developed composite material can play potential role in biomedical applications.

Keywords: Hydroxyapatite, XRD, PEG, Biological activity.

I. INTRODUCTION

In biomedical industry, calcium phosphates are primarily used as bone substitutes due to their biocompatibility, low density and chemical stability[1]. Hydroxyapatite having a composition of $Ca_{10}(PO_4)_6(OH)_2$, a kind of calcium phosphate is one of the most widely investigated bioceramic materials and used for bone and tooth substitution due to the structural similarity to the mineral part of calcified tissues[2].

During the past few decades, considerable research work has been focussed towards the synthesis of different bioceramics for orthopedic applications[3-5]. Cytotoxic effect is not exhibited by HA. Moreover HA can directly bond to the bone. Unexpectedly, due to low reliability, particularly in wet environments, the HA cannot presently be used for heavy load bearing applications, like artificial teeth or bones. HA is synthesized by various methods such as hydrothermal method, hydrothermal-micro emulsion synthesis, chemical precipitation technique, biomimetic preparation, and sol–gel method [6-8]. Chemical (or wet) precipitation technique is the most popularly used technique for the synthesis of HA [9]. The raw materials used in this technique at a reasonable cost controlling the process parameters such as particle size and shape, particle distribution and agglomeration are possible to improve the properties of HA ceramic. Nanocrystalline HA powders exhibit greater surface area. Moreover, nanophase ceramics clearly represent a unique and promising class of orthopedic/dental implant formulations with improved osteointegrative properties. However, the brittleness and poor performance of mechanical stability of pure HA limit its use for the regeneration of non-load-bearing bone defects and tissue engineering applications. Composite biomaterials like metal and polymer matrix are used to improve the mechanical compatibility of nano HA. Generally, composite biomaterials are prepared by using biocompatible/biodegradable and synthetic/natural polymers. It has been broadly used in orthopedic surgery applications such as bone defect filler and as a coating for metallic prostheses to enhance their biological properties. In orthopedic surgery, the major parts of post-surgical infections are associated with the presence of implant materials, and the problem usually needed the removal of the prosthesis. Several in vitro studies reported that the silver, copper and zinc ions in the implant coatings play main role in prevention or minimization of initial bacterial adhesion [10]. Copper and zinc ions in the higher amounts are potentially toxic so the uses of those ions in the small quantities are essential for various metabolic processes in most of the living organisms [11]. Bone formation in vitro and in vivo, zinc has also a stimulatory effect in this process [12]. There are three main mechanisms for the antimicrobial activity of copper and zinc ions. Firstly, metal ions bind to proteins deactivate them. Secondly, metal ions can interact with microbial membrane, which causes structural change and permeability. Finally, metal ions interact with microbial nucleic acids, preventing microbial replication. Several new studies of research, antimicrobial activity of copper and zinc-doped hydroxyapatite were reported [13].

In addition to this, the right choice of the composition of both filler and polymer matrix are essential in addition to the processing method to obtain suitable biopolymer composites [14]. Recently, attempts have been made to develop nanocomposites, wherein mineralized nano HA particles are embedded in polyethylene glycol (PEG) polymeric matrices. Extensive studies have been made on both natural (collagen, gelatin and silk fibroin) and synthetic (polyethylene, polyamide, chitosan, polystyrene, poly[vinyl alcohol], poly[ethylene glycol/PEG] polymers to overcome the mechanical problems associated with bioceramics in bone tissue engineering applications [15-19]. Among these different raw materials, PEG remains one of the widely used polymer group of biomaterials applied for medical implants. In accordance with the related synergistic effect resulting from the combination of HA and PEG, the present work describes the synthesis of a nanostructured HA/Cu/Zn/PEG composite under controlled environment, evaluate their physical-chemical characteristics and discuss their biological potential as smart materials for biomedical applications.

II. EXPERIMENTAL

2.1. Chemicals

The chemicals used in this study were calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$, 98%, Sigma aldrich) and Copper nitrate hexahydrate ($Cu(NO_3)_2{\cdot}6H_2O$, 97%, Merck), Zinc nitrate ($Zn(NO_3)_2{\cdot}6H_2O$, 98.9%, Sigma aldrich), dipotassium hydrogen phosphate K_2HPO_4, 99%, Sigma aldrich), PEG (M.W 200) and ammonia (NH_3, 25%, Sigma aldrich). All reagents used were of analytical reagent grade without further purification and deionized water was employed as the solvent throughout the experiments.

2.2. Synthesis

Pure HA and Cu/Zn/PEG–HA were synthesized by Precipitation method. In brief, for the synthesis of pure HA, solutions of 0.5 M $Ca(NO_3)_2{\cdot}4H_2O$ and 0.3 M K_2HPO_4 were separately prepared. Then, the prepared K_2HPO_4 solution was slowly added drop wise into the $Ca(NO_3)_2{\cdot}4H_2O$ solution to produce a colloidal solution. The pH of the solution was adjusted to 10 by the addition of 0.1M NH_3.

The above reaction mixture was then stirred for 4 h and the pH was constantly adjusted and maintained at 10 during the reaction of the synthesis. NH_4^+ and NO_3^- ions were removed by washing the precipitate repeatedly with deionized water, filtered and then dried at 120◦C for about 24 h in hot air oven. Finally, dried cakes were compressed to obtain white powders. The dried white powder was calcined and sintered in a muffle furnace at 800 ◦C for 3 h. In the same way we have also prepared copper and zinc substituted HA. And also synthesized mineral [Cu (0.1M)/Zn (0.1M)] and polymer[PEG(0.1M)] substituted HA by following the above method. The reaction of pure HA can be described as follows:

$$10Ca(NO_3)_2{\cdot}4H_2O+6K_2HPO_4+NH_3 \rightarrow Ca_{10}(PO_4)_6(OH)_2+6H_2O+12KNO_3$$

Characterization techniques

The FTIR spectral characterization was performed for as-synthesized HA and Cu/Zn/PEG–HA samples by using FTIR spectrophotometer with a number of scans between 32 and 4 cm^{-1} resolutions using KBr(JASCO 6100 FTIR spectrometer) and then pressed into discs for the analysis. The phase composition, purity and the crystallinity of the as prepared HA, Cu/Zn/PEG–HA nanoparticles were determined by the XRD (Seifert, X-ray diffractometer Siemens D500 Spectrometer) in the range between $20◦ \leq 2 \leq 60◦$ with Cu K radiation generated at 35 kV and 25 mA. The crystallite size of the samples was calculated from the XRD pattern.

The morphology and elemental composition of the as-synthesized samples of HA, Cu/Zn/PEG–HA were determined using FESEM (Philips XL-305 FEG) operated at an accelerating voltage of 15 kV equipped with EDAX.

Antibacterial activity

The in vitro antibacterial activity of the as-synthesized HA, Cu/Zn/PEG–HA nanoparticles has been investigated against clinically isolated bacterial pathogen i.e., Staphylococcus epidermidis. The samples were dissolved in 1 ml of DMSO separately and mixed well which were used to perform antimicrobial screening tests. The inoculums of all microorganisms were prepared from fresh overnight broth cultures (Tripton soy broth with 0.6% yeast extract – Torlak, Belgrade) that were incubated at 37 ◦C. The resulting broth cultures were used for both tests. The agar diffusion test was performed at Muller-Hinton agar (Institute for Immunology and Virology, Torlak, Belgrade). The culture turbidity was adjusted to 0.5 McFarland equivalents (1.5 x 108 CFU).

The antibacterial activity of leaf extracts of selected plants were determined using agar well diffusion method.. A suspension of test culture (50 µl) was swabbed on the Muller 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 / volumes (25, 50, 75 µl/µg) of each sample was separately loaded into wells and allowed to diffuse at room temperature. 25 µl of ciprofloxacin (1 µg/µl) for bacteria was served as positive controls. The bacterial plates were incubated at 37° C for 24 hours. After appropriate incubation period, the diameter of zone of growth inhibition was measured (in mm).

Cell culture and in vitro cytotoxicity assay of HA/Cu/Zn/PEG :

Human cervical cancer cells (HeLa cells) were purchased from American Type of Culture collection (Manassas, VA). An in vitro cytotoxicity test method was performed for the test sample as per ISO 10993:5.

The culture medium from the L929 monolayer was replaced with fresh medium. Test sample in duplicates were added to the cells. And the cells were incubated in the growth medium containing different concentrations of HA/Cu/Zn/PEG. Afterwards , cells were washed and treated with MTT solution (1 mg/ mL, final concentration) for 4 h. Finally, the supernatant was removed, and DMSO (150 lL) was added to solubilize the formed formazan salt and read at 570 nm using a visible spectro photometer. Cytotoxicity and cell viability were calculated by using the formula:.

Cytotoxicity = [(AbsControl – AbsTreated)/ AbsControl] * 100

Cell viability= (AbsTreated / AbsControl) * 100

The cell cytotoxic level was quantified as a percentage compared to blank.

III. RESULTS AND DISCUSSION

3.1 FTIR

FTIR spectra of pure HA and Cu/Zn/PEG-HA nanocomposite are shown in Fig.1(a-d). The spectral data for HA (Fig. 1a) clearly revealed the characteristic absorption corresponding to phosphate and hydroxyl groups. The FTIR spectra of pure nano HA and nano PEG/HA composites are as shown in Fig. 1a and 1c. The peak at 471-472.06 cm^{-1} corresponds to PO_4^{3-} group in HA. The bands at 1036 to 1048 and 566 to 570 cm^{-1} are attributed, respectively to ν3 and ν4 P-O stretching vibration of regular tetrahedral PO_4^{3-} groups. The band observed at 633 cm^{-1} corresponds to O-P-O bending and ν1 symmetric P-O stretching modes. The ν1 symmetric stretching mode of phosphate group was observed at 963.26cm^{-1}. The x band observed at 1385 cm^{-1} is due to the stretching mode of carbonate, which may be due to the acquisition of air during mineral precipitation. Similarly, the bands observed at 1413 and 857 to 874 cm^{-1} are assigned to carbonate ions. The bands observed in the region between 2067 and 2069 cm^{-1} are related to their harmonic overtones or combination bands. The lattice H_2O exists in the range of 1603 to 1608 cm^{-1}, while the bands observed at 3394 to 3572.44 cm^{-1} overlap the –OH group. The band at 2855 and 2925 cm^{-1} corresponds to C-H stretching of PEG. A new peak of stretching is observed at 2926 cm^{-1}, when the PEG was added. This indicates the chemical bond interactions between HA and PEG.

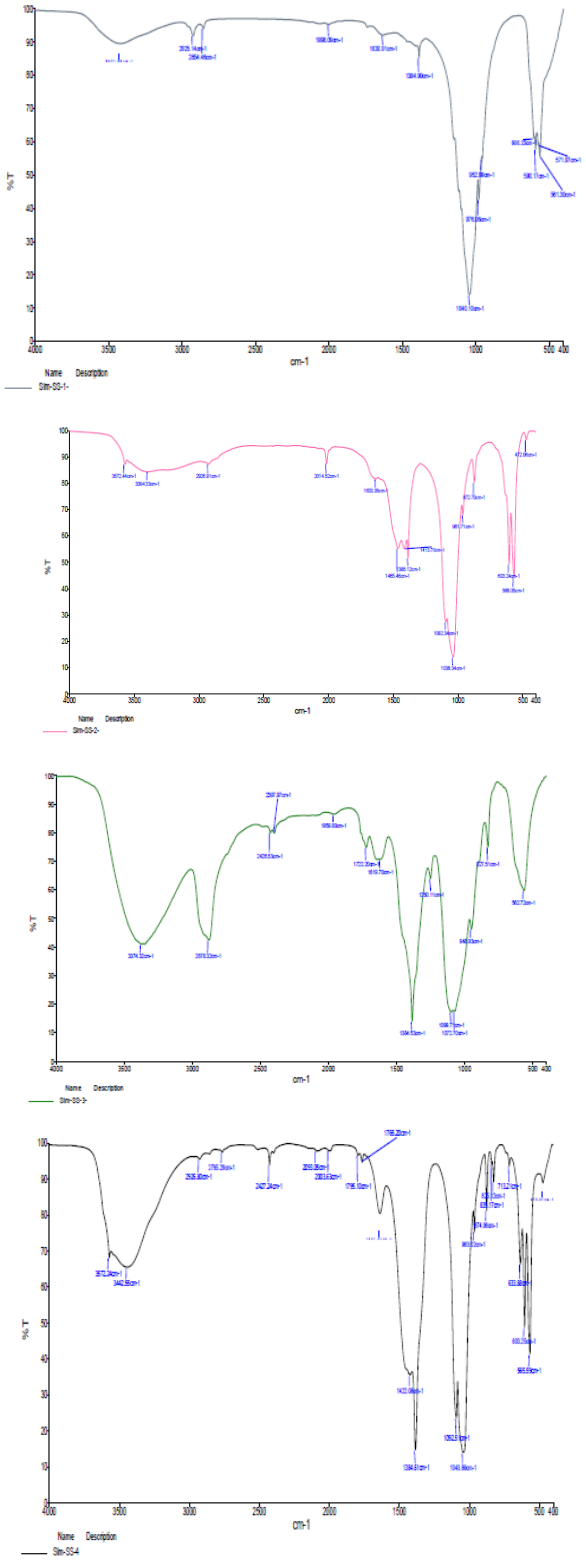
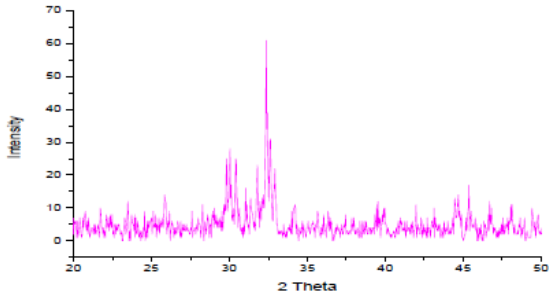


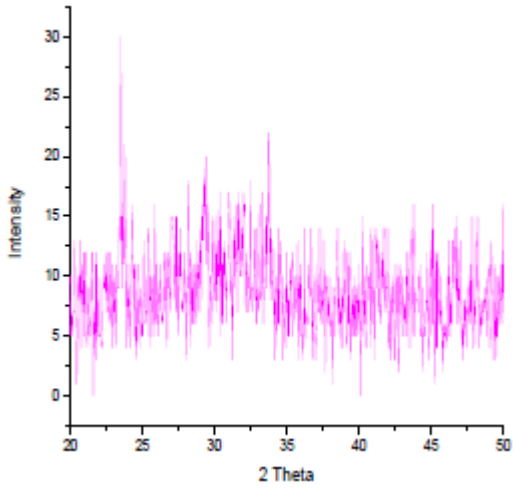
Fig. 1. FTIR spectra of (a) pure HA, (b) HA/Cu/Zn, (c) HA/PEG, (d) HA/Cu/Zn/PEG.

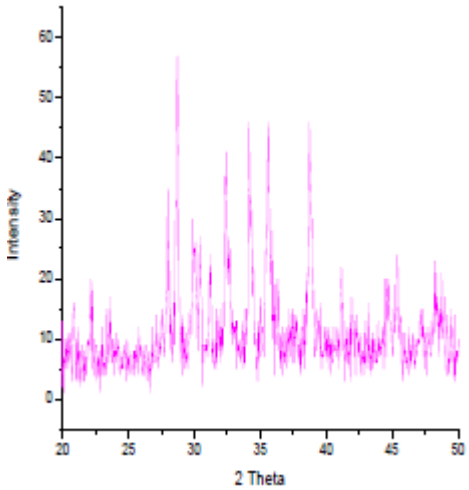
3.2 XRD

X-ray diffraction pattern for the pure HA, HA/Cu/Zn, HA/PEG and HA/Cu/Zn/PEG composites, are shown in Fig (2a-d). Well-resolved characteristic peak of more intensity was obtained for the 2 theta values are 31.77,

37.9, 30, 31.5, 35.0, 29.8, 31.2. From these values sharp intense peak of 31.77, 29.8 values exhibit pure HA. The polymer composite peaks are observed at 37.90˚. The broad peaks and very wide baseline show the presence of inorganic component. The patterns are in good agreement with JCPDS (09-0432) and confirmed to the pure phase of HA polymer matrix and composite. The particle size of HA crystalline size is (20 - 100 nm) in length and width of (2 - 4 nm).







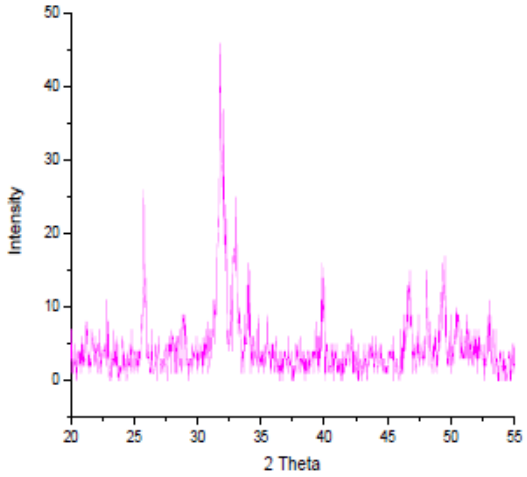
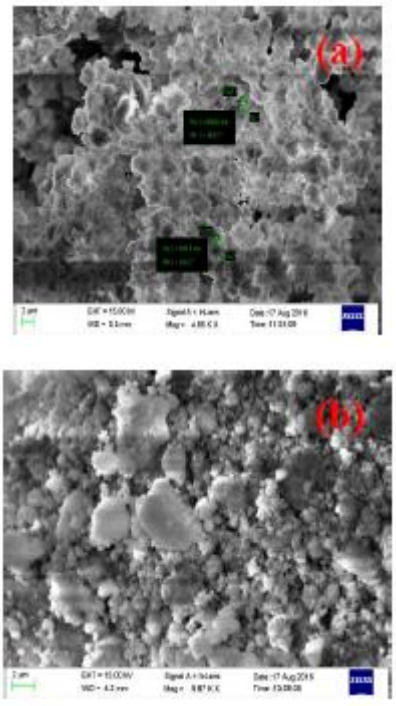


Fig. 2 .XRD pattern of (a) pure HA, (b) HA/Cu/Zn, (c)HA/Cu/Zn/PEG, (d) HA/PEG

3.3 SEM

The surface morphology and crystallite morphology of the synthesized materials were investigated by scanning electron microscope (SEM) operating at an accelerating voltage of 20 KV. The SEM image of pure HA(a), HA/Cu/Zn(b) , HA/PEG(c), HA/Cu/Zn/PEG(d) nanoparticles were shown in Fig.3(a-d). SEM micrograph shows plate like structure with nano rod size ranging between 2-20 µm. It can be seen that all the samples consist of similar agglomerates that are composed of fine crystallites that cannot be seen individually in microphotographs because of their exceptionally small size.



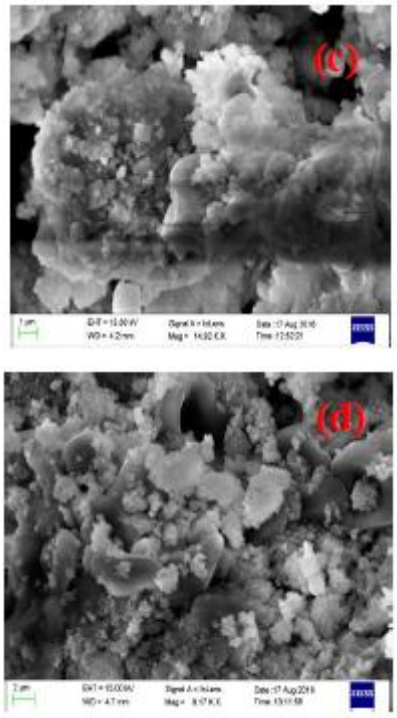
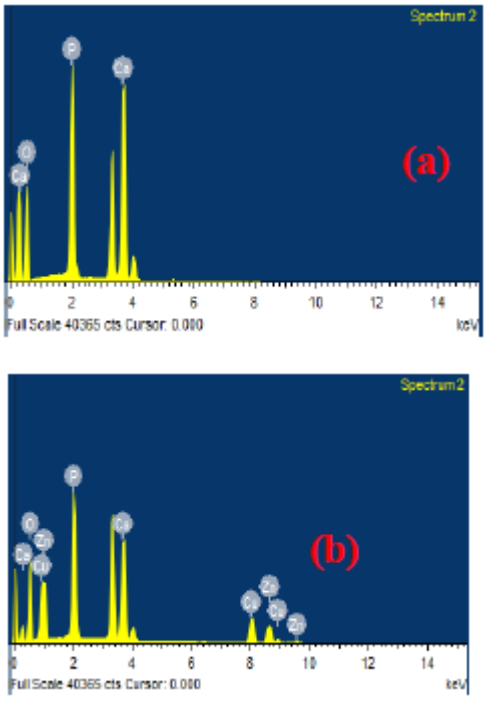


Fig. 3. FESEM image for (a) pure HA, (b) HA/Cu/Zn, (c) HA/PEG, (d) HA/Cu/Zn/PEG.

The elemental composition of pure HA(a), HA/Cu/Zn(b) , HA/PEG(c), HA/Cu/Zn/PEG(d) nanoparticles were investigated using EDAX study with optimal concentration (0.1 M),and their spectra are shown in Fig.4(a-d). The EDAX pattern confirms the presence of calcium (Ca), copper (Cu), zinc (Zn), phosphorus (P) and oxygen (O) and carbon(C) in the structure of the as-developed nano composite.



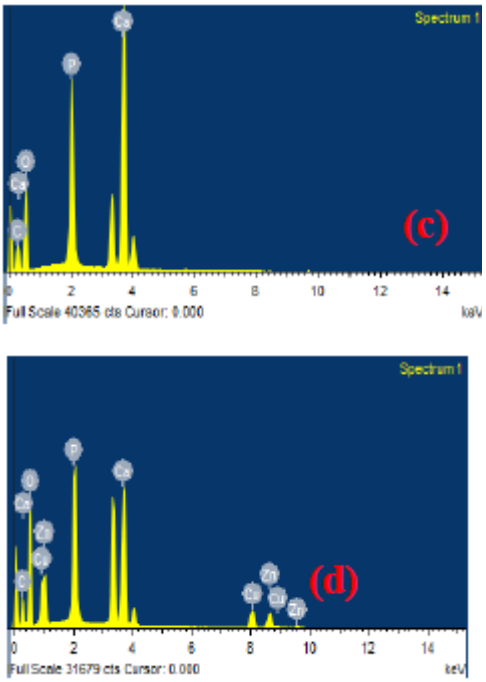


Fig.4. EDAX image for pure (a) HA, (b) HA/Cu/Zn, (c) HA/PEG, (d) HA/Cu/Zn/PEG.

3.4 Antimicrobial study:

Dissolution studies on antimicrobial materials are a necessary step in assessing the correctness of their use. Antimicrobial materials with low solubility have good antibacterial properties for a longer period. The results of antimicrobial disk diffusion tests showed that it affects S. epidermidis. The average inhibition zones were 2 and 3 mm for S. epidermidis. The results of the quantitative antimicrobial tests carried out in liquid medium for all metal-doped HA samples are shown in Fig.5(a-d) .

Antimicrobial activity of HA/Cu/Zn/PEG is more when compared to HA/Cu/Zn and HA/PEG, Where as the Pure HA shows lower inhibition. It is proposed that greater quantity of incorporated Zn(II) ions are in the surface of ZnHA sample particles. Several authors established that zinc ions and copper ions are mostly incorporated in the surface of hydroxyapatite and hinder the crystal growth.

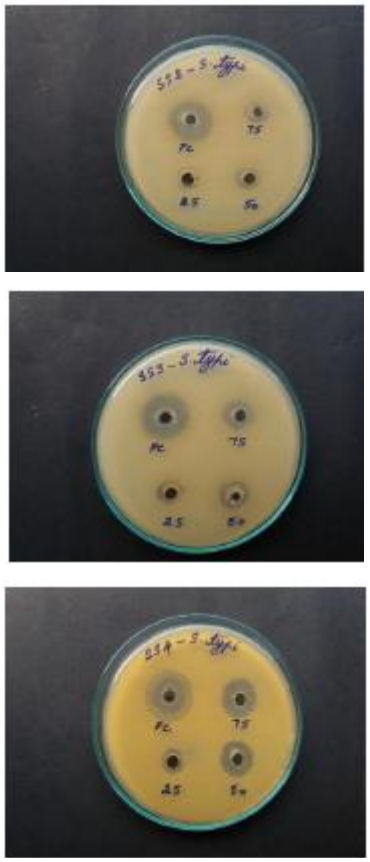
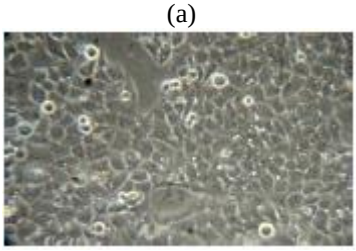


Fig.5.Antimicrobial image for (a) pure HA, (b) HA/Cu/Zn, (c)HA/PEG, (d)HA/Cu/Zn/PEG.

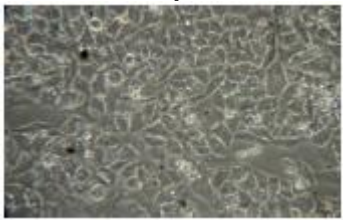
3.5 Cytotoxicity:

The cytoxicity levels of pure HA and HA/Cu/Zn/PEG nanohybrids were evaluated against HeLa cell lines using MTT assay. The method is based on the reduction of the yellow tetrazolium salt of MTT to violet crystals product by the mitochondrial succinate dehydrogenase in viable cells. The results of in vitro cytotoxicities of pure HA and HA/Cu/Zn/PEG nanohybrids against HeLa cells for 24h, are demonstrated in Fig.6. The pure HA showed no toxicity, which indicates that it did not induce any effect upon cellular viability and proliferation at 24 h against the Hela cell lines. It can be observed that the incubation time (24h) increased the cytotoxicities of HA/Cu/Zn/PEG against the selected cell lines. The cell lines showed a dose-dependent cytotoxicity toward HA/Cu/Zn/PEG (Fig.6). HA shows no cytotoxicity against HeLa cells at 24 h contact at different concentrations, but HA/Cu/Zn/PEG shows moderate cytotoxicity against HeLa cells at 24 h than pure HA.

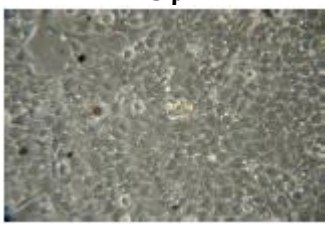
(a)



50µl

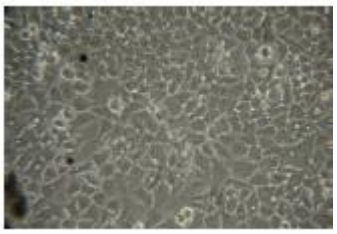


75 µl

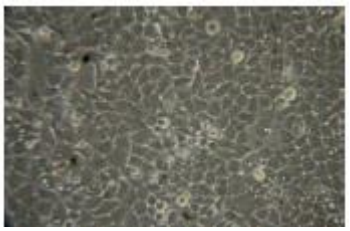


100 µl

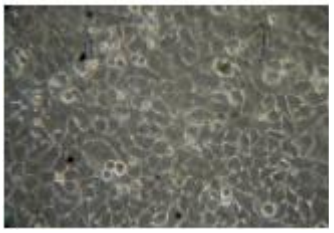
(b)



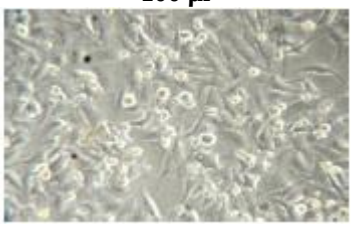
50µl



75 µl



100 µl



control

Fig.6. (a) Pure HA, (b) HA/Cu/Zn/PEGnanohybrids images, cytotoxicity against HeLa cells at 24 h

3.6 Cell viability:

The cell viability of pure HA and Cu/Zn/PEG/HA nanocomposites were tested against HeLa cell lines. Minimum essential medium is supplemented with foetal bovine serum. For this method, Trypan blue dye was

used. The results of in vitro cell viabilities of pure HA and Cu/Zn/PEG/HA nanocomposites against HeLa cells for 24h are shown in Fig.7 (a-b). The pure HA themselves did not show any harmful effect towards HeLa cells in a concentration range 50-100 µl and even at such higher concentration as 100 µl, HA still no suppression on cells. The cell viabilities for Cu/Zn/PEG/HA nanomaterials at the concentration of 50-100 µl are shown in Fig.7b.The overall look on the data revealed that Cu/Zn/PEG/HA nanocomposite had greater suppression efficiency on HeLa cells. It was observed that Cu/Zn/PEG/HA nano composite with good controlled release property also exhibited moderate anticancer activity in vitro bioassay test.

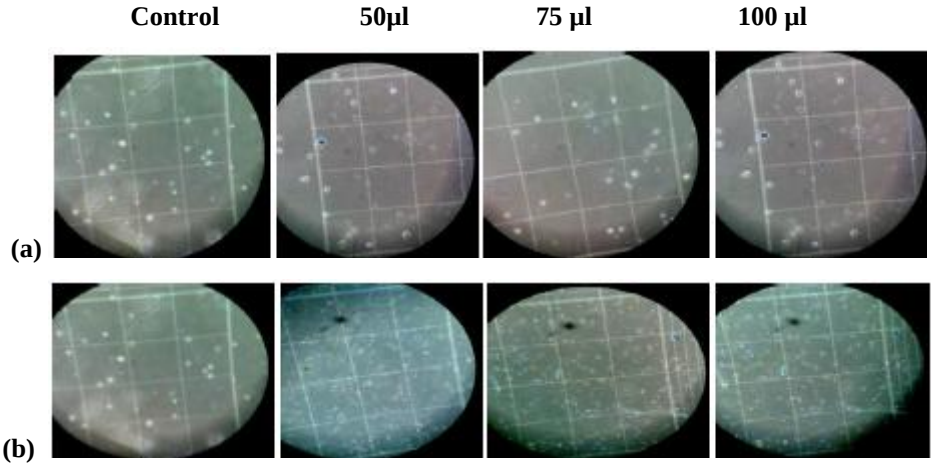


Figure.7. (a) Pure HA, (b) HA/Cu/Zn/PEGnanohybrids images, cell viability against HeLa cells at 24 h

IV. CONCLUSION

The analysis of XRD, FTIR and SEM showed that particles of all samples are of nano size and homogeneous in composition. Chemical analysis showed that the copper and zinc ions are fully incorporated into the PEG doped HA samples. The antibacterial results reveal that the as-synthesized HA/Cu/Zn/PEG powder exhibited a strong antibacterial activity against the S. epidermidis. Finally it can be concluded that the antimicrobial efficiency of the materials is a function of the types and quantities of metal ions as well as strain of microorganism. In vitro studies of the synthesized HA/Cu/Zn/PEG nanomaterial suggested their promising activity against human cervical cancer cells (HeLa cells). This facile strategy for the preparation of nanohybrids would find widespread applications in diverse areas.

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