Supplementary Information

Synthesis, Characterization and *in vitro* screening of nano-Hydroxyapatite/Chitosan/Euryale ferox nanoensemble- An inimitable approach for Bone Tissue Engineering

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1. Experimental

1.1. Synthesis of nano-hydroxyapatite

Wet chemical synthesis was performed based on the use of $Ca(NO_3)_2.4H_2O$ and $(NH_4)_2HPO_4$ (DAHP) precursors for the preparation of n-HA nanocrystals where 0.6mol of $Ca(NO_3)_2.4H_2O$ was added drop-wise to 0.4mol of DAHP at constant stirring in order to obtain Ca/P ratio of 1.67. The pH value of the mixture was maintained above 10 throughout the precipitation process by the addition of ammonia (25%) solution. The resultant precipitate obtained by vacuum filtration was rinsed with deionised water and oven dried at 85 ˚C.[1,2]

2. Characterizations

2.1 Agar Diffusion Method

The inoculum was prepared by diluting the overnight cultures with sterile normal saline to a 0.5 McFarland standard. The agar petri plates were prepared by spreading the 1×10^8 CFU per 50 µL of mature broth culture of specific bacterial strains with a sterile L-shaped glass rod. Now, the stock solution of formulations of n-HA/CS/EF nanocomposite suspended in sterile PBS was used to evaluate the antibacterial activity. The experimental procedure was performed under sterile conditions using bio-safety level 2 (BSL-2) hoods. The petri plates were incubated at 37 ˚C overnight. The susceptibility of our formulation was determined on the basis of zone of clearance developed against human pathogenic strains E.*coli* and S.*aureus*. The inhibition zone was analysed thrice and the average was calculated for the antibacterial activity and compared with the reference drug vancomycin as a control.[3]

2.2. RBC Lysis test

In vitro erythrocyte lysis test was carried out as a primary toxicity test of the as-synthesized nanocomposite formulations, which is evaluated by quantifying the haemoglobin released as a result of membrane leakage or disruption caused by incorporation of low doses of the n-HA/CS/EF nanocomposite. In brief, centrifugation of the fresh blood procured from a healthy rabbit collected in anticoagulant solution (EDTA) was performed at 1000g for 10 min at 4^{o}C. Both buffy coat and plasma were discarded. Washed erythrocytes were diluted with isotonic buffer (20 mM PBS) to prepare 50% haematocrit. n-HA/CS/EF nanocomposite was incubated in RBC suspension with different concentrations at 37 ˚C for 1 h to determine the degree of haemolysis.[4] After the centrifugation of incubated solutions at 1500g for 15min, the supernatant obtained was characterized by UV-visible spectroscopy (λ_{max}= 576nm). The haemolysis percent was measured by the following equation:

$$\% \text{ Haemolysis} = \{(Abs(T)- Abs(C) / Abs(100\%) – Abs (C)\}* 100$$

where, Abs(T) = Absorbance of the supernatant from samples incubated with n-HA/CS/EF.

Abs(c) = Absorbance of the supernatant from controls (normal saline).

Abs(100%) = Absorbance of the supernatant of controls incubated in the presence of 1% Triton® X-100, which causes complete lysis of RBCs (total lysis).

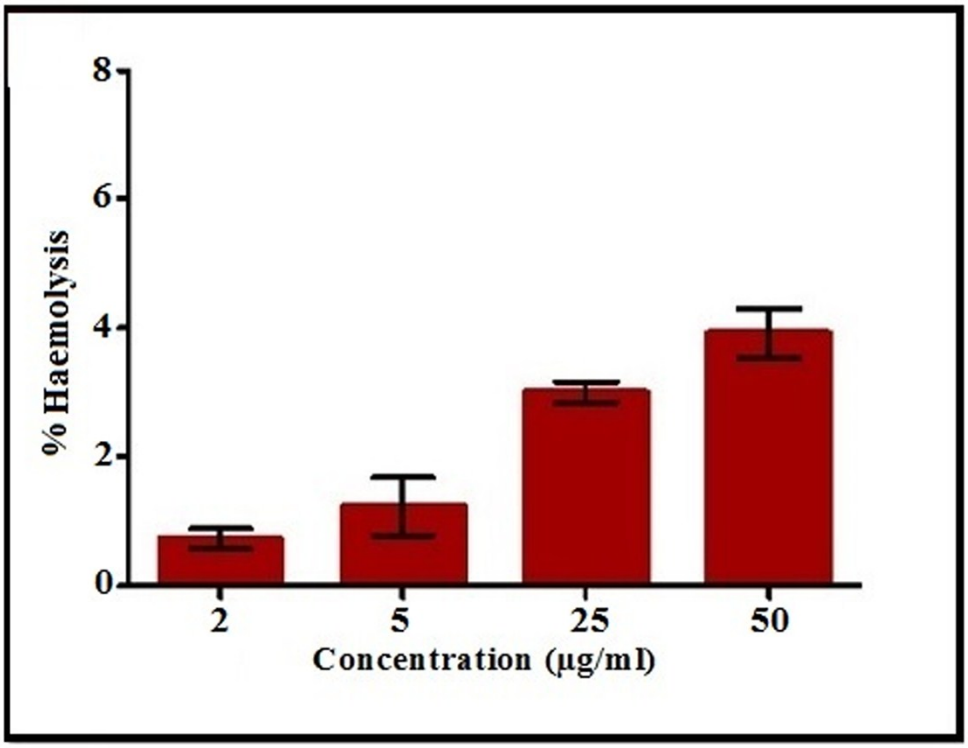

Fig. S1. RBC lysis of n-HA/CS/EF nanocomposite

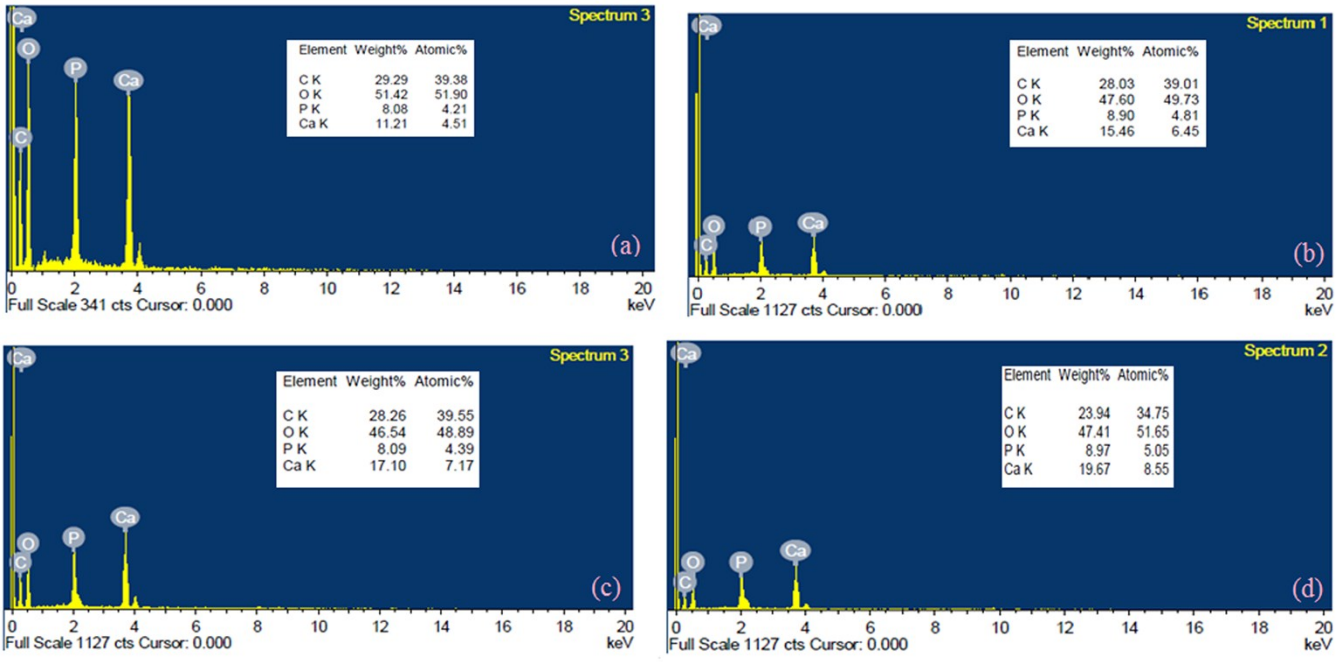

Fig. S2. EDX of (a) n-HA/CS (b) n-HA/CS/EF nanocomposite and their respective SBF study after 30 days (c-d).

References

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