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Comparative *in vitro* Cytotoxicity and Antioxidant Activity of Biologically Derived Nanohydroxyapatite and Commercial Nanohydroxyapatite

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ABSTRACT

Background: The poultry industry generates vast amounts of waste, including eggshells, which can be utilized as a source of nanohydroxyapatite (nHAp). This sustainable approach can reduce waste disposal costs, minimize environmental pollution and provide valuable biomaterials for various applications.

Methods: Indian Hen eggshells, hybrid Hen eggshells and a commercially available nHAp sample (as a reference) were used in this study. The biologically derived nHAp samples were synthesized *via* a precipitation method and the antioxidant activity and cytotoxicity of the nHAp samples were evaluated *via* the DPPH (1,1-Diphenyl-2-picrylhydrazyl) assay and MTT (Cell sensitivity) assay, respectively.

Result: The results of the DPPH assay revealed that 3.125 µg/mL ascorbic acid was equivalent to 250 µg/mL Indian Hen eggshell-derived nHAp (IE1), whereas 1.56 µg/mL ascorbic acid was equivalent to 50 µg/mL hybrid Hen eggshell-derived nHAp (HE1). The reference sample showed that 0.78 µg/mL ascorbic acid was equivalent to 1 µg/mL ascorbic acid. The MTT assay revealed that all three nHAp samples were noncytotoxic and presented varying IC_{50} values (IE1: 1797 µg/mL, HE1: 1348 µg/mL, reference: 1972 µg/mL), indicating potential suitability for biomedical applications.

Key words: Antioxidant activity, Bone tissue engineering, Cytotoxicity, Hybrid hen eggshells, Indian hen eggshells, Nanohydroxyapatite.

INTRODUCTION

In the sphere of biomedical research, creating innovative biomaterials is vital for addressing challenging healthcare issues. Hydroxyapatite (HAp) is one such biomaterial that has drawn much attention because of its remarkable properties, particularly in regard to hard tissue implants. The different components of HAp as biomaterials are explained in this introduction, along with the intricacies of oxidative stress and its effects during bone implantation, the importance of antioxidants and HAp limits. In addition to outlining the goals and possible contributions of this research, it highlights a novel metal doping method to increase HAp performance.

A common biomaterial for hard tissue implants is HAp, a calcium phosphate crystal with the chemical formula $Ca_{10}(PO_4)6(OH)_2$ (Gülçin *et al.,* 2010). It is highly biocompatible and biodegradable because of its crystalline structure, which resembles the mineral makeup of bone tissue. Because of these intrinsic properties, HAp is an ideal choice for orthopedic and dental applications, where it can be used as a base for dental restorations, coatings for orthopedic implants and bone transplants.

As an alternative to bone transplantation, HAp has become more significant in orthopedics because of its capacity to promote osseointegration and bone regeneration. HAp enhances the compatibility of orthopedic implants with host bone tissue when used as a covering material, which promotes recovery. HAp is utilized in dentistry for restorative procedures and dental implants since it is both biocompatible and aesthetically similar to human teeth. HAp has also shown promise in drug delivery systems, where it functions as a carrier for pharmaceuticals, guaranteeing regulated release for better therapeutic

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results. Oxidative stress is a fundamental physiological condition that results from an imbalance between the body's defense mechanisms and the generation of reactive oxygen species (ROS) (Schieber *et al.,* 2014).

ROS include a variety of species, such as hydrogen peroxide (H2O2), hydroxyl radicals (•OH) and superoxide anions (O2•-). Although ROS are necessary for regular physiological functions, excess ROS can result in oxidative stress, which can harm cells and cause a variety of diseases (Barnes, 2013; Kramer *et al.,* 2014; Machado *et al.,* 2012).

Because ROS are produced after bone implantation as part of the body's immune response and wound healing processes (Mahanty *et al.,* 2023), serve a dual function in tissue repair and can cause oxidative stress and inflammation if their levels are not properly regulated (Jiang *et al.,* 2002), it is crucial to comprehend how ROS and bone implantation interact in order to improve implant outcomes. Implant-related oxidative stress can result in oxidative damage, which can affect the success of the implant and the healing process (Krishani *et al.,* 2023).

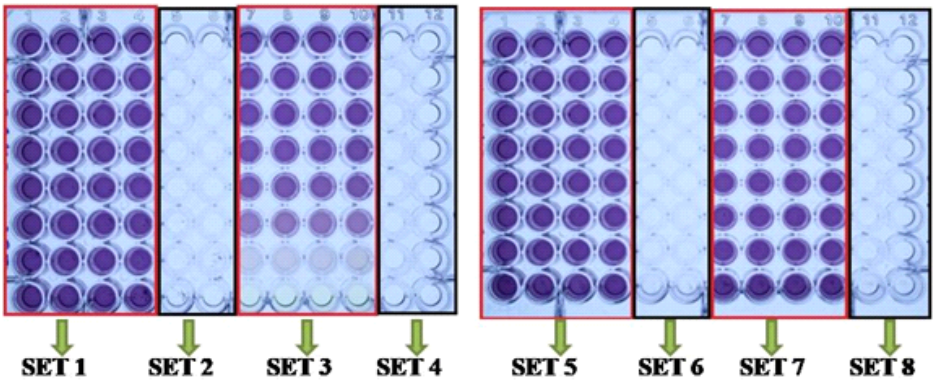
The body uses antioxidants as a natural defense mechanism against oxidative stress (Taladrid *et al.,* 2023). By providing electrons to ROS, they neutralize them and stop oxidative damage to biological components. Enzymatic antioxidants such as catalase and superoxide dismutase work in tandem with nonenzymatic antioxidants such as glutathione, polyphenols and vitamins C and E to preserve redox balance (Poljsak *et al.,* 2013) (Hasanuzzaman *et al.,* 2020).

To combat the oxidative stress caused by the implantation procedure and subsequent tissue healing, antioxidants are essential during bone implantation. The importance of antioxidant techniques in implantology is highlighted by the fact that imbalances in the antioxidant defense system can lead to increased ROS levels and compromised healing. HAp has limitations in regard to hard tissue implants, despite its clear advantages. Its low mechanical strength is a major drawback that restricts its applicability in load-bearing applications. Additionally, over time, HAp brittleness may result in wear or fracture. Additionally, because ROS generation is elevated during implantation procedures, HAp is unable to effectively counteract oxidative stress, which is a significant issue. The antioxidant activity of nHAp can be evaluated *via* the DPPH radical scavenging assay, which provides insight into its potential to neutralize free radicals. Building on this concept, we investigated the biocompatibility and osteogenic potential of nHAps using the MG-63 human osteoblast-like cell line (Kumar *et al.,* 2024).

A popular human osteoblast-like cell model, the MG-63 cell line, is frequently used to study the activity of bone cells and their interactions with biomaterials. Nanohydroxyapatite (nHAp) is commonly used in combination with MG-63 cells to evaluate its osteogenic potential and biocompatibility. By using an *in vitro* method, scientists can clarify how nanosized hydroxyapatite interacts with bone cells, offering important new information on its possible use in bone tissue engineering (Acharya *et al.,* 2022).

In this study, we performed a DPPH radical scavenging assay and an MTT assay for three samples of nano-hydroxyapatite. The first sample was synthesized from Indian hen eggshell (IE1), the second was synthesized from hybrid or broiler hen eggshell (HE1) *via* the precipitation method and the third sample was the reference sample nano-Xim HAp 202 (a gift sample from Fliidinova, Portugal).

In this study, we performed a DPPH radical scavenging assay and an MTT assay for three samples of nanohydroxyapatite. The first sample was synthesized from Indian hen eggshell (IE1), the second was synthesized from hybrid or broiler hen eggshell (HE1) *via* the precipitation method described in the article (Patil *et al.*, 2025), and the third sample was the reference sample nano-Xim HAp 202 (a gift sample from Fliidinova, Portugal).



SET: 1) Reference sample with DPPH; 2) reference sample without DPPH; 3) ascorbic acid (Standard) with DPPH; 4) ascorbic acid (Standard) without DPPH; 5) HE 1 sample with DPPH; 6) HE 1 sample without DPPH; 7) IE 1 sample with DPPH; 8) HE 1 sample without DPPH.

Fig 1: 96-well plates for the DPPH assay after incubation.

MATERIALS AND METHODS

Materials

2,2-diphenyl-1-picrylhydrazyl (DPPH) Dimethyl Sulfoxide (DMSO), Sabouraud dextrose agar Plate-(SDA) (Sisco Research Laboratories Pvt. Ltd. (SRL)-India), Methanol and ascorbic acid (S D fine-chem limited), MG63 (Procured from NCCS Pune) cell line, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS) (Purches from HIMEDIA). (SRL Chem, Cat no.-19427) plates. Fungal Culture (*C. albicans*, MTCC 854) Procured from Microbial Type Culture Collection and Gene Bank (MTCC)-Chandigarh and Amphotericin B-(Amphocare) The study of sample preparation of n-HAp was conducted in

Table 1: Absorption values of standard and test samples by the microplate reader.

Sample	Test replicates (Absorption value)					Blank (Absorption value)		
conc.	1	2	3	4	Average	1	2	Average
Ascorbic acid (Standard)								
0	0.677	0.67	0.66	0.647	0.6635	0.088	0.078	0.083
0.78	0.619	0.618	0.629	0.607		0.044	0.043	0.0435
1.56	0.617	0.65	0.607	0.654		0.079	0.057	0.068
3.125	0.576	0.579	0.602	0.609		0.05	0.05	0.05
6.25	0.562	0.564	0.544	0.566		0.046	0.056	0.051
12.5	0.438	0.463	0.45	0.452		0.04	0.041	0.0405
25	0.196	0.212	0.19	0.215		0.035	0.038	0.0365
50	0.156	0.137	0.095	0.102		0.045	0.051	0.048
Control value of ascorbic acid= 0.6635--0.083= 0.5805								
IE1								
0	0.633	0.649	0.646	0.644	0.643	0.052	0.054	0.053
1	0.617	0.606	0.619	0.634		0.034	0.035	0.0345
10	0.643	0.616	0.641	0.613		0.051	0.051	0.051
50	0.649	0.649	0.671	0.628		0.077	0.082	0.0795
100	0.682	0.679	0.678	0.696		0.121	0.128	0.1245
250	0.869	0.846	0.875	0.852		0.263	0.36	0.3115
500	0.931	0.921	0.909	0.902		0.367	0.379	0.373
1000	1.204	1.204	1.226	1.216		0.704	0.652	0.678
Control value of IE1= 0.643--0.053= 0.590								
HE1								
0	0.628	0.632	0.625	0.634	0.62975	0.056	0.052	0.054
1	0.625	0.62	0.617	0.599		0.044	0.043	0.0435
10	0.621	0.626	0.613	0.633		0.059	0.055	0.057
50	0.614	0.631	0.618	0.635		0.057	0.067	0.062
100	0.642	0.646	0.657	0.638		0.093	0.088	0.0905
250	0.675	0.665	0.677	0.672		0.128	0.126	0.127
500	0.716	0.714	0.702	0.704		0.176	0.176	0.176
1000	0.901	0.884	0.906	0.905		0.362	0.377	0.3695
Control value of HE1= 0.62975--0.054= 0.57575								
Reference sample								
0	0.665	0.651	0.654	0.675	0.66125	0.083	0.092	0.0875
1	0.613	0.621	0.622	0.612		0.051	0.046	0.0485
10	0.619	0.611	0.618	0.613		0.053	0.05	0.0515
50	0.605	0.603	0.602	0.608		0.047	0.044	0.0455
100	0.626	0.622	0.612	0.602		0.07	0.054	0.062
250	0.619	0.611	0.657	0.64		0.12	0.048	0.084
500	0.617	0.627	0.636	0.649		0.063	0.121	0.092
1000	0.625	0.623	0.616	0.605		0.087	0.084	0.0855
Control value of the reference sample = 0.66125--0.0875=0.57375.								

Note: The control value of the standard or sample was obtained *via* subtraction of the average absorption value of the blank from the average absorption value of the sample containing a zero-concentration standard or test sample.

Pharmaceutical Department of Satara College of Pharmacy, Satara, Maharashtra and study conducted from November 2022 to January 2025.

2,2-Diphenyl-1-picrylhydrazyl assay

In a 96-well plate, 0.1 ml of 0.1 mM DPPH solution was mixed with 5 µl of a different stock of the test chemical (as stated in Table 1). The reaction was set up in quadruplicate and duplicate blanks were made with 0.2 ml of methanol and 10 µl of standard/sample at various concentrations (Fig 1 and Table 1). Wells without reagents (DPPH) were referred to as blanks, while wells without treatment were referred to as controls (Zero-concentration samples). The plate was left in the dark for half an hour. A microplate reader (µMark, Bio-Rad) was used to measure decolorization at 517 nm at the final stage of incubation. A total of 20 µl of deionized water was added to the reaction mixture as a control.

Table 2: Corrected absorption values of standards and test samples.

Sample conc.	1	2	3	4
Corrected absorption values of ascorbic acid (Standard)				
0	0.594	0.587	0.577	0.564
0.78	0.5755	0.5745	0.5855	0.5635
1.56	0.549	0.582	0.539	0.586
3.125	0.526	0.529	0.552	0.559
6.25	0.511	0.513	0.493	0.515
12.5	0.3975	0.4225	0.4095	0.4115
25	0.1595	0.1755	0.1535	0.1785
50	0.108	0.089	0.047	0.054
Corrected absorption values of IE1				
0	0.58	0.596	0.593	0.591
1	0.5825	0.5715	0.5845	0.5995
10	0.592	0.565	0.59	0.562
50	0.5695	0.5695	0.5915	0.5485
100	0.5575	0.5545	0.5535	0.5715
250	0.5575	0.5345	0.5635	0.5405
500	0.558	0.548	0.536	0.529
1000	0.526	0.526	0.548	0.538
Corrected absorption values of HE1				
0	0.574	0.578	0.571	0.58
1	0.5815	0.5765	0.5735	0.5555
10	0.564	0.569	0.556	0.576
50	0.552	0.569	0.556	0.573
100	0.5515	0.5555	0.5665	0.5475
250	0.548	0.538	0.55	0.545
500	0.54	0.538	0.526	0.528
1000	0.5315	0.5145	0.5365	0.5355
Corrected absorption values of reference				
0	0.5775	0.5635	0.5665	0.5875
1	0.5645	0.5725	0.5735	0.5635
10	0.5675	0.5595	0.5665	0.5615
50	0.5595	0.5575	0.5565	0.5625
100	0.564	0.56	0.55	0.54
250	0.535	0.527	0.573	0.556
500	0.525	0.535	0.544	0.557
1000	0.5395	0.5375	0.5305	0.5195

Note: Corrected absorption values of the standard and sample were obtained by subtracting the average absorption value of the blank at each concentration (from Table 1) from the respective absorption value of the standard or sample. These corrected values used for calculating the % RSA.

The formula was used to determine the percentage of radical scavenging activity (RSA)(Shi *et al.,* 2023; Abdulkadir *et al.,* 2020; Singh *et al.,* 2024).

$$\frac{\text{Abs control - Abs sample}}{\text{Abs control}} \times 100$$

Abs sample = Absorbance of sample.
Abs control = Absorbance of control.

Compared with that of the control, the scavenging activity was displayed as "% inhibition". IC_{50} was computed with graph pad prism 6 (software). A graph was prepared between the X-axis (sample concentration) and the Y-axis (% inhibition with respect to the control).

Cell sensitivity assay

An MTT assay was used to assess the cytotoxicity of the samples to the MG63 cell line. For 24 hours, the cells (10000 cells/well) were cultivated in 96-well plates at 37°C with 5% CO_2 in DMEM supplemented with 20% FBS and 1% antibiotic solution. The cells were treated with varying quantities the next day (concentrations are specified in Table 4). The cells without MTT were referred to as the blank and the untreated cells were referred to as the control. After a 24-h incubation period, the cell culture was supplemented with MTT solution and incubated for an additional 2 h. After the culture supernatant was removed at the end of the experiment, the cell layer matrix was

Table 3: Representative % RSA of standard and test samples.

Sample conc.	1	2	3	4	% RSA (Mean of N*)
			Ascorbic acid (Standard)		
0	-2.325581	-1.11972	0.602929	2.842377	0.00
0.78	0.8613264	1.033592	-0.86133	2.92851	0.99
1.56	5.4263566	-0.2584	7.149009	-0.94746	2.84
3.125	9.3884582	8.871662	4.909561	3.703704	6.72
6.25	11.972438	11.62791	15.07321	11.28338	12.49
12.5	31.524548	27.21792	29.45736	29.11283	29.33
25	72.523686	69.76744	73.55728	69.25065	71.27
50	81.395349	84.66839	91.90353	90.69767	87.17
			IE1		
0	1.6949153	-1.01695	-0.50847	-0.16949	0.00
1	1.2711864	3.135593	0.932203	-1.61017	0.93
10	-0.338983	4.237288	0	4.745763	2.16
50	3.4745763	3.474576	-0.25424	7.033898	3.43
100	5.5084746	6.016949	6.186441	3.135593	5.21
250	5.5084746	9.40678	4.491525	8.389831	6.95
500	5.4237288	7.118644	9.152542	10.33898	8.01
1000	10.847458	10.84746	7.118644	8.813559	9.41
			HE1		
0	0.3039514	-0.39079	0.825011	-0.73817	0.00
1	-0.998697	-0.13026	0.390795	3.517152	0.69
10	2.0408163	1.172384	3.430308	-0.04342	1.65
50	4.1250543	1.172384	3.430308	0.477638	2.30
100	4.2118975	3.517152	1.6066	4.906644	3.56
250	4.8198003	6.556665	4.472427	5.34086	5.30
500	6.2092922	6.556665	8.640903	8.29353	7.43
1000	7.6856274	10.6383	6.817195	6.990881	8.03
			Reference sample		
0	-0.653595	1.786492	1.263617	-2.39651	0.00
1	1.6122004	0.217865	0.043573	1.786492	0.92
10	1.0893246	2.48366	1.263617	2.135076	1.74
50	2.4836601	2.832244	3.006536	1.960784	2.57
100	1.6993464	2.396514	4.139434	5.882353	3.53
250	6.7538126	8.148148	0.130719	3.093682	4.53
500	8.496732	6.753813	5.185185	2.91939	5.84
1000	5.9694989	6.318083	7.538126	9.455338	7.32

Note: 1) N* Mean absorption of four samples.

dissolved in 100 µl of DMSO and the absorbance was measured at 540 nm (Imam *et al.,* 2011) (Morgan *et al.,* 2003). An ELISA plate reader (iMark, Bio-Rad, USA) was used. Images were taken with an AmScope digital camera (10 MP Aptima CMOS) under an inverted microscope (Olympus ek2). The mean ± SEM (standard error of the mean) was used to represent the 50% inhibitory concentration (IC_{50}). To calculate the percentage of viable cells in the MTT assay, we used one formula. In the MTT assay, we used the formula to determine the percentage of live cells (Van *et al.,* 2011; Fotakis *et al.,* 2005; Tihauan *et al.,* 2020; Ngurthankhumi *et al.,* 2024).

$$\frac{A_{test}}{A_{Control}} \times 100$$

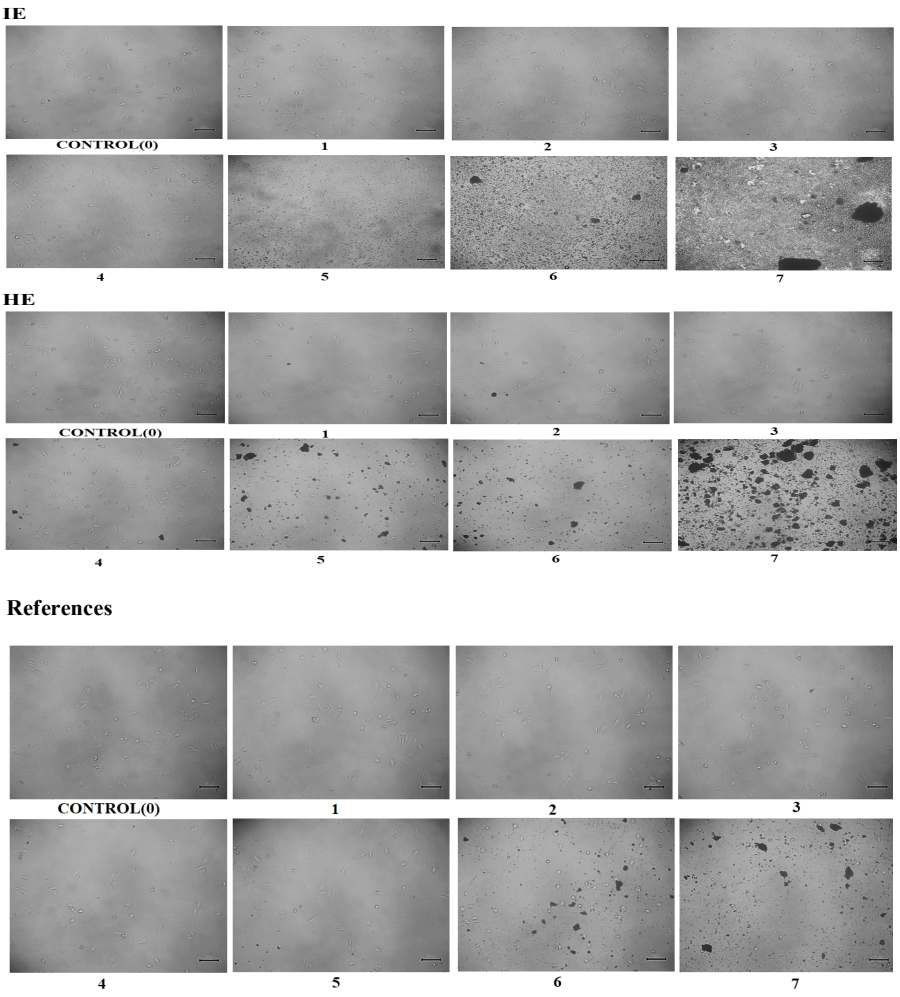
A_{test} = Test sample's absorbance.
$A_{Control}$ = Control sample's absorbance.

RESULTS AND DISCUSSION

2,2-Diphenyl-1-picrylhydrazyl assay (DPPH assay)

The antioxidant activity (DPPH assay) of the samples was calculated on the basis of the experimental results and the 50% inhibitory concentration (IC_{50}). Compared with standard ascorbic acid (Table 3), 50% of the inhibitory concentration was above the dosage limit, *i.e.*, 1000 µg/ml. It was discovered that 3.125 µg/ml ascorbic acid was equal to 250 µg/ml ascorbic acid in sample IE1. Similarly, 50 µg/ml HE1 was comparable to 1.56 µg/ml ascorbic acid, while 1 µg/ml reference sample was identified as being equivalent to 0.78 µg/ml ascorbic acid (Table 3).

The results of the DPPH assay suggest that the HAp samples from Indian hen eggshells and hybrid hen eggshells have antioxidant activity comparable with that of the reference sample, which may be attributed to the presence of bioactive compounds such as proteins and minerals.



Controls consisted of samples at concentrations of 1 (1 µg/ml), 2 (10 µg/ml), 3 (50 µg/ml),4 (100 µg/ml), 5 (250 µg/ml), 6 (500 µg/ml) and 7 (1000 µg/ml). Images were taken with an AmScope digital camera (10 MP Aptima CMOS) under an inverted microscope (Olympus ek2).

Fig 2: Images showing the viability of the MG63 cell line at different concentrations.

The reference HAp sample presented relatively low antioxidant activity, which may be due to the absence of these bioactive compounds.

Cell sensitivity assay (MTT assay)

On the basis of the MTT assay results, the negligible cytotoxic activity of samples IE 1, HE1 and reference was estimated when the MG63 cell line was exposed to varying concentrations of the samples (Fig 2). The results revealed that the IC_{50} values of the HAp samples were significantly different. The IE1 sample has an IC_{50} value of 1797 μg/mL, the HE1 sample has an IC_{50} value of 1348 μg/mL and the reference HAp sample has an IC_{50} value of 1972 μg/mL, indicating that all the samples have lower cytotoxicity (Table 6), may be more biocompatible and suitable for biomedical applications.

This study successfully synthesized and characterized nanohydroxyapatite (nHAp) from Indian and hybrid hen eggshells, evaluating its antioxidant activity and cytotoxicity in comparison to a commercial reference sample. The results indicate that biologically derived nHAp holds great promise as a biocompatible and sustainable biomaterial for various biomedical applications.The DPPH assay revealed that the biologically derived nHAp samples exhibited significant free radical scavenging activity, comparable to the commercial reference. This antioxidant potential may be attributed to the presence of bioactive components such as trace proteins, calcium and phosphate ions, which may enhance radical scavenging. Given that oxidative stress plays a key role in implant-related inflammation and failure, the ability of nHAp to neutralize free radicals could be advantageous in biomedical applications, particularly in bone tissue

Table 4: Absorption values of the samples in the ELISA plate reader.

Sample	Test replicates					Blank		
conc.	1	2	3	4	Average	1	2	Average
				IE1				
0	0.139	0.159	0.146	0.144	0.147	0.041	0.042	0.0415
1	0.143	0.146	0.129	0.128		0.04	0.04	0.04
10	0.12	0.13	0.136	0.142		0.042	0.042	0.042
50	0.107	0.131	0.146	0.132		0.04	0.04	0.04
100	0.13	0.124	0.127	0.139		0.043	0.043	0.043
250	0.13	0.126	0.122	0.128		0.042	0.042	0.042
500	0.13	0.133	0.128	0.121		0.044	0.044	0.044
1000	0.123	0.119	0.109	0.122		0.043	0.043	0.043
Control value IE1= 0.147-0.0415=0.1055								
				HE1				
0	0.145	0.138	0.125	0.161	0.14225	0.041	0.041	0.041
1	0.132	0.137	0.148	0.126	0.13575	0.04	0.041	0.0405
10	0.139	0.138	0.131	0.139	0.13675	0.043	0.045	0.044
50	0.116	0.138	0.134	0.133	0.13025	0.039	0.047	0.043
100	0.113	0.142	0.123	0.125	0.12575	0.044	0.047	0.0455
250	0.126	0.125	0.134	0.128	0.12825	0.056	0.044	0.05
500	0.102	0.108	0.129	0.12	0.11475	0.043	0.044	0.0435
1000	0.11	0.113	0.111	0.12	0.1135	0.045	0.043	0.044
Control value HE1= 0.14225-0.041=0.10125								
				Reference				
0	0.143	0.135	0.134	0.145	0.13925	0.044	0.041	0.0425
1	0.124	0.137	0.137	0.144	0.1355	0.04	0.04	0.04
10	0.135	0.134	0.137	0.14	0.1365	0.043	0.042	0.0425
50	0.128	0.139	0.126	0.135	0.132	0.04	0.04	0.04
100	0.126	0.123	0.132	0.139	0.13	0.043	0.043	0.043
250	0.118	0.136	0.119	0.124	0.12425	0.043	0.042	0.0425
500	0.113	0.121	0.117	0.122	0.11825	0.044	0.044	0.044
1000	0.106	0.1	0.121	0.123	0.1125	0.043	0.043	0.043
Control value of reference= 0.13925-0.0425=0.09675								

Note: The control values of the samples were obtained by subtracting the average blank absorption value from the average absorption value of the samples containing zero concentrations of the test samples.

Table 5: Corrected absorption values of the test samples.

Sample conc.	1	2	3	4
		IE 1		
0	0.0975	0.1175	0.1045	0.1025
1	0.103	0.106	0.089	0.088
10	0.078	0.088	0.094	0.1
50	0.067	0.091	0.106	0.092
100	0.087	0.081	0.084	0.096
250	0.088	0.084	0.08	0.086
500	0.086	0.089	0.084	0.077
1000	0.08	0.076	0.066	0.079
		HE1		
0	0.104	0.097	0.084	0.12
1	0.0915	0.0965	0.1075	0.0855
10	0.095	0.094	0.087	0.095
50	0.073	0.095	0.091	0.09
100	0.0675	0.0965	0.0775	0.0795
250	0.076	0.075	0.084	0.078
500	0.0585	0.0645	0.0855	0.0765
1000	0.066	0.069	0.067	0.076
		Reference		
0	0.1005	0.0925	0.0915	0.1025
1	0.084	0.097	0.097	0.104
10	0.0925	0.0915	0.0945	0.0975
50	0.088	0.099	0.086	0.095
100	0.083	0.08	0.089	0.096
250	0.0755	0.0935	0.0765	0.0815
500	0.069	0.077	0.073	0.078
1000	0.063	0.057	0.078	0.08

Note: Corrected absorption values of samples were obtained by subtracting the average absorption value of the blank at each concentration (from Table 4) from the respective absorption value of the sample. These corrected values were used for calculating the percentage of viable cells.

Table 6: Per cent viability of the MG 63 cell line with different concentrations of the test samples.

Sample conc.	1	2	3	4	% viable cells (Mean of N*)
			IE1		
0	92.417062	111.3744	99.05213	97.1564	100
1	97.630332	100.4739	84.36019	83.41232	91.46919
10	73.933649	83.41232	89.09953	94.78673	85.30806
50	63.507109	86.25592	100.4739	87.20379	84.36019
100	82.464455	76.77725	79.62085	90.99526	82.46445
250	83.412322	79.62085	75.82938	81.51659	80.09479
500	81.516588	84.36019	79.62085	72.98578	79.62085
1000	75.829384	72.03791	62.55924	74.88152	71.32701
			HE1		
0	102.71605	95.80247	82.96296	118.5185	100
1	90.37037	95.30864	106.1728	84.44444	94.07407
10	93.82716	92.83951	85.92593	93.82716	91.60494
50	72.098765	93.82716	89.87654	88.88889	86.17284
100	66.666667	95.30864	76.54321	78.51852	79.25926

Table 6: Continue..

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250	75.061728	74.07407	82.96296	77.03704	77.28395
500	57.777778	63.7037	84.44444	75.55556	70.37037
1000	65.185185	68.14815	66.17284	75.06173	68.64198
			Reference		
0	103.87597	95.60724	94.57364	105.9432	100
1	86.821705	100.2584	100.2584	107.4935	98.70801
10	95.607235	94.57364	97.67442	100.7752	97.15762
50	90.956072	102.3256	88.88889	98.19121	95.09044
100	85.788114	82.68734	91.98966	99.22481	89.92248
250	78.036176	96.64083	79.06977	84.23773	84.49612
500	71.317829	79.58656	75.4522	80.62016	76.74419
1000	65.116279	58.91473	80.62016	82.68734	71.83463

Note: 1) N* Mean absorption of four samples.

2) Corrected absorption value taken from Table 5 to Calculate % *via*bility of the MG 63 cell line of respective samples.

engineering and implant coatings.The MTT assay demonstrated that all three nHAp samples were non-cytotoxic to MG63 osteoblast-like cells, with IC_{50} values above 1000 µg/mL. This suggests excellent biocompatibility, making biologically derived nHAp a *via*ble alternative to synthetic hydroxyapatite. The slight differences in IC_{50} values among the samples may be due to variations in elemental composition and crystal structure, which can influence their interactions with cells. The hybrid hen-derived nHAp (HE1) exhibited slightly greater bioactivity, potentially due to its mineral content. Compared to the commercial reference sample, the biologically derived nHAp samples exhibited comparable antioxidant activity and minimal cytotoxicity, suggesting that eggshell-derived nHAp could serve as a sustainable alternative to conventionally synthesized hydroxyapatite. Additionally, this method provides an eco-friendly solution for waste management, repurposing poultry industry by-products into valuable biomedical materials. Given these findings, biologically derived nHAp could be explored for use in bone regeneration, dental applications and drug delivery systems. Future research should focus on *in vivo* biocompatibility studies, mechanical property enhancements and surface modifications to improve osteogenic potential. Moreover, metal doping strategies (*e.g.*, incorporating magnesium or zinc) could further enhance its bioactivity. Overall, this study highlights the potential of biologically derived nHAp as a sustainable and effective biomaterial for biomedical applications. With further optimization and validation through clinical studies, it could serve as a cost-effective alternative for bone implants, coatings and tissue engineering scaffolds.

CONCLUSION

This study introduces a novel and sustainable approach for synthesizing nanohydroxyapatite (nHAp) from Indian and hybrid hen eggshells, transforming poultry waste into a high-value biomedical material. Unlike conventional chemical synthesis, this eco-friendly method provides a cost-effective and resource-efficient alternative while preserving bioactivity. The antioxidant potential observed in the biologically derived nHAp highlights its ability to mitigate implant-related oxidative stress, a key factor in improving tissue regeneration. Additionally, the non-cytotoxic nature of the synthesized nHAp, confirmed through MTT assays, establishes its biocompatibility and safety for biomedical applications such as bone tissue engineering, implant coatings and dental restorations. This research is distinctive in demonstrating that waste-derived nHAp can match commercial hydroxyapatite in bioactivity, offering a sustainable solution for biomedical engineering. The integration of natural bioactive compounds may further enhance its biological properties, distinguishing it from synthetic alternatives. By bridging the gap between waste management and advanced biomaterials, this study provides a strong foundation for future *in vivo* research and clinical applications. Its potential impact on regenerative medicine and implantology makes it highly relevant for publication in biomaterials and pharmaceutical sciences journals.

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Disclaimers

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Conflict of interest

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