Antibacterial and cytotoxic efficacy of Nano-Hydroxyapatite Synthesized from Eggshell and Sheep bones bio Waste

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Abstract

Background: Through a new material pattern addition approach, NHA is synthesized using eggshell materials and sheep bone under different calcination temperatures (respectively at 900 °C and 800 °C) using a sol-gel approach. NHA/Es and NHA/Sb, due to their biocompatibility and bioactivity, are widely used in applications such as antibacterial and cytotoxic of PDL cells and teeth and as hard tissue.

Results: We investigated the physicochemical properties of NHA/Es and NHA/Sb by performing FTIR, energy scattering X-ray analysis, SEM, and XRD. The formation of NHA/Es, NHA/Sb particles occurs due to the usage of Eggshell materials and sheep bones as a pattern in NHA synthesis, which are less crystalline. Results of surface morphology analysis of NHA particles indicate that these particles are discrete and rod-shaped at low temperatures (850 °C), whereas larger particles are formed at higher temperatures. Therefore, antibacterial activity against four gram-negative bacteria were investigated through MIC and MBC methods, which included E. coli, Pseudomonas aeruginosa, Candida, Saccharomyces and gram-positive bacteria including Staphylococcus aureus and Enterococcus faecalis.

Conclusions: The reason for the compatibility of calcined NHA/Sb powder as a substance used in biomedical applications is that this powder has shown strong antibacterial efficacy against all bacterial strains with a range of inhibitory zones. The nature and structure of PDLs have been studied in many studies; While we focus on the quantitative analysis of the structural properties of PDL cells and their cytotoxic activity through the use of bio waste materials.

1. Introduction

The penchant in hydroxyapatite as a biological material is due to the fact that the main mineral phase forms the teeth and bones and represent the hard tissue [1–4]. The Interest in hydroxyapatite as a biological material is due to the fact that the main mineral phase forms the teeth and bones and represent the hard tissue [5–10]. studies show that hydroxyapatite nanoparticles have better biocompatibility and higher capacity in absorption. Eggshell are among the world-consuming foods, so there are many waste. This makes egg skin due to abundance, much cheaper than other materials. In the synthesis of compounds, physicochemical properties such as particle size, surface area and morphological porosity must be considered [11–13]. The assessment of hydroxyapatite bio waste reproducing the tissue is clinically needed as an alternative to autologous- and heterologous-derived scaffolds [14–19]. One of the bioceramics used as a material in the construction of filler bones and teeth, is hydroxyapatite [20–25]. As hydroxyapatite is similar to bone composition, so it can be said that the hydroxyapatite bone filler can be occupied by the bone network. Stimulation of bone growth is due to the osteoconductive property of hydroxyapatite. Also hydroxyapatite has high biocompatibility. Chemical formula of HA with ratio of molar Ca/P 1.67, is Ca_{10}(PO_4)_{6}(OH)_2 [26–28]. Biocompatible, pores, osteoconductive and bioactive are among the characteristics of hydroxyapatite [29–32]. A natural and artificial source of calcium can be used to make Hap [33–35]. In general, for the synthesis of NHAp, the source of artificial calcium (CaO, Ca(NO_3)_2, Ca(OH)_2, CaCO_3 and CaCl_2) is used [36, 37]. The periodontal
ligament, usually PDL for short, is a group of specialized connective tissue fibers that attach the tooth to the alveolar bone in which it is located. They are also found in collagen fiber packages (collagen types I and III). It enters the root cement on one side and the alveolar bone on the other. Osteoblasts, fibroblasts, semenoblasts and stem cells are among the heterogeneous PDL cells [38–40]. Worldwide, 90% of adults are affected by periodontal disease caused by bacteria. Factors such as loss of connective tissue and bone support and eventually loss of teeth occur due to severe periodontitis. Fibroblasts in the maintenance and regeneration of periodontal tissue, have an important role to play and are also considered as pdl cells [41–44]. So, the aim of this study was to use hydroxyapatite as a new pattern for measuring its antibacterial effects on the properties of NHA and is also used as a source of calcium obtained from eggshells and sheep bones, leading to the synthesis of new biowaste materials. To evaluate the effect of NHA samples on the properties of the synthesized material, a comparative study was performed with the obtained NHA samples. Nano-hydroxyapatites are responsible for antibacterial properties by providing bio waste materilas data. To prevent any infection with biomedical implants and injection materials, synthesized antibacterial hydroxyapatite is a good candidate because it causes failure in bone repair. Figure 1 shows some of the applications of hydroxyapatite.

2. Material And Methods

2.1 Synthesis Hydroxyapatite

To produce calcium oxide, first sheep bone of duck and egg shell were collected and cleaned of bio-waste materials, then at 900 °C and 800 °C, egg shells and sheep bone of duck were calculated, respectively. Through calcium and phosphate precursor reactions by comparing egg shell molar concentrations (Ca / P: 1.88) and (Ca / P: 1.53), hydroxyapatite synthesis was performed. By wise drop methods, 0.5 M Ca (OH) solution with 0.3 M phosphoric acid was later added, then trap in closed chemical glass to use aluminum foil to produce the suspension. 1 M NaOH was gradually added to the suspension until it reached a pH 10. To produce NHAp powder, the solution was aging overnight. The HAp crystal is kept in the furnace until it cools and is weighed with an analytical balance until its mass is proven Fig. 2. Following a series of thermal processes, hydroxyapatite powder was obtained from sheep bone of duck. In all parts of a sheep, the fresh sheep bones were cut into smaller pieces and thoroughly cleaned to remove macroscopic sticky impurities. In order to removal of sheep bone marrow and tendons more easily, sheep bone samples were boiled in distilled water for 8 hours. Then, as the sheep bone continues to boil in water, the sheep bone is de-proteinized. Drying of the boiled sheep bone samples was performed at 200 °C overnight. Deproteinized sheep bone calcination occurs for 3 h at 800 °C. It is impossible for prion or disease-causing agents to survive at this temperature. Finally, the resulting product was crushed into small pieces. Also for 24 h milled in a ball mill pot. By vibro-milling method (McCrone Micronizing Mill) re-milling each 20 grams of HA/Sb powder, was performed using ethanol as the milling medium Fig. 3. With some instrument, the NHAp crystal can be identified. By X-ray diffraction, the phase combination of the HAp crystal was studied. The functional group was studied with FTIR, FESEM, PSA, EDX and map to examined the surface of morphological characteristics.
2.2 Microstructural evaluation

To study the structural changes of NHA samples, scanning electron microscope (VEGA3-TESCAN, Czech) was used. So, samples for better electrical conductivity, was covered with a very thin layer of gold by sputtering method. The SEM images were taken at different magnification. To find the element distribution through the microstructure of the samples, EDS analysis with a spectrometer (RONTEC, USA) was used.

2.3. Fourier transform infrared spectroscopy (FTIR)

The FTIR spectrum was obtained to identify the bonds and chemical groups present on the surfaces of the samples using the Tensor II spectrometer and the MAUNA-IR 750 spectrometer. Infrared spectroscopy (FTIR) test was used in the frequency range of 4000 – 400 cm$^{-1}$ in the passing mode.

2.4. Phase evaluation

NHA samples analyzes to identify phases of presence was performed using XRD method. The structural properties of the samples were investigated by XRD spectra by device the Bruker model (D8 Advance, Germany) was performed with a wavelength of 1.5405 CuKα angstrom in the angle range of $2\theta = 20^{\circ}$-60°. JCPDS reference cards and Xpert high score software were used to identify the patterns.

2.5. Antibacterial Activity

2.5.1. MIC

In this section, to evaluate the antibacterial susceptibility of the samples, all measures were performed according to the instructions of the Institute of Clinical and Laboratory Standards. Quantification of the MICs of experimental compounds was performed by microdilution broth assay. In liquid medium (brain-heart infusion), the effect of compounds on the growth rate of microorganisms (Pseudomonas aeruginosa, Enterococcus faecalis, Escherichia coli, Staphylococcus aureus, Saccharomyces and Candida) was investigated. Therefore, by BHI or liquid medium, all 96 wells were filled at 90 µl. Then compounds with a decreasing concentration from 1000 µg / mL to 7.8 µg / mL, were added to the culture medium at 90 µl. In the next level, transfer the 10µl microorganisms with 600 nm OD (0.5 McFarland), was done to the wells comprising the drug. One negative control row (BHI) and one positive control row (BHI and microorganisms), in this process was considered. Eventually, for 24 hours the plates were incubated at 37 °C, then were read on the spectrophotometer (BioTek, Winooski, VT, USA) in the range of 600 nm. Since this method was repeated three times, the results obtained are given in the following equation:

$$V\% = \frac{A_t - A_b}{A_c - A_b} \ast 100$$
A<sub>t</sub> is the amount of adsorption of tested wells and V % the viability percent of microorganisms. The absorption values of positive and negative control wells are shown as A<sub>c</sub> and A<sub>b</sub>, respectively.

### 2.5.2 MBC

In BHI for 24 h culture of all microorganisms was performed; Then for each microorganism a stock with a concentration of 10^6 – 10^5 CFU / mL was prepared. In summary, to calculate the MBCs, wells selected as MICs and all wells that had no visible growth were cultured in nutrient agar; Then, after storage for 24 hours in a 37 ° C incubator, the growth of microorganisms was examined. The lowest concentration amount of the sample as the MBC was considered because it created less than four visible colonies. Each test was repeated three times.

### 2.6. Mtt assay

The cytotoxicity of hydroxyapatite samples was analyzed on the PDL cell line by the MTT assay. For this purpose, in 6 mL of alpha-MEM culture medium comprising 10% FBS and 2% Penicillin-Streptomycin, in the form of 36 × 103 cells were suspended. Then to 60 wells from a 96-well plate, 100 µl of the cell suspension was transferred by a sampler. Thus, 60,000 cells were seeded in each well, and the rest of the wells contained only the culture medium as control wells (without cells and samples). The plate was then incubated at 37°C with 5% CO2 to stabilize the cells. After 24 h, 50 µL was removed from the previous culture medium, 50 µL of the sample containing each of the nanoparticles was replaced at concentrations of 1000, 500, 250, 100, and 25. So plate again was returned to the incubator. In the first well, the culture medium was poured without nanoparticles, which is the positive control, and wells without suspension were used as control (negative control). After 24 h, 80 µL was removed from each cell-containing well and replaced by 80 µl of an MTT-containing culture medium at 0.5 mg/mL, and the plate was incubated for 3 h. Then, 80 µl was removed from cell-comprising wells, and to wells from DMSO at the rate of 200 microliters was added. In order for Formazan crystals to completely dissolve in DMSO, the plate was shaken and incubated for another 30 minutes. The plate was placed on a shaker for 30 min, and eventually the absorbance of each well at 570 nm was measured by a microplate reader (BioTek Power Wave XS2). According to the following formula cell viability was calculated:

\[
Viability(\%) = \frac{A_t - A_b}{A_c - A_b} \times 100
\]

In this formula, V% is the percentage of cell viability, and absorption values in experimental, blank and control wells are shown as At, Ab and Ac, respectively.

### 2.7 Statistical calculations and analyzes

In the present study, data were analyzed using SPSS IBM software. Data from each group were presented as mean ± standard deviation, to analyze the results of biological experiments. Statistical analysis of
data was performed using ANOVA. Then, complementary analysis was performed using Tukey test to compare between groups. The statistically significant level was considered $P < 0.05$.

3. Results

3.1. Characterization of synthesized powders

Figure 4 shows the XRD patterns of NHA powders prepared in the present study, which has the corresponding peaks of hydroxyapatite and shows that the hydroxyapatite powder produced is free of impurities. Egg shell materials and sheep bones induce more dispersion of the $\text{PO}_4^{3-}$ ions nuclei, which possess strong polarizability and thus are excel lent -absorbing factors. As a outcome, reaction of HA/ES is more intense than NHA/Sb as can be noticed from the superior crystallinity XRD pattern. Te Ca/P ratio and elemental EDX of the pelletized samples were obtained by an area scan using semi quantitative EDX analysis (Fig. 5). Te Ca/P ratios were 1.84 and 1.53 for the pellets obtained from the NHA/Es, NHA/Sb respectively. The resulting ratios associate with the theoretical value of 1.84 and 1.53 corresponded to the XRD patterns. The elemental Map of the carbon, calcium, phosphorus and oxygen showed a good distribution throughout the pellets. In Fig. 5 (a and b), the EDX elemental analysis of NHA/Es, NHA/Sb are demonstrated; in the insert, the elemental distribution rate can be seen. As illustrated, NHA/Es, NHA/Sb comprises 10.66, 46.44, 14.86, and 28.03 W%/A% (carbon, oxygen, Phosphorus, and calcium), and 8.576, 51.42.0.38,15.60,24.02 carbon, oxygen, Silicon, Phosphorus, and calcium, respectively. In this matter, the Silicon caused half of the weight of the composition showing its superior effect in boosting the HA of the composition that could increase the overall attenuation rate of the materials. The phosphate functional at HA with stretching vibrations are at $1000–1086$ medium and at $1021$ cm$^{-1}$ and also bending vibrations perceived at $562–604$ cm$^{-1}$. From the FTIR spectrum was used to detect the presence of carbonate in the perceived crystals in the HAp and carbonate apatite samples.

In this section, developed nanomaterials were well-characterized using diverse analyses. In Fig. 6 (a), a view of the FT-IR spectrum of (I) NHA/Es and NHA/Sb can be seen. As shown in part (I), NHA/Es and NHA/Sb are successfully synthesized and presented a fingerprint of Bio waste materials. In this section, the peak between regions $530–630$ cm$^{-1}$ corresponds to the stretching vibration of the $\text{PO}_4^{3-}$ functional group related to Asymmetric bending vibration. This peak for developed Hydroxyapatite appeared at a wavenumber of $561$ cm$^{-1}$. Moreover, other peaks within the FT-IR spectrum of Hydroxyapatite are attributed to sp2 alkene of C-H band related to disubstituted-E ($872–984$ cm$^{-1}$), C-H Asymmetric stretching vibration ($1022–1071$ cm$^{-1}$), hydroxyl functional groups (-OH) ($3520$ cm$^{-1}$). In part (I, II), the HA can be seen; in this parts, are successfully decorated with NHA/Es, NHA/Sb spectrum nanoparticles. Other peaks within this spectrum correspond to Out of plane bending mode CO23 ($854$ cm$^{-1}$). The ion stretching vibration around ($3622$ cm$^{-1}$), confirms the presence of a hydroxyl group. Likewise, the other stretching vibrations for carbonyl and phosphate groups were also observed as reported earlier.
Moreover, in Fig. 7 (a and b), FESEM images of NHA/Es can be seen. Using FESEM analysis, the surface morphology of NHA/Es crystals was studied. The results of characterization FESEM showed that hydroxyapatite is regular in shape and without pores. The best hydroxyapatite with temperature sintering at 900°C showed oval shaped with pores without agglomerated. As depicted, NHA/ES exhibits well-defined particles with uniform size distribution and high crystallinity. Successful formation of NHA/Sb through the protocol used, was confirmed by the data together with the previously obtained data. Therefore, the images obtained from FESEM showed that NHA/Es have flake, aggregated hexagonal particles and rod-like structures, respectively. When adding egg shells, some of them stick together as aggregates (mostly like hexagons). FE-SEM images of NHA/Sb levels are shown in Fig. 7 (c and d), which have been subjected to hydrothermal operation for 72 h. On Sb, a fine nanoscale lattice structure is formed, that this structure composed of many feather-like, elongated features placed perpendicularly to the surface. Conversely, rod-like apatite nanoparticles in a loosely compact film covered the NaOH-treated Sb samples. This study found that the morphology of apatite deposits was closely related to the surface modification conditions and the underlying substrate material and topography. Besides, in Fig. 8, the chemical structure of NHA/Es and NHA/Sb proposed crystalline structure can be seen. These MAP obtained data, are in accord with obtained data from EDX analysis, in which the chemical structure of the NHA/Es and NHA/Sb derived compounds were found to be cubic, more details can be seen in Fig. 7. TEM analysis was performed to observe their morphologies in detail and to evaluate the particle size. Figures 8 (a, b) and (c, d) present the results of the TEM analysis of the samples NHA/Es and NHA/Sb, The NHA samples has a nanorod-like morphology with length in the range of 10–70 nm (Fig. 8), with regular particle size distribution. A complementary analysis was performed in the NHA Bio waste. As it can be observed, the NHA used in this study possess a rod morphology and a particle size. In this way, PSA analysis was used to evaluate the particle size distribution., PSA analysis was conducted. PSA analysis for NHA/Es and NHA/Sb can be seen in Fig. 9 respectively. As can be seen in Fig. 9 particle size analysis in the ranges of 10 and 70 nm.

4. The Antibacterial Studies

Survival of Staphylococcus aureus under HA/Es samples was lower than HA/Sb. Therefore, this sample has more antibacterial properties against Staphylococcus aureus than HA/Sb; HA/Es is antibacterial up to the concentration of 500 micrograms per milliliter. In Fig. 10, HA/Es samples also had a greater antibacterial effect on Enterococcus faecalis than HA/Sb. This sample is antibacterial up to the concentration of 500 µg/mL, while the HA/Sb samp4we4wele showed antibacterial properties only at the initial concentration of 1000 µg/mL. The HA/Sb sample has good (significant) antibacterial properties on E.coli bacteria so that this property preserves up to the concentration of 250 micrograms per milliliter, and only about 5% of E.coli bacteria survive in this concentration. In the NHA/Es sample, this effect is even greater, as this sample has antibacterial properties up to a concentration of 125 micrograms per milliliter, and only about 6% of bacteria survive under the influence of this concentration sample. The best performance of these samples was on Pseudomonas aeruginosa. The NHA/Sb sample and the NHA/Es sample were antibacterial up to 125 µg/mL concentrations and 62.5 µg/mL, respectively. Therefore,
NHA/Es sample has more antibacterial properties than NHA/Sb sample against the mentioned bacteria. Also, these two samples had the highest antibacterial effect on Pseudomonas aeruginosa. The MICs and MBC / MFCs values of the compounds against microorganisms are given in Table 1.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>NHA/Sb</th>
<th>NHA/Es</th>
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<tbody>
<tr>
<td></td>
<td>MIC (µg/mL)</td>
<td>MBC (µg/mL)</td>
</tr>
<tr>
<td>S. aureus</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>125</td>
<td>250</td>
</tr>
<tr>
<td>E.coli</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Candida</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Saccharomyces</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>

5. Cytotoxicity Assessment

Using MTT method, cytotoxicity of two samples of hydroxyapatite on PDL cell line was investigated. The results are shown as the average ± viability (%) in Fig. 11, which shows the viability (%) of PDL cells after 24 h of exposure. As shown in the Fig. 11, the hydroxyapatite (eggshell) sample has a viability rate of 92.38% at the highest concentration, and cell viability increases with decreasing the concentration. At the lowest concentration, viability increases significantly to > 100% due to cell induction. A viability rate of 98.95% is observed for the hydroxyapatite (sheep) sample at the highest concentration, which increased to 108.13% with decreasing the concentration because of cell induction. The high viability rate at all concentrations indicates the very low toxicity of hydroxyapatite samples in exposure to PDL cells.

Here, the control sample was compared with the other two samples, namely hydroxyapatite (sheep) and hydroxyapatite (eggshell). Control and hydroxyapatite: p-value = 0.341 (sheep is > 0.05). Control and hydroxyapatite: p-value = 0.764 (eggshell is > 0.05). Then, the other samples were compared with each other, except the control. Hydroxyapatite (sheep) and hydroxyapatite (eggshell): p-value = 0.042 (there is a significant difference as eggshell is < 0.05).

6. Discussion

In recent, studies have been conducted on the bio compatibility and antibacterial effects of hydroxyapatite. In one study investigated the effect of nano hydroxyapatite (nHA) suspensions on the proliferation and cell cycle of human periodontal ligament (HPDL) cells and provided evidence for the use of nHA in periodontal therapy. The results show that nHA has no toxicity to HPDL cells. In addition, the
study found that the smaller the diameter of the nHA particles, the higher the proliferation of the cells.[45] In another study, flower-like hydroxyapatite nanostructures were obtained from eggshells by a simple and rapid microwave method. The MTT toxicity test of floral hydroxyapatite was studied in 3T3-L1 mouse fibroblasts at doses of 50, 100 and 200 µg/mL. According to the results, there was no significant difference between the growth rate of cells treated with nHA and the control after 48 hours.[46] In one study, hydroxyapatite (HAp) nanorods were prepared by green synthesis using leaf extracts of Azadirachta indica and Coccinia grandis for antibacterial applications in the orthopaedic field. The antibacterial test of the prepared samples was performed on two of the most common bacteria found in bones (E. coli and S. aureus). MBC results showed a significant inhibition diameter of 19–26 mm.[47] Hydroxyapatite \([\text{HA, Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]\), the major mineral component of natural bone and dental tissue, has attracted considerable attention due to its good biocompatibility and is one of the most commonly used restorative materials. Hard tissue has become a clinical treatment.[48]

All these reports highlight the antibacterial properties and bio compatibility of nano hydroxyapatites. In this study, NHA/Es and NHA/Sb synthesised on PDL cells were investigated. According to the results of MTT hydroxyapatites NHA/Es and NHA/Sb in the highest concentration, 92.38% and 98.95% survival were observed, respectively. As the concentration decreased, the viability increased and reached more than 100% for both types of hydroxyapatites, showing that the synthesized hydroxyapatites have excellent biocompatibility. The antibacterial properties of the synthesised samples were also evaluated for their efficacy against bacteria (S. aureus, Enterococcus faecalis, Pseudomonas aeruginosa, E.coli, Candida and Saccharomyces). According to the MIC results, the highest antibacterial property of the NHA/Sb sample is due to Pseudomonas aeruginosa bacteria at a concentration of 125 µg/mL. Similarly, the highest antibacterial property of the NHA/Es sample is due to E.coli bacteria at a concentration of 62.5 µg/mL. The antibacterial properties and excellent biocompatibility of the synthesized hydroxyapatites make them suitable candidates for various dental applications, drug delivery, implants, etc.

7. Conclusion

The results of this study showed that the synthesis of hydroxyapatite can be done from ES and Sb ducks by wet precipitation methods. One of the factors affecting the degree and phase of NHA crystallization is temperature of sintering. It seems to be the most appropriate ratio for this method, to be \(\text{nNHAp} = \text{CNC}\) ratio, because by FESEM analysis, which shows the importance of particle size and distribution, is provided. Furthermore, the results of superior antibacterial and antifungal performance obtained from MIC, MBC and MFC NHA / Es and NHA / Sb evaluations against selected bacteria and yeasts compared to NHA / ES and NHA / Sb, proved. Moreover, the superior antibacterial properties of NHA/Es by assessment of MIC/MBC performance of chemically synthesized NHA/Es and NHA/Sb, against gram-negative and gram-positive bacteria confirmed. Also compared to NHA/Sb particles, showed the highest inhabitation rate. The results of this study showed that the extraordinary role of NHA / Es and NHA / Sb in improving the antibacterial / antifungal performance of nanoparticles is higher than that of many recently developed biowaste nanoparticles. More experiments and clinical research in the future are likely
to take place on a wide scale. The goal of this research is to examine the cytotoxic activity of the NHA/Es and NHA/Sb materials has been examined using approach to assess its low cytotoxic activity against PDL cells.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Nano hydroxyapatite</td>
<td>NHA</td>
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<tr>
<td>Nanohydroxyapatite egg shell</td>
<td>NHA/Es</td>
</tr>
<tr>
<td>Nanohydroxyapatite sheep bone</td>
<td>NHA/Sb</td>
</tr>
<tr>
<td>Periodontal ligament</td>
<td>PDL</td>
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<tr>
<td>Fourier transform infrared spectroscopy</td>
<td>FTIR</td>
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<tr>
<td>Scanning electron microscopy</td>
<td>SEM</td>
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<tr>
<td>X-ray diffraction</td>
<td>XRD</td>
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<tr>
<td>Minimum Inhibitory Concentration</td>
<td>MIC</td>
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<tr>
<td>Minimum Bactericidal Concentration</td>
<td>MBC</td>
</tr>
<tr>
<td>Bovine fetal serum</td>
<td>BFS</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>DMSO</td>
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<tr>
<td>one-way analysis of variance</td>
<td>ANOVA</td>
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<tr>
<td>Transmission Electron Microscopy</td>
<td>TEM</td>
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<td>Energy dispersive spectroscopy</td>
<td>EDS</td>
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**Declarations**

**Ethics approval and Consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

Data available in a publicly accessible repository.

**Competing interests**
The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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**Authors' contributions**

Conceptualization, S.M.M; writing—original manuscript, Kh.Y, N.V.R, N.O. and A.G.; writing—review and editing, F.F., M.Y.K., Y.Gh. and W.H.C.; visualization, S.A.H.; supervision, S.M.M. and W.-H.C.; All authors have read and agreed to the published version of the manuscript.

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**References**


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Figures
Figure 1 shows some of the applications of hydroxyapatite.
Figure 2

Synthesis of NHA/Sb Powder
Figure 3

Synthesis of NHA/Es Powder

Figure 4
XRD patterns of NHA powder (a) NHA/Es and (b) NHA/Sb powders

Figure 5

EDAX patterns of NHA powder (a) NHA/Es and (b) NHA/Sb powders
Figure 6

FTIR patterns of HA powder (a) NHA/Es and (b) NHA/Sb powders
Figure 7

SEM images of NHA powders (a,b) NHA/Es and (c,d) NHA/Sb powders
Figure 8

TEM images of (a,b) NHA/Es and (c,d) NHA/Sb powders

Figure 9

PSA images of NHA/Es and NHA/Sb powders (a) NHA/Es and (b) NHA/Sb powders
Figure 10

Effects of nanoparticles on viability percentages of different microorganisms in tested concentrations. Each bar represents the mean± SD (standard deviation) of three independent tests.
Figure 11

The cytotoxic effects of samples on PDL cell lines cells under different concentrations.