

CHARACTERIZATION OF EGGSHELL NANOPARTICLES AS REINFORCEMENT MATERIAL IN BIODEGRADABLE METAL MATRIX COMPOSITES FOR BIOMEDICAL APPLICATION

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This paper investigated the potential utilization of eggshell nanoparticles bio-resources as reinforcement material in Biodegradable Metal Matrix Composites (BMMC) for biomedical applications using characterization techniques. The eggshell nanoparticles were produced via chemical (sol-gel) and mechanical (ball mill) routes and thereafter calcined at 1000 °C for 3 h under 11 °C/min heating rate. The resulting calcined products were then characterized using a Transmission Electron Microscope (TEM/EDX), X-ray Diffraction (XRD), and X-ray fluorescence (XRF). The antibacterial, antifungal, and antimicrobial activities of the functionalized bandage characteristics of the eggshell calcined nanoparticles from both routes were performed. Results indicated that eggshell nanoparticles were successfully produced from sol-gel and ball mill methods with characteristics 2.102 ± 0.757 and 10.793 ± 7.540 nm mean particle size respectively. The XRF and XRD analyses show the presence of hard metallic oxides such as CaO (91.37-92.87) wt%, MgO (02.66-2.92 wt%), Fe₂O₃ (0.23-0.36 wt%), ZnO (0.18-0.22) wt% and hard ceramic phases (calcite, lime and portlandite) for both the sol-gel and ball milled eggshell nanoparticles. In addition, Eggshell nanoparticles produced from both routes displayed an enhanced antibacterial, and antifungal activity and a higher zone of inhibition on functionalized bandage against all the tested bacteria at 150 µg/ml concentration. Meanwhile, the sol-gel sample performed better compared to the ball-milled sample at all concentrations with greater antibacterial efficacy on Pseudomonas aeruginosa and Escherichia coli bacteria strains. The presence of hard phases and enhance antibacterial performance of eggshell nanoparticles suggest it, as a promising reinforcement material for improve mechanical and biological performance when use as reinforcement material for BMMC development.

Keywords: Eggshell, Nanoparticles, metal matrix composites, Biodegradable metal, Biomaterials

INTRODUCTION

The increasing global surge in municipal and industrial wastes coupled with inappropriate disposal management are currently constituting a threat to the public's health and environmental degradation. This menace is driven by the increasing growth of population, food, and beverage processing industries across the globe (1, 2). A major representative of this class of waste is eggshells. It is the hard covering part of the chicken egg usually discarded after consumption of the edible portion. The eggshell which makes up of about 11% of the egg's total weight acts as a barrier against physical and atmospheric interferences to the egg yolk and albumen. It is a cheap natural source of calcium, in carbonate form ($\geq 94\%$) and other mineral elements such as magnesium, potassium, strontium, sulphur, and

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organic matter in minute quantity (3, 4). These elements play a crucial role in bone and body development. Consequently, the mammoth eggshell wastes generated due to high consumption rate are discarded into the environments and dumpsites with serious obnoxious smells and environmental pollution (5, 6). For instance, the 63,7 metric tons global egg production reported (7) in 2010 had risen to 141.3 metric tons in 2024 (2), representing about 122 percent increment, and the trend continues.

Several uses and applications of eggshell have been unveiled in the literature including milk tablet supplement production (8), calcium supplement making (9), synthesis of hydroxyapatite and calcium nitrate nanosheet for bone graft substitute application (10–13), catalyst for biodiesel production (14), as heavy metal removal in the treatment of water (15) bio-filler for polymer and metal matrix composite production (16, 17). Despite the several useful elemental compositions and a wide range of applications, the indiscriminate discarding of eggshells still remains a global concern and had attracted researchers' interest in creating more windows of useful applications for managing of eggshells waste in the society. This may include as reinforcement material, for the development of biodegradable metal matrix composites useful in biomedical applications. Biodegradable metals such as magnesium, zinc, and iron offer a hopeful solution to the challenge usually encountered with non-degradable orthopedic metal implants by circumventing the risk and challenges often bored by bone-fractured patients during the second surgery operation. Biodegradable metal, once implanted in the human body provides temporal support and nurtures the fractured bone site during the recuperative process for new bone generation. It remains in the body permanently and only deteriorates *in vivo* harmlessly at a rate equal to the bone healing pace (18, 19). Hence, it vanished totally when the bone had fully regenerated.

Although, among the degradable metals, magnesium is ranked first on the researchers' table representing the most investigated biodegradable metallic implant followed by zinc and iron. However, research has revealed that, premature loss of mechanical integrity of magnesium due to its high degradation rate *in-vivo* in the physiological environment. This deteriorates the implant-bone joint interface before the heal-up time of the fractured bone hence, constituting its major unresolvable limitation in the biomedical field (20–23). On the contrary, zinc had a moderate degradation rate but poor mechanical properties while iron was characterized by an insufficient degradation rate below the clinical requirements for load-bearing applications (24). Thus, researchers are now intensifying efforts to explore several approaches to combating the rapid degradation rate and poor mechanical properties of biodegradable metal. Several techniques have been explored to enhance the structural and biological performance of biodegradable metals. These approaches include alloying, deformation process, surface modification, structural design, thermal treatment, and formation of composite materials. In all, compositing stands a better chance due to its tunability advantage for the enhancement of both mechanical and biological properties of biodegradable metals (25, 26). In addition, the incorporation of bio-functional ceramic nanoparticles in the matrix phase signifies an evolving alternative strategy for improving the mechanical and biological performance of biodegradable metals due to increasing surface area and dislocation density at the grain boundaries (27). Though still evolving, very little work has been found on biodegradable metals matrix composite in the literature compared to alloying approaches despite its high dual-potential at enhancing the biological and structural performance of the biodegradable metals (27–30). The selection of reinforcement material in biodegradable metal matrix composite fabrication is highly conservative and it requires a careful selection to ensure only a bioactive with good antibacterial material is chosen as the reinforcement phase. Consequently, very few reinforcement materials (hydroxyapatite, gra-

phene, bioactive glass, and bridges carbon nanotubes) have been explored in the literature for the development of biodegradable matrix composite (27, 28, 31). The continuous search for more sustainable and cost-effective materials development (32) in many critical engineering applications such as the biomedical field, coupled with a bio-safe chemical composition of the eggshell makes it a good potential reinforcement candidate in biodegradable metal matrix design however, experimental data to support this proposition are rarely found in the literature. In addition, knowledge of its antibacterial, antifungal, and functionalization in wound healing is limited. In addition, the processing path of this nanoparticle reinforcement material may also influence its performance. Among the several routes for the production of nanoparticles, the sol-gel and mechanical methods constitute a major route from the economic, high precision, and quantity perspectives (33). However, chemical and antibacterial comparison analysis of mechanically and sol-gel-produced eggshell calcined nanoparticles for biomedical application has not been reported in the literature to the best of the author's knowledge. Thus, this paper investigates the potential utilization of eggshells as reinforcement materials in biodegradable metal matrix composites for biomedical applications.

MATERIALS AND METHOD

MATERIALS AND EQUIPMENT

The materials used in this work include hydrochloric acid with $36\% \pm 1\%$ purity concentration, sodium hydroxide with 97% purity, and commercial calcium oxide which were procured from Lab Trade Scientific Medical Equipment and Chemical Supplies, Ilorin, Kwara State, Nigeria. Waste chicken eggshells were collected from food vendors within Malete township, Kwara state. Major equipment used in this work were tube furnace, a vibratory sieving machine at the Department of Material Science and Engineering, Kwara State University, a ball mill at Federal Institute of Industrial Research, Oshodi (FIRRO), Lagos, scanning electron microscope, digital weighing balance, EMPYREAN X-ray Diffraction (National Geosciences Research Laboratories, Kaduna), JEM-ARM200F-G TEM machine (Rolab research laboratory, Ibadan), EDX 3600B X-ray Fluorescence spectrometer at the National Agency for Science and Engineering Infrastructure, Akure, Nigeria.

PREPARATION OF EGGSHELL POWDER (ESP)

The procured eggshells (Fig. 1a) were sorted to remove unwanted materials such as stones, food items, and greens that might have gotten stuck with the eggshells during collection and thereafter crushed. The crushed and sorted eggshell was poured into a large bowl containing clean tap water and detergent. It was washed (Fig. 1b) and rinsed properly for 4 times. The cleaned eggshells were sun-dried for three days (Fig. 1c). The dried eggshell was converted into powder using a stainless-steel electric blender (Fig. 1d) to avoid contamination with impurities. The powdered eggshell powder was sieved using a vibratory sieve shaker (Fig. 1e) arranged in decreasing order of fineness and ran for 15 mins following a standard procedure described in the literature (34). The eggshell powder that passed through the 0.063 mm equivalent to <63 microns (Fig. 1f) was used in this study.

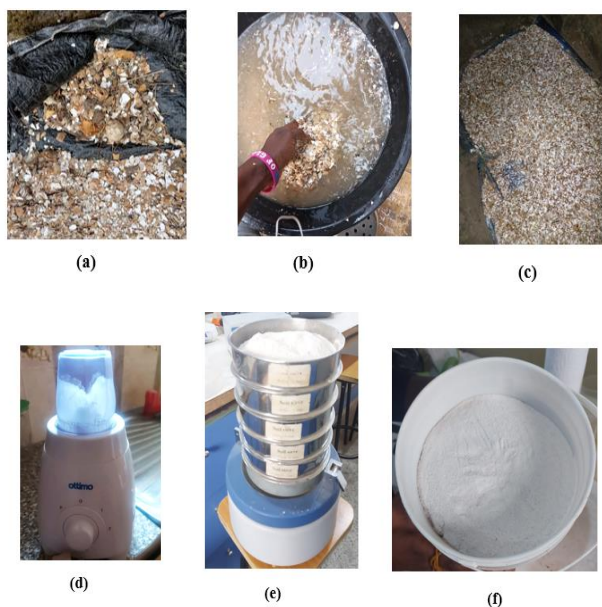


Figure 9. Preparation of eggshell powder

PREPARATION OF NANOPARTICLE OF EGGSHELL

Both the chemical (sol-gel) and mechanical (ball-milled) methods were employed in this work for the preparation of eggshell nanoparticles. For the sol-gel route, 12.5g of eggshell powder (<63 microns) as precursor was weighed and transferred into a beaker containing 250 ml of prepared dilute 1M HCl placed on a magnetic stirrer was stirred (Fig. 2a) until complete dissolution and homogenous colloidal solution was obtained meanwhile, the clamped burette filled with prepared dilute 250 ml 1M NaOH solution was kept on standby. Upon obtaining a homogenous mixture, NaOH in the clamped burette was introduced into the stirring eggshell powder solution in drops at a rate of 59 drops/min for 1h 35 min until the 250 ml volume of prepared dilute NaOH was exhausted after which it was aged for 24 h. Thereafter, the filtrate was decanted to separate the Ca(OH)_2 gel from the NaCl solution (Fig. 2b), the gel obtained was oven dried (Fig. 2c) for 3 h at 100 °C before the final product (Fig. 2d) was been kept in an air-tight container.

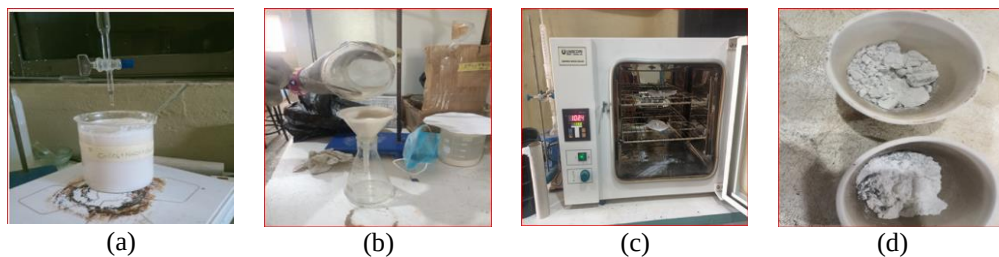


Figure 2. Production of eggshell nanoparticle via the sol gel route.

Furthermore, the mechanical (ball mill) technique was also adopted to produce eggshell nanoparticles using the crushed eggshell particles (ESp) <63 microns sieved product. The as-sieved ESp was thereafter ball milled using Model 87002 Limoges-France, A50 ball mill machine (Fig. 3a) with a ball-to-powder ratio of 3:1, at a speed of 80 rpm for 76 h and was kept under an air-tight container to prevent air infiltration. The final products as obtained from both approaches (sol-gel and ball-mill) were finally calcined in a tube furnace at 1000 °C for 3 h at 11 °C/min heating rate to obtain the eggshell nanoparticles (Fig. 3b).



Figure 3. Ball milling process (a) and eggshell nanoparticles (b)

CHEMICAL AND MORPHOLOGICAL CHARACTERIZATION OF EGGSHELL NANOPARTICLES

The chemical and morphological characterization of eggshell nanoparticles was performed using Transmission Electron Microscope TEM, X-ray Fluorescence (XRF), and X-ray Diffraction (XRD) techniques. The TEM analysis was performed by placing eggshell nanoparticle carbon-coated TEM grids and analyzed by high-resolution JEM-ARM200F-G TEM equipped with Energy Dispersive Spectroscopy (EDS) at Rolab Research Laboratory, Ibadan to examine the particle size and morphology shapes and distribution. The percentage chemical composition of the synthesized eggshell nanoparticle was examined using an EDX 3600B X-ray Fluorescence spectrometer at the National Agency for Science and Engineering Infrastructure, Akure, Nigeria. XRD analysis was performed in an Empyrean X-ray diffractometer at the National Geoscience Research Laboratory (NGRL), Kaduna using copper radiation $\text{CuK}\alpha$, $\lambda = 0.15418$ nm, under an operating voltage of 45 kV and 40 mA current. The scan was $2\theta = 10^\circ - 70^\circ$, with 0.026° at 30 s per step and an acquisition rate of $2.5^\circ \text{ min}^{-1}$. The mean crystallite size (D) of the eggshell nanoparticle was determined using Scherrer's equation [1], and using data obtained from the XRD analysis:

$$D = \frac{K\lambda}{\beta \cos \theta} \quad [1]$$

where: D = Crystallites size (nm); $K = 0.9$ (Scherrer constant); $\lambda = 0.15406$ nm (Wavelength of the x-ray sources); β = Structural broadening; FWHM (radians) and θ = Peak position (radians). Also obtained from the XRD analysis was the lattice strain of produced nanoparticles' crystal (ϵ) as given by Equation [2].

$$\epsilon = \frac{\beta}{4 \tan \theta} \quad [2]$$

where: β = Structural broadening; FWHM (radians) and θ = Peak position (radians).

ANTIMICROBIAL ASSESSMENT OF EGGSHELL NANOPARTICLE

The antibacterial, antifungal, and antimicrobial activities of the functionalized bandage were used to assess the antimicrobial characteristics of the produced eggshell nanoparticles. It is worthy to note that, all experiments were independently conducted in triplicate and results were expressed as mean $\pm$ standard error using built-in functions and data analysis toolpak add-in feature in excel soft-ware.

The antibacterial activity of the synthesized nanoparticles was assayed using the broth culture method (35). Approximately 0.1 mL of each 24 h broth culture was adjusted to a 0.5 McFarland standard (1×10^6 cfu/mL) for isolated strains of multidrug-resistant gram-positive and gram-negative bacterial isolates (*Escherichia coli*, *Klebsiella oxytoca*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) obtained from the Kwara State University Teaching Hospital, Ilorin, Nigeria were employed in this study. This standardized culture was used to inoculate 9 ml of peptone broth, with the addition of 1 mL of each of the eggshell nanoparticles produced and calcined at 1000 °C containing graded concentrations of 50, 75, 100, 125 and 150 μ g/mL. The control had no nanoparticles added. They were incubated at 37 °C for 24 h. Their optical density readings were taken afterward at 600 nm, and the percentage growth inhibition was determined from the Equation [3]. Ciprofloxacin 30 μ g/mL was chosen as the positive control for the study of antibacterial activity and compared the results.

$$\% \text{ Growth inhibition} = \frac{OD \text{ Control} - OD \text{ Test}}{OD \text{ Control}} \times 100 \quad [3]$$

In addition, the antifungal effectiveness of the eggshell nanoparticles against specific fungi isolated from spoiled food samples was evaluated using the method described in the literature (36). The nanoparticles at two different concentrations (100 and 150 μ g/mL) were integrated into potato dextrose agar (PDA) plates. Subsequently, 6 mm agar plugs from 72h cultures of *Aspergillus flavus*, *Aspergillus fumigatus*, and *Aspergillus niger* were placed on the PDA plates containing the nanoparticles. Control plates were prepared with fungal agar plugs but without the nanoparticles. All plates were then incubated at 28 ± 2 °C for 72 h. The diameters (D) of fungal growth were measured in millimeters, and the percentage of growth inhibition was calculated using Equation [4]:

$$\% \text{ Growth inhibition} = \frac{OD \text{ Control} - OD \text{ Test}}{OD \text{ Control}} \times 100 \quad [4]$$

Furthermore, the antimicrobial potential of the functionalized bandage was assessed against the previously mentioned bacterial strains using the agar diffusion method, as outlined earlier (37). Fabric discs with a diameter of 14 millimeters were cut and treated with the synthesized eggshell nanoparticles. Muller-Hinton agar plates were prepared and inoculated with 24-hour-old cultures of the test organisms using a sterile swab. The functionalized fabric discs were then placed aseptically on the agar plates and incubated for 24 h. The zones of inhibition at different concentrations (50, 75, 100, 125 and 150 μ g/mL) of eggshell nanoparticle were observed and measured according to the method described earlier (37).

Moreover, the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of sol-gel synthesized and ball-milled calcium nanoparticles were determined using the standard broth dilution method, in accordance with the Clinical and

Laboratory Standards Institute (CLSI M07-A8) protocol (38). Calcium nanoparticles were prepared at concentrations of 50, 75, 100, 125, and 150 $\mu\text{g/mL}$ in Nutrient Broth. Bacterial cultures of *Escherichia coli*, *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were grown overnight and adjusted to a 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL). The MIC and MBC determination was performed by adding 1 mL of the nanoparticle solution to each test tube, followed by 0.1 mL of the bacterial suspension. The tubes containing only nutrient broth and bacteria served as the positive control, while tubes containing only nutrient broth and nanoparticles (without bacteria) served as the negative control. All tubes were incubated at 37 °C for 24 h. For MBC determination, samples from tubes with no visible growth were plated onto nutrient agar and incubated for another 24 h at 37 °C. The MBC was defined as the lowest concentration at which no bacterial colonies were observed (39). The MIC was recorded as the lowest concentration of calcium nanoparticles that showed no visible turbidity (indicating inhibition of bacterial growth) after incubation while the MBC was recorded at the lowest concentration at which no bacterial colonies were observed, indicating 99.9% bacterial kill. All experiments were performed in triplicate for accuracy (40).

RESULTS AND DISCUSSION

CHARACTERIZATION OF EGGSHELL NANOPARTICLES PRODUCED FROM DIFFERENT ROUTES

The eggshell nanoparticles produced from both the sol-gel and ball-milled method were characterized and compared with commercially available calcium oxide using various characterization tools. High-resolution imaging Transmission Electron Microscopy (TEM) was used to determine and examine the size and shape of eggshell nanoparticles produced through the sol-gel and ball milled technique as illustrated in Figure 4a and b and compared with the commercially available nanoparticle calcium oxides (Fig. 4c).

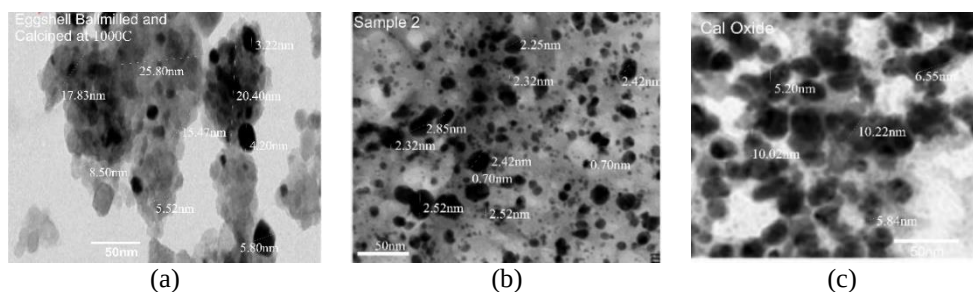


Figure 4. TEM image of synthesized eggshell nanoparticle

The image was obtained at 50-nm magnification as discernible in Figure 4. The ball-milled eggshell-produced nanoparticles have a significant agglomeration in an irregular shape with the mean particle size of 10.79 nm which varies from 3.29 to 17.79 nm in distribution. These results corroborated with the earlier findings (41), where 59.91 to 96.03 nm particle size with accompanied agglomeration was reported. The sol-gel-produced eggshell nanoparticle shows no agglomeration and was characterized with uniform near-net spheroid shapes of 2.10 nm mean particle size on 0.75 nm upper and lower limit variations. This

characteristic lower particle size of nanoparticles as obtained in this study may be attributed to the smaller particle size of the precursor ($<63\ \mu\text{m}$) and higher relative calcination temperature ($1000\ ^\circ\text{C}$) used during preparation compared to ($<100\ \mu\text{m}$) precursor particle size and $900\ ^\circ\text{C}$ calcination temperature earlier reported (42, 43). In their work, calcium oxide nanoparticles synthesized by sol-gel techniques yielded 24.34 and 50-198 nm particle sizes as reported (42, 43). Comparatively, the TEM results of the commercially available calcium oxides are similar to that of the sol-gel-produced eggshell nanoparticles with the exception that it has a more uniform spherical shape and relatively bigger particle size. These findings confirm that the ball milled and sol-gel route processing conditions as employed in this study were effective in producing nano-sized particles of eggshells. Furthermore, a quantitative representation of the particle size variation comparison as depicted in Figure 5 shows that the sol gel method with the lowest particle size and variations can be used to obtain a more uniform and smaller nanoparticle size of eggshell.

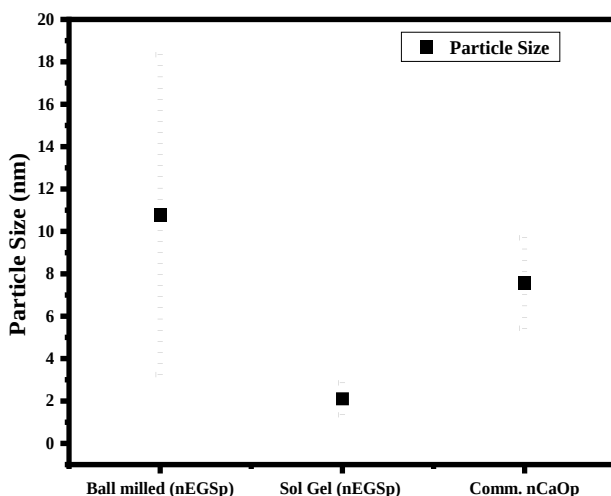


Figure 5. Variation of eggshell nanoparticle size produced from different routes

The EDS elemental composition of the eggshell nanoparticle obtained in this work is presented in Table 1. From the results (Table 1), the eggshell and commercially available calcium oxide nanoparticles contain calcium, oxygen, and carbon as the main constituents at varying ranges. Meanwhile, sodium, magnesium, and potassium elements were also found in both the sol-gel and ball-milled-produced eggshell nanoparticles. The commercially available calcium oxides have the highest (78.30 wt.%) amount of calcium followed by that produced by the sol-gel approach. The least was found in the ball-milled produced eggshell nanoparticles. This characteristic variation in calcium, oxides, and carbon as obtained in this work suggests the purity of calcium oxides obtained using different production routes. This implies that the sol-gel-produced eggshell nanoparticles have the purest form of calcium oxide after the commercially available one. The presence of other mineral elements (sodium, magnesium, silicon, phosphorus, iron, and potassium) in both the sol-gel and ball-milled produced eggshell nanoparticles which can aid in bone metabolism (44) could be an added advantage when incorporated as a reinforcement material for the development of biodegradable metal matrix composites.

The XRF analysis showing the chemical composition of the sol-gel and ball-milled produced eggshell nanoparticles compared with commercially available calcium oxide was depicted in Table 2. From the results, the ball milled and sol-gel eggshell nanoparticles are composed of 91.34 and 92.54 wt.% calcium oxides respectively as well as other mineral compounds such as magnesium oxide, iron III oxide, zinc oxide, tin oxide, and silicon iv oxides which are hard metallic oxide phases (34) in varying proportions. The presence of these metallic hard oxide phases when incorporated as reinforcement in a biodegradable metal matrix can raise load bearing capacity of the matrix to enhance the structural integrity of the resulting matrix composites.

Table 2. Elemental compositional analysis

Elemental Composition	Commercial CaO (wt.%)	Sol-gel @ 1000 °C(wt.%)	Ball milled @ 1000°C (wt.%)
Ca	78.30	67.4	66.33
O	21.40	17.17	19.55
C	1.30	3.62	3.10
Na	-	1.53	0.32
Mg	-	2.41	2.27
P	-	1.52	1.77
K	-	1.25	1.28
Al	-	0.85	0.76
Si	-	3.20	3.06
Fe	-	1.05	1.56

The high percentage of calcium oxide found in commercially available samples in this study could be attributed to the presence of calcium and oxygen in larger amounts as depicted by the EDS analysis (Table 3) compared to other samples.

Table 3. Elemental compositional analysis

Eggshell sample	Elemental Composition (wt%)									
	Ca	O	C	Na	Mg	P	K	Al	Si	Fe
Commercial CaO	78.30	21.40	1.30	-	-	-	-	-	-	-
Sol-Gel @ 1000 °C	67.40	17.17	3.62	1.53	2.41	1.52	1.25	0.85	3.20	1.05
Ball-milled @ 1000 °C	66.33	19.55	3.10	0.32	2.27	1.77	1.28	0.76	3.06	1.56

Furthermore, the X-ray diffraction (XRD) phase analysis of the eggshell nanoparticle produced by sol gel and ball milling techniques compared with that of market available calcium oxides are shown in Figure 6. The XRD peaks as discernible in Figure 6 corresponds to either of lime, portlandite or calcite phase at different diffraction angles. However, only two main phases (lime and portlandite) were conspicuously observed in commercially available calcium oxide while the eggshell nanoparticle produced from both routes possesses calcite phase in addition compared to the control sample. The lime phase was mainly detected at diffraction angle of $2\theta = 37.30^\circ$ and 53.8° (Fig. 6).

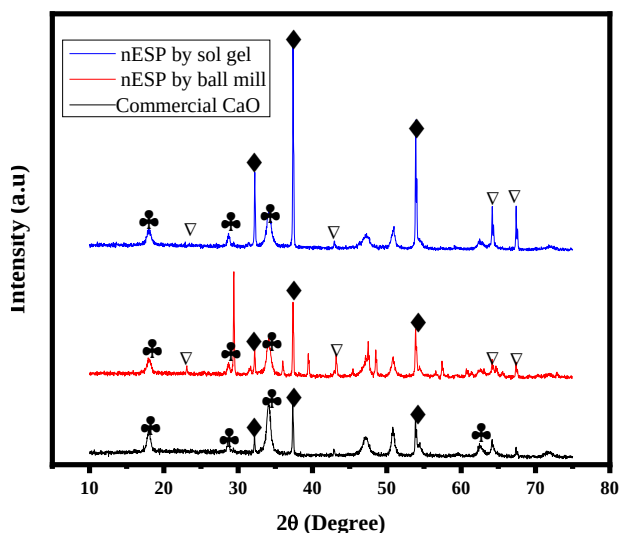


Figure 6. XRD Analysis of synthesized of eggshell nanoparticle

The peaks of portlandite (slake lime) phase were strongly exhibited at 2θ angles of 18.00° , 28.70° and 34.10° while the calcite phase was found at 23.10° and 43.20° diffraction angles. In contrast with the commercially available calcium oxide, the peaks of calcite found in either of sol gel and ball milled produced eggshell nanoparticle may be as a results of high carbon content originated from the natural source of the eggshell that contains organic matters which are not easily decomposed by the calcination temperature (44) thus, the formation of a hard and thermally stable calcite phase as depicted in Figure 6 for both the sol gel and ball milled produced eggshell nanoparticles. The above characteristic peaks as obtained for lime, portlandite and calcite phases were similar and amongst the peaks reported earlier (45) for lime, portlandite and calcite phase.

The crystallite size and lattice strain of the eggshell nanoparticle as illustrated in Figure 7 was determined by Scherrer equation (Equation [1]) and Equation [2] based on broadening of the diffraction peaks obtained from XRD analysis. The result shows that, the crystallite particle size varies from one diffraction peak to another at a certain diffraction angle for both the sol-gel sample and the ball-milled specimen. The minimum crystallite size varies from 4.08 nm to 32.49 nm while a range of 8.12 nm to 44.17 nm was obtained for the ball-milled eggshell nanoparticle. Precisely, the sol-gel-produced eggshell nanoparticle has a mean crystallite particle size of 18.39 nm while that of ball milled produced sample was 25.55 nm. This result affirms and validates the particle size ranges obtained from the TEM analysis and presented in Figures 4 and 5. However, the results obtained from TEM analysis are lower than that obtained from the X-ray diffraction peaks. This variation may be attributed to the fact that, the TEM focused on a small portion compared to a larger focus area of the X-ray diffraction light beams on sample crystals. In addition, the crystallite lattice strain of the eggshell nanoparticle obtained from both routes were as presented in Figure 7b with a higher magnitude of 8.912×10^{-3} for sol-gel sample while the ball-milled sample was estimated to be 7.267×10^{-3} . This implies that the sol-gel sample with a smaller particle size is under higher strain conditions compared to the sample produced by the ball-milled process with a relatively larger particle size (Figure 5).

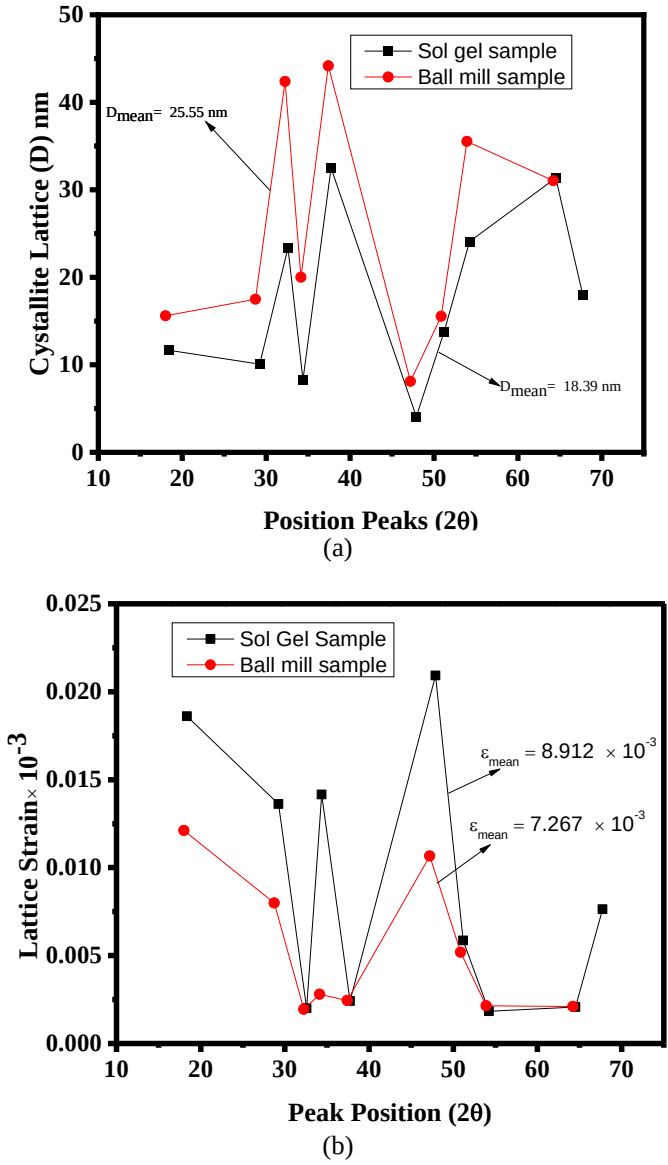


Figure 7. Crystal lattice size (a) and crystal lattice strain (b) variation with eggshell nanoparticles

ANTIBACTERIAL ACTIVITY OF EGGSHELL NANOPARTICLE

The antibacterial activity of eggshell nanoparticles against four isolated strains of multi-drug-resistant *Escherichia coli* (EC), *Klebsiella oxytoca* (KO), *Staphylococcus aureus* (SA), and *Pseudomonas aeruginosa* (PA) were investigated under five different concentrations (50, 75, 100, 125 and 150 µg/ml) with the resulting outcomes of percentage growth

inhibition were presented in Figure 8, for both the sol-gel (Fig. 8a) and ball milled (Fig. 8b) produced eggshell nanoparticles in comparison with Ciprofloxacin as positive control.

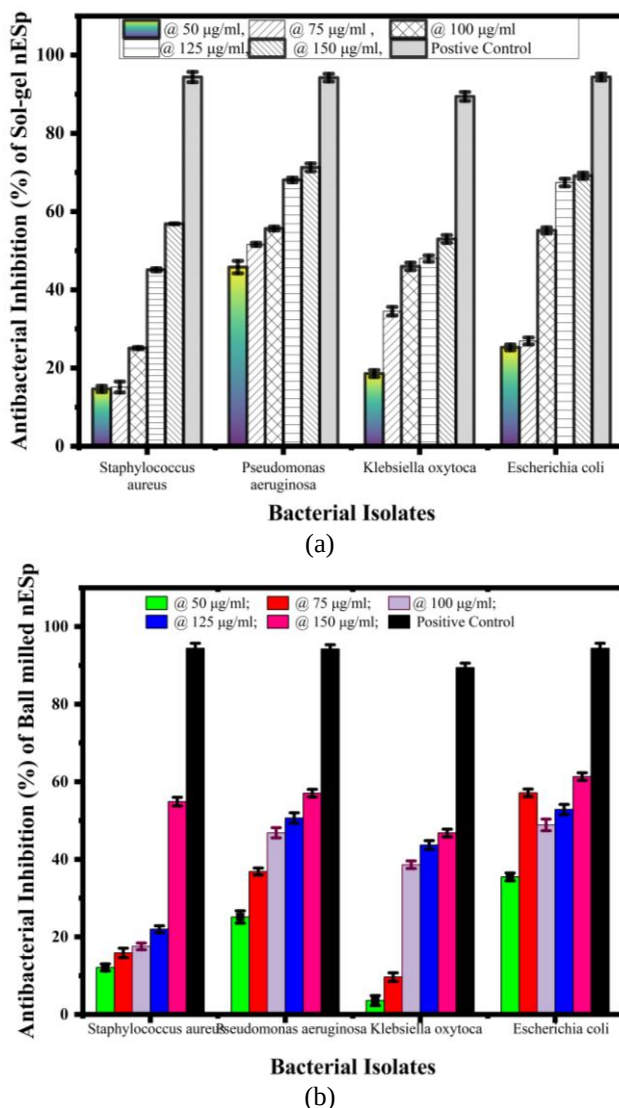


Figure 8. Antibacterial activity of Sol-gel (a) and ball-milled (b) produced eggshell nanoparticles

The results indicated a clear concentration-dependent inhibition characteristic of the eggshell nanoparticles that are destructive to all the tested bacterial strains at varying concentrations for both the sol-gel and ball-milled samples. The growth inhibition is higher at higher concentrations compared to lower concentrations of nanoparticles. For instance, SA Isolate showed growth inhibition of $14.61 \pm 0.80\%$ and $12.11 \pm 1.60\%$ at $50 \mu\text{g/ml}$ for sol-gel and ball-milled samples respectively. While at $150 \mu\text{g/ml}$ concentration, the growth inhi-

bition of SA bacterial strains rose to $56.86 \pm 0.10\%$, and $54.86 \pm 1.11\%$ respectively for sol-gel and ball-milled produced samples. Similar growth inhibitions of 45.80 ± 1.6 , 25.12 ± 1.20 , and 71.29 ± 1.01 , $57.04 \pm 1.12\%$ for the sol-gel and ball-milled samples respectively were obtained for PA isolates at 50 and 150 $\mu\text{g/ml}$ concentrations. Investigation with KO isolate depicts 18.58 ± 0.90 , 3.56 ± 1.3 , and 52.93 ± 0.87 , $46.77 \pm 1.0\%$ growth inhibitions at 50 and 150 $\mu\text{g/ml}$ using the sol-gel and ball-milled eggshell nanoparticles respectively. Equally, 25.25 ± 0.75 , 35.45 ± 1.03 , and 69.14 ± 0.82 , $61.35 \pm 0.98\%$ growth inhibitions were noticed with sol-gel and ball-milled produced samples at 50 and 150 $\mu\text{g/ml}$ concentrations. This noticeable antibacterial efficacy, of both the sol-gel and ball-milled eggshell nanoparticles especially at higher concentrations against the bacteria isolates, is due to the nanometer-scale size and enhanced surface reactivity of the eggshell nanoparticles, which promote close interaction with bacterial membranes, leading to structural damage, disruption of ion balance, and eventual cell lysis.

Based on these results, the sol-gel prepared sample has the most harmful effect on all the bacterial strains investigated with the peak antibacterial activities recorded against the PA isolate. On the contrary, the least inhibition potential was observed in the KO isolate for both the sol-gel and ball-milled prepared samples. This implies that the eggshell nanoparticle has the least antibacterial effect on *Klebsiella oxytoca* and more antibacterial potential with *Pseudomonas aeruginosa*. The better antibacterial effect of the sol-gel-produced sample against the ball-milled eggshell nanoparticle could be attributed to slow and controlled gelation process of the sol-gel precipitation process using dropwise titration of dilute NaOH into a homogeneous eggshell-HCl solution coupled with aging and calcination process that favored the formation of uniformly dispersed, ultrafine crystalline Ca(OH)_2 nanoparticles with improved physicochemical properties, such as increased surface area as evidenced in the TEM particle size analysis (Fig. 4). This characteristic feature can induce the lattice strain and surface defects formation for increased generation of reactive oxygen species (ROS), thereby increasing bactericidal activity of eggshell nanoparticle produced by sol-gel method. These results are in line with the earlier submissions (21, 47, 48) where it was reported that the accumulation of metal oxide against the *E.Coli* and other bacterial strains, caused a damaging effect on the cell membrane. The heightened susceptibility of *P. aeruginosa* and *E. coli*, at lower and higher concentrations can be attributed to thinner peptidoglycan layer in the cell wall which can catalyze nanoparticle penetration and internal damage while the relatively lower inhibition observed against *K. oxytoca* may be traced to its inherent resistance mechanisms, such as efflux pump expression and biofilm development, which are well-documented in literature (49).

Although, the sol-gel and ball-milled produced eggshell nanoparticles as obtained in this study demonstrated lower inhibition efficacy compared to positive control (Ciprofloxacin which recorded over 89% across all isolates) but, their biogenic origin, sustainability, and promising efficacy support their potential as alternative or supplementary antibacterial agents. These findings are consistent with earlier study (50), where it was reported that calcium-based nanoparticles synthesized through sol-gel approaches showed broad-spectrum of antimicrobial activity.

ANTIFUNGAL ACTIVITY OF EGGSHELL NANOPARTICLE

The antimicrobial activity of eggshell nanoparticles against fungi strains, including *A. niger*, *A. fumigatus*, and *A. flavus*, as measured by percentage growth inhibition zone is presented in Table 3 and Figure 9.

Table 3. Antifungal efficacy of eggshell nanoparticle

Antimicrobial Activity on Fungi Strains (%) at different Concentrations (µg/ml)				
Fungi Isolates	Sol gel sample @100 (µg/ml)	Sol gel sample @150 (µg/ml)	Ball milled sample @100 (µg/ml)	Ball milled sample @150 (µg/ml)
<i>A. niger</i>	No zone	40	No zone	No zone
<i>A. fumigatus</i>	18	No zone	27	21
<i>A. flavus</i>	50	56	43	75

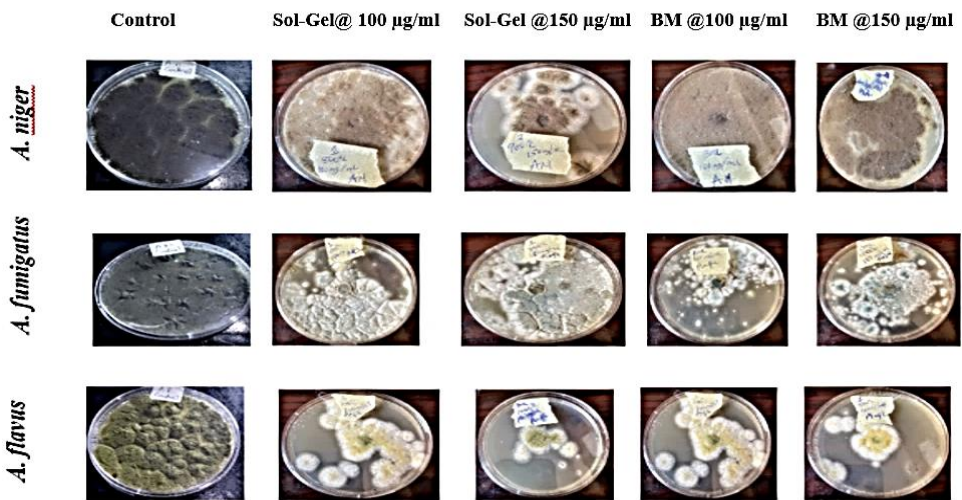


Figure 9. Anti-fungal growth inhibition of eggshell nanoparticle

It was observed that the eggshell nanoparticles have some anti-fungi potential which were more noticeable at higher concentrations especially for *A. flavus* fungi strains. For instance, *A. niger*, isolate, showed no zone of inhibition at 100 µg/ml for the sol-gel produced sample (Fig. 9), while at 150 µg/ml concentration, it exhibited growth inhibition of 40% (Fig. 9). On the contrary, there were no zones of inhibition detected for the ball-milled prepared samples at both 100 and 150 µg/ml concentrations (Fig. 9). Also, analysis with *A. fumigatus* isolate exhibited growth inhibition zone of 18% at 100 µg/ml (Fig. 9), whereas no zone of growth inhibition was obtained with 150 µg/ml of sol-gel produced sample (Fig. 9). Similar characteristics were also observed with the ball-milled prepared sample where a growth inhibition zone decrease from 27 to 21% at 100 and 150 µg/ml concentrations (Fig. 9), respectively. In addition, for *A. flavus* isolate, a zone of inhibition at varying degrees was displayed both concentrations for both the sol-gel and ball-milled produced samples (Table 3 and Figure 9). The sol-gel produced nanoparticle showed a 50% growth inhibition at 100 µg/ml and 56% inhibition at 150 µg/ml concentrations. While for the ball-milled nanoparticle, a growth inhibition of 43 and 75% inhibition were exhibited at 100 and 150 µg/ml nanoparticle concentrations. This indicates that, *Aspergillus flavus* was the most susceptible among the tested fungi isolates, with the highest inhibition zone rate at 150 µg/ml concentration for both the sol-gel and ball milled produced eggshell nanoparticle. This sug-

gests that, both methods of synthesis produced active nanoparticles though, the ball-milled eggshell nanoparticle at higher concentrations were more effective against *A. flavus*. In contrast, *Aspergillus niger* exhibited strong resistance, with no inhibition observed in three of the treatment concentrations. This may possibly due to its thick, melanized cell wall that restricts nanoparticle penetration. These findings corroborated with earlier findings (51) where it was reported that nanoparticle efficacy varies significantly with fungal species due to differences in spore surface composition and cell wall integrity. Furthermore, the enhance antifungal activity observed at higher concentrations, particularly in *A. flavus*, aligns with earlier submission (52) who attributed such effect to enhanced generation of reactive oxygen species with accompanied disruption of cell membrane. This trend of anti-fungi performance of eggshell nanoparticles has also been reported in the literature (53) where a moderate anti-fungi efficacy was reported of CaCO_3 nanoparticle against the tested fungi isolates.

FUNCTIONALIZATION OF BANDAGE

The antibacterial activity of functionalized bandages treated with both sol-gel synthesized and ball-milled calcium nanoparticles at 5 different (50, 75, 100, 125 and 150 $\mu\text{g/ml}$) concentrations as depicted in Figure 10 showed dose-dependent inhibition against all the tested bacterial isolates. The zones of inhibition increased with increasing nanoparticle concentrations. In both formulations, *Escherichia coli* exhibited the highest susceptibility, particularly to sol-gel synthesized nanoparticles, with a maximum inhibition zone of 24.50 mm at 150 $\mu\text{g/ml}$. *Staphylococcus aureus* showed relatively moderate susceptibility across both nanoparticle types, though the inhibition patterns were comparable.

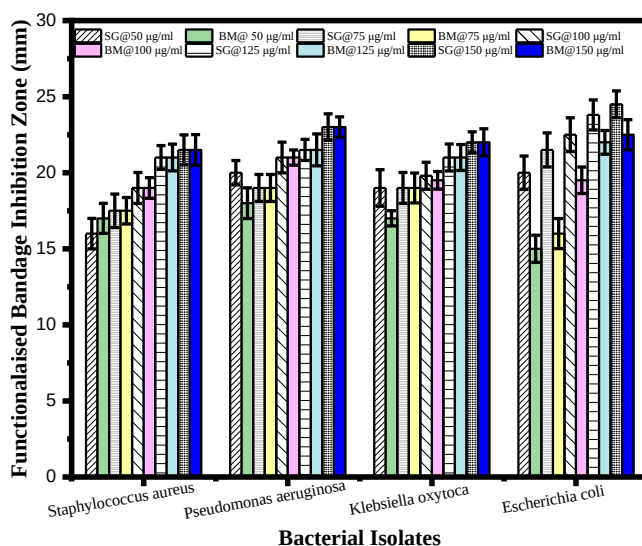


Figure 10. Functionalization of bandage Zone of inhibition of different Bacterial Isolates

As evident, Figure 10 displayed zones of inhibition ranged from 16 mm to 21.5 mm across different concentrations and processing routes of eggshell nanoparticles for *Staphylococcus aureus* strain. Although, there was no significant difference in performance com-

paring the nanoparticle processing route when the same concentrations were used on the same isolate of *Staphylococcus aureus*. Similar characteristic trends were also observed with other bacterial strains with the exception of *Escherichia coli* bacterial strains whose performance is dose and processing route dependent. For *Pseudomonas aeruginosa*, it exhibited zone of inhibition ranging from 18 mm to 23 mm while, *Klebsiella oxytoca* displayed inhibition range from 17 to 22 mm. But *Escherichia coli* showed performance difference with processing route at varied concentrations exhibiting enhanced activity (24.5 mm) compared to 22.5 mm noticed for the sol-gel over the ball-milled nanoparticle type. The enhanced performance of sol-gel synthesized nanoparticles can be attributed to their smaller, more uniform particle size and higher surface area, which may improve interaction with bacterial membranes and enhance reactive oxygen species (ROS) generation leading to bacterial cell death. These findings align with recent studies, with earlier work (54–56) where a greater bactericidal efficacy of nanoparticles integrated into fabric matrices due to better physico-chemical characteristics was reported, suggesting their potential for biomedical applications like wound dressings. By implication of this result, it is hopeful that, the incorporation of eggshell nanoparticles as reinforcement material in a biodegradable metal matrix composite can enhance the wound healing pace of the bone fracture site via the prevention of bacterial growth at the osteogenesis stage.

EGGSHELL NANOPARTICLE MIC AND MBC DETERMINATION

The MIC and MBC results revealed that sol-gel eggshell nanoparticles were generally more effective than ball-milled produced nanoparticles against the tested bacterial isolates. Notably, *Escherichia coli* showed the highest susceptibility to sol-gel with a MIC of 75 µg/mL and MBC of 150 µg/mL, compared to 100 µg/mL and >150 µg/mL respectively for ball-milled eggshell nanoparticles. This suggests that the sol-gel route enhance nanoparticle dispersion and surface reactivity, thereby increasing antimicrobial activity. In contrast, *Staphylococcus aureus* exhibited relatively higher resistance to both nanoparticle types, aligning with earlier studies which report that Gram-positive bacteria often possess thicker peptidoglycan layers that may reduce nanoparticle penetration (57, 58). The superior performance of sol-gel sample may be attributed to their smaller particle size and higher surface area resulting which has been reported to enhance bactericidal action by increasing reactive oxygen species generation and membrane disruption (55). This finding underscored the significance of processing route on nanoparticle bioactivity and suggest sol-gel technique may offer a more promising route for synthesis of effective antibacterial agents from biogenic source.

CONCLUSIONS

In this paper, eggshell nanoparticles from chicken hen eggs were prepared using the sol-gel and ball milling routes. The resulting nanoparticle calcined at 1000 °C for 3 hours from both techniques was characterized to examine the chemical compositions, morphology, phase, and antibacterial properties for possible utilization as reinforcement material in biodegradable metal matrix composite development useful in biomedical applications. Based on the results and analyses, the following conclusions were hereby presented:

1. Eggshell nanoparticle from chicken hen eggshells was successfully prepared from both the sol-gel and ball-mill techniques as confirmed by the TEM and XRD result analysis.

2. The sol-gel method yielded the finest eggshell nanoparticle with mean particle and crystallite sizes of 2.102 ± 0.757 and 18.39 nm respectively without agglomeration. While 10.793 ± 7.540 and 25.550 nm mean particle and crystallite sizes of eggshell nanoparticles were obtained from the ball mill technique.
3. The presence of hard metallic oxides such as CaO (91.37-92.87 wt.%), MgO (2.66-2.92 wt.%), Fe_2O_3 (0.23-0.36 wt.%), and ZnO (0.18-0.22 wt.%) and hard ceramic phases (calcite, lime, and portlandite) as detected by XRF and XRD analyses suggest that, the inclusion of eggshell nanoparticle as reinforcement in biodegradable matrix can improve the load bearing capacity of the resulting composites.
4. Eggshell nanoparticles produced from both the sol-gel and ball-milled method displayed an enhanced antibacterial, and antifungal activity and a higher zone of inhibition on functionalized bandage against all the tested bacteria at $150 \mu\text{g/ml}$ concentration. Furthermore, the sol-gel sample performed better compared to the ball-milled produced sample at all concentrations with greater antibacterial efficacy on *Pseudomonas aeruginosa* and *Escherichia coli* bacteria strains.
5. The eggshell nanoparticle produced from sol- the gel route characterized with the least particle size (nm) without agglomeration, high hard metallic oxides and ceramic phases with better enhanced antibacterial activities against all the tested bacterial strains can be a promising reinforcement material for the development of biodegradable metal matrix composite with enhance structural and biological performance for biomedical applications.

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