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Antibacterial and Antibiofilm Activity of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell

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ABSTRACT

Background: The Calcite Calicium Carbonate Nanoparticles ($CaCO_3$NPs) synthesized from the chicken eggshell (CES), this was done by crushing the shell and sieving.

Objective: The aim of the current study is to find a way to benefit from CES residues and the possibility of exploiting them in the production of $CaCO_3$NPs, which is used in many medical applications.

Material and methods: A hundred burn wounds samples were collected from several clinical sources. A total of 60 bacterial isolates were obtained from the burn wounds. $CaCO_3$NPs has effective antibacterial activity; the inhibition zone was 12 mm for *Acinetobacter baumannii*, *Proteus mirabilis* and *Staphylococcus aureus*, 13 mm for *Escherichia coli*, 11 mm for *Klebsiella pneumoniae*, 14 mm for *Burkholderia cepacia*, and *Pseudomonas aeruginosa*.

Results: The results showed that the MIC for *A. baumannii* was 25 µg/ml. The same MIC values were for *K. pneumoniae* and *S. aureus*, while *B. cepacia*, *E. coli*, *P. mirabilis*, and *P. aeruginosa* have the MIC was 6.25 µg/ml. The present study shows the efficiency of $CaCO_3$NPs to prevent the biofilms of Gram negative and Gram-positive isolates.

Conclusion: Synthesized $CaCO_3$NPs from the chicken eggshell have biological effectiveness and it can be used in dental fillings and bone repair.

Keywords: Burn wound, Pathogenic bacteria, Chicken eggshells, $CaCO_3$NPs, Antibacterial activity, Antibiofilm activity, University of Baghdad.

INTRODUCTION

Chicken eggs are a vital portion of the daily human food worldwide and serve as an economical and high-grade nutritious food [(1)]. An eggshell is the external shell of a rigid-covered chicken egg, which contains generally of $CaCO_3$ with certain proteins, additional minerals and organic membranes [(2)]. The total egg producing has grown from 61.7 million tonnes in 2008 to 76.7 million tonnes in 2018, as maintained by data from the FAO [(3)].

The producing eggs in Iraq increases yearly; the amount of eggs was 55,920 tonnes in 2020. The ES waste is only used at the level of scientific research, and the remaining quantities of ES waste are thrown into landfills sites without any pretreatment [(4)].

Managing the large amounts of ES rubbish per year produced in the world is a trouble as generally this material is only thrown at landflls with smell output and microbial propagation. Environmental pollution resulting from throwing ES in the landfill, such as the release of nushadir (NH_3) and kibritid alhaydrujin (H_2S) and unpleasant odors, which attract rodents and insects [(5)].

$CaCO_3$ as an intact natural mineral has been used in a broad scale of applications including medicine, manufacturing, and nanotechnology [(6)]. $CaCO_3$ of eggshell is fundamentally used as a diluent in rigid potion forms in a medicinal excipient [(7)]. $CaCO_3$ it's also used as consumption as a ground for pharmaceutical and dental productions, buffering and disintegration aid in dissolvable tablets, as well as a food additional and calcium enhancer [(8)].

The current study aimed to find a way to benefit from ES residues and the possibility of exploiting them in the production of Calcium carbonate nanoparticles ($CaCO_3$NPs), which is used in many medical applications.

MATERIALS AND METHODS

Sample Collection: A hundred burn wounds samples were collected from several clinical sources, including the Burn Center / Yarmouk Teaching Hospital, educational laboratories / Burns Specialized Hospital in Medical City, Al-Kadhimiya Teaching Hospital, Imam Ali Hospital (peace be upon him) and Al-Sadr Hospital at different ages and for the period between October 17th, 2021 to January 10th, 2022. The burn wounds samples were cultured on different media for the purpose of isolation and initial diagnosis of pathogens bacteria.

Isolation and identification of pathogens bacteria: Burn wounds samples were inoculated on different media and incubated at 37ºC for 24 hours. For further phenotypic characteristics, isolates were sub cultured on (Eosin methylene blue, CHROMagar Orientation). Then, isolates were subjected to Gram stain and biochemical tests including IMViC (indole, VP, MR, citrate), oxidase, catalase, urease and Kligler iron agar tests. Identification of isolates was confirmed by Vitek2 compact system according to manufacturer's instruction [(9)].

Preparation of chicken eggshells powder: The chicken eggs powder prepared according to [(10)].

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Synthesis of $CaCO_3$NPs from chicken eggshells powder: The CES powder was dried in hot air oven at 50°C for 12 h and transformed into nanomaterials utilizing a mechanical process in the existence of a Laboratory Ball Mill (IndiaMART, India) for three hours subsequently reserved at 50°C in a sterilized bottle previous to usage. The eggshell powder was calcinated in a furnace (Nabertherm™ Muffle Furnace, Denmark) at 850°C for an hour to produce calcium carbonate nanoparticles [11].

Preparation of $CaCO_3$NPs Colloidal: $CaCO_3$NPs powder was dissolve in deionized distilled water using a propane sonicator (Cole-Parmer Ultrasonic Processor Stainless Steel Temperature Probe) and use the suspension to test the characterization of the $CaCO_3$NPs and antibacterial activity.

Characterization of Calcite ($CaCO_3$NPs) Synthesized from Chicken Eggshell

1. UV-Vis Spectra analysis: The $CaCO_3$NPs synthesized using Chicken Eggshell was slightly distribute in double distillation water. The colloidal put in a cuvette and measure the absorbance from (300-800 nm).

2. Fourier Transform Infra-Red Spectroscopy (FT-IR): The Fourier Transform Infra-Red Spectroscopy was used to determine the effective compounds present in the calcium carbonate nanoparticle sample prepared using the chicken eggshell according to [12].

3. X-ray diffraction (XRD): One gram to two grams of eggshell powder was used for X-ray diffraction analysis to determine the peaks of $CaCO_3$NPs and to determine their size using the Dobby-Scherer equation [13].

4. Scanning electron microscope (SEM) imaging: Scanning Electron Microscope (SEM) was used to determine the morphologies of the $CaCO_3$NPs sample and the grain size of each sample was measured by quantitative analysis using the image application.

5. Zeta potential Measurements: Zeta potential measurements were performed to discover the surface charges obtained by $CaCO_3$NPs, which can be used to acquire more of insights into the stability of the obtained colloidal $CaCO_3$NPs [14].

6. Determination of antibacterial activity: The antibacterial activity of calcite (CaCO3) nanoparticles synthesized from chicken eggshells was done according to [15].

7. Minimum inhibitory concentration (MIC): The MIC of calcite ($CaCO_3$) nanoparticles synthesized from chicken eggshells was done according to [16].

8. Determination of antivirulence efficacy: The detection of the anti-biofilm efficacy of calcite ($CaCO_3$) nanoparticles synthesized from chicken eggshell against pathogenic bacteria using Microtiter plate assay as reported in [17].

Ethical approval

The Ethical and Scientific Committee in the College of Education for Pure Sciences -Ibn Al-Haitham, University of Baghdad, Iraq agreed to give their permission to the cross-sectional study that was conducted with approval number RECACPUB-3102020D. Signed consent was obtained from each participant.

Statistical analysis

The collected data were introduced and statistically analyzed by utilizing the Statistical Package for Social Sciences (SPSS) version 22 for windows. Qualitative data were defined as numbers and percentages. Quantitative data were tested for normality by Kolmogorov-Smirnov test. Normal distribution of variables was described as means and standard deviation (SD). P value ≤0.05 was considered to be statistically significant.

RESULTS

Isolation and identification

A total 60 bacterial isolates were obtained from the burn samples. The predominant bacteria isolated were Staphylococcus aureus (25%), followed by Acinetobacter baumannii (20%), Pseudomonas aeruginosa (16.7%), Klebsiella pneumoniae (13.3%), Escherichia coli (11.7%), Proteus mirabilis (10%) and Burkholderia cepacia (3.3%) (**Table 1**).

Table1. The number and percentage of Bacteria isolated from burn samples.

Bacterial isolates	Number	Percentage
Staphylococcus aureus	15	25
Acinetobacter baumannii	12	20
Pseudomonas aeruginosa	10	16.7
Klebsiella pneumoniae	8	13.3
Escherichia coli	7	11.7
Proteus mirabilis	6	10
Burkholderia cepacia	2	3.3
Total	60	100%

There are significant differences between the number of samples (P ≤0.05).

The UV-Vis analysis of the Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

The results of the UV-Vis analysis of the Calcite (CaCO3) Nanoparticles Synthesized from Chicken Eggshells showed in figure 1. The $CaCO_3$NPs were confirmed by the absorbance peak obtained at 301 nm, indicating that the $CaCO_3$NPs were sparse in the aqueous solution with no evidence of accumulation.

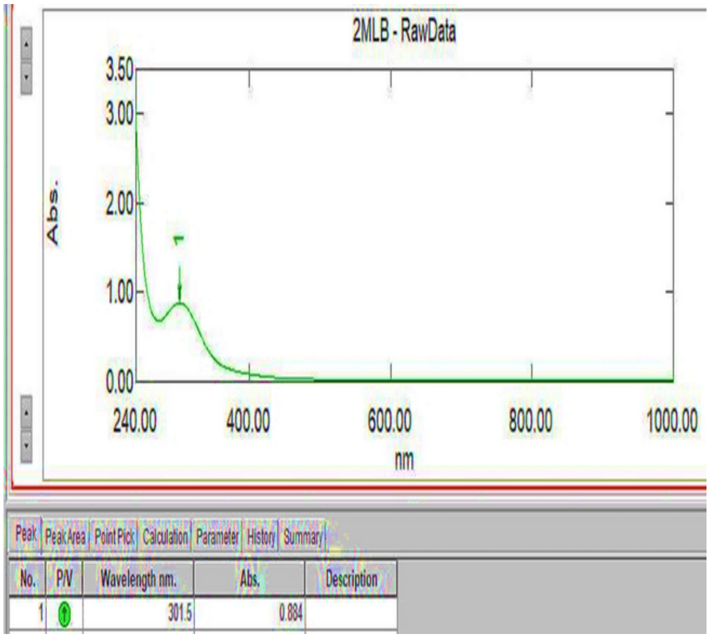

Figure 1. The UV-Vis analysis of the Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

The FTIR spectra of the Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

Figure 2 shows various absorption bands at multiple locations present in the FTIR spectra due to different vibrational modes of various chemical groups of C-O, C=O, C=C, and Ca-CO on the surface of the particles. The absorbance spectrum of calcite (CaCO3) nanoparticles presented six bands at 2501 cm−1, 1790 cm−1, 1408 cm−1, 1080 cm−1, 850-cm-1, and 710-cm-1, which are typical for the calcite phase.

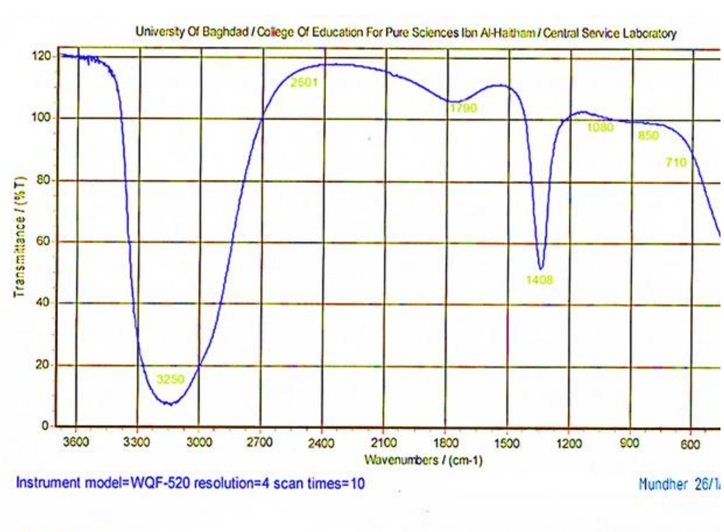

Figure 2. FTIR spectra of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

PXRD analysis of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell

The diffraction peaks of Calcite (CaCO3) nanoparticles synthesized from chicken eggshells are positioned at 2-theta values. **Figure 3** possess angles at 2-theta values 23.0°, 29.4°, 35.9°, 39.4°, 43.1°, 47.1°, 47.4°, 48.5°, 57.4° and 58.0° which correspond to peaks (0 1 2), (1 0 4), (1 1 0), (1 1 3), (2 0 2), (0 1 8), (0 1 6), (012), (1 2 2) and (0 10), respectively. This set of values is characteristic of calcite (CaCO3) when compared with standard data (JCDPS No. 05-0586). The average particle size of CaCO3NPs was calculated using the Debye - Scherrer equation, it was 32 nm.

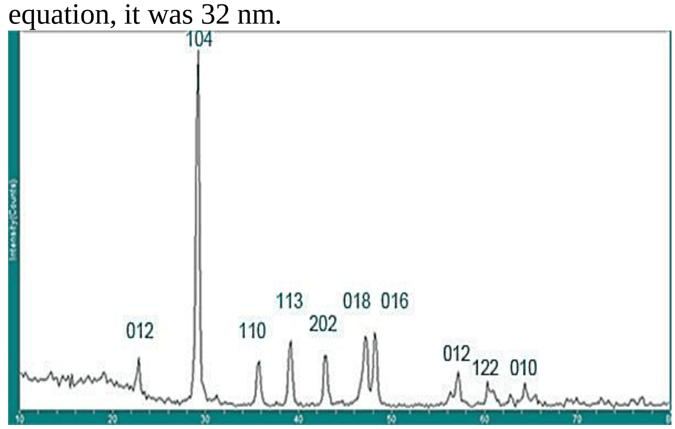

Figure 3. PXRD analysis of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

SEM analysis of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

Figure 4 shows the SEM image of Calcite $CaCO_3$NPs synthesized from the chicken eggshells. It detects that $CaCO_3$NPs appear rhombohedral cubic and particles form clusters. The sizes of particles range from 31-34 nm.

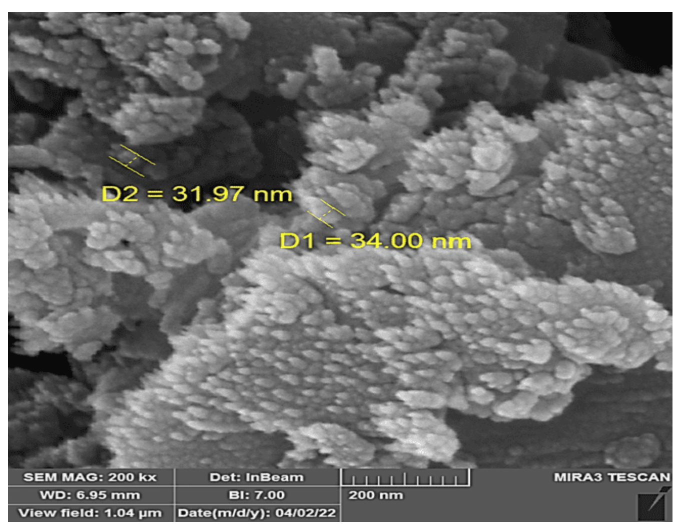

Figure 4. SEM analysis of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

Zeta potential measurement of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

Zeta potential of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell colloidal was -30 mV indicates that the particles are stable and do not agglomerate **Figure 5**.

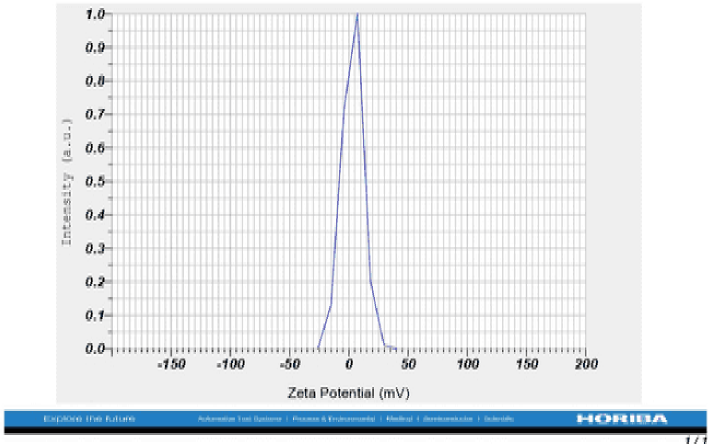

Figure 5. Zeta potential measurement of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell.

The Antibacterial Activity of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell

The results in **Table 2** and **Figures 6 and 7** appear that Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell has efficient antibacterial efficacy on the bacteria. The inhibition zone was 12 mm for *Acinetobacter baumannii*, *Proteus mirabilis*, and *Staphylococcus aureus*, 13 mm for *Escherichia coli*, 11 mm for *Klebsiella pneumoniae*, 14 mm for *Burkholderia cepacia* and *Pseudomonas aeruginosa*. The antimicrobial activity of eggshell powder had the least effective against the tested isolates, as it ranged between 7-8 mm, and the deionized distilled water had no antibacterial activity.

Table 2. The Antibacterial Activity of the Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell against the tested bacteria as demonstrated by diameters of the inhibition zone (mm).

Isolated bacteria	Zone of Inhibition		
	Deionized distilled water	Calcite ($CaCO_3$) Nano-particles powder	Calcite ($CaCO_3$) Nano-particles colloidal
Acinetobacter baumannii (No.1	0	7	12
Escherichia coli (No.5)	0	8	13
Burkholderia cepacia (No.2)	0	8	14
Klebsiella pneumoniae (No.4)	0	8	11
Proteus mirabilis (No.2)	0	7	12
Pseudomonas aeruginosa (No.1)	0	8	14
Staphylococcus aureus (No.4)	0	8	12

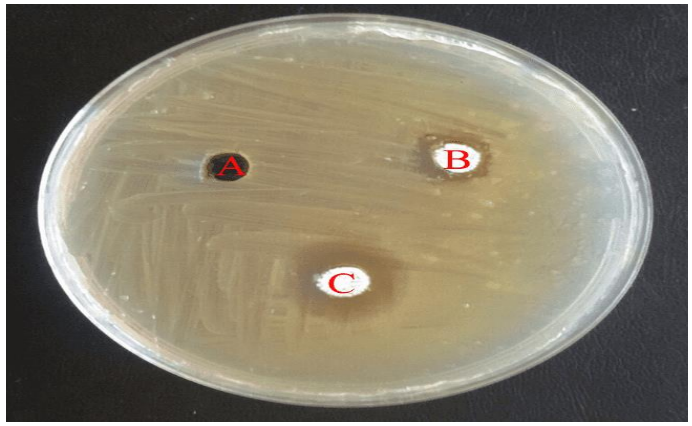

Figure 6. The antibacterial effect of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell (A) Deionized distilled water (B) $CaCO_3$NPs powder (C) $CaCO_3$NPs colloidal uses the test bacterium MDR *Acinetobacter baumannii*.

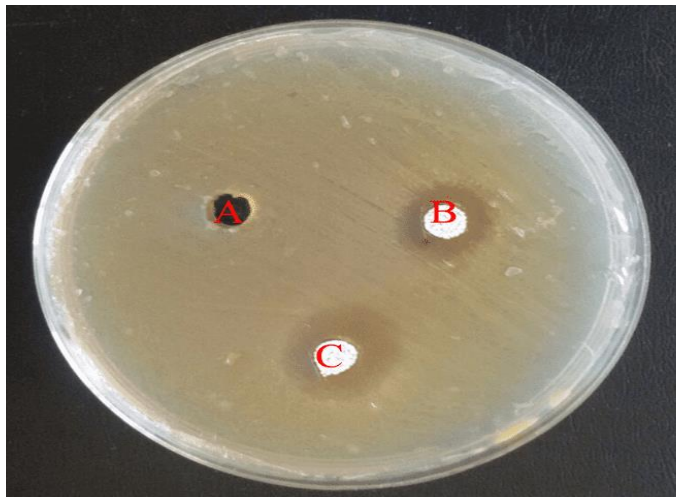

Figure 7. The antibacterial effect of Calcite ($CaCO_3$) Nanoparticles Synthesized from Chicken Eggshell (A) Deionized distilled water (B) $CaCO_3$NPs powder (C) $CaCO_3$NPs colloidal uses the test bacterium *Staphylococcus aureus.*

Minimum inhibitory concentration (MIC)

Seven concentrations of calcium carbonate nanoparticles (3.125, 6.35, 12.5, 25, 50, 100, and 200) µg/ml were used to determine the minimum inhibitory concentration. The results showed that the MIC for *Acinetobacter baumannii* was 25 µg/ml.

The same MIC values were for *Klebsiella pneumoniae* and *Staphylococcus aureus*, while *Burkholderia cepacia*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* have the MIC was 6.25 µg/ml (**Table 3 and Figure 8**).

Table 3. The antibacterial activity of Calcite ($CaCO_3$) Nanoparticles concentrations against Gram negative and Gram positive bacteria.

Isolates	Inhibition zone						
	$CaCO_3$NPs Concentrations						
	3.12 µg/ml	6.25 µg/ml	12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
Acinetobacter baumannii (No.1)	0	0	0	12	16	18	17
Burkholderia cepacia (No.2)	0	5	9	10	15	16	17
Escherichia coli (No.5)	0	7	8	13	17	17	16
Klebsiella pneumoniae (No.4)	0	0	0	13	18	18	17
Proteus mirabilis (No.2)	0	6	9	13	16	18	18
Pseudomonas aeruginosa (No.1)	0	5	8	13	17	19	16
Staphylococcus aureus (No.4)	0	0	0	14	17	18	17

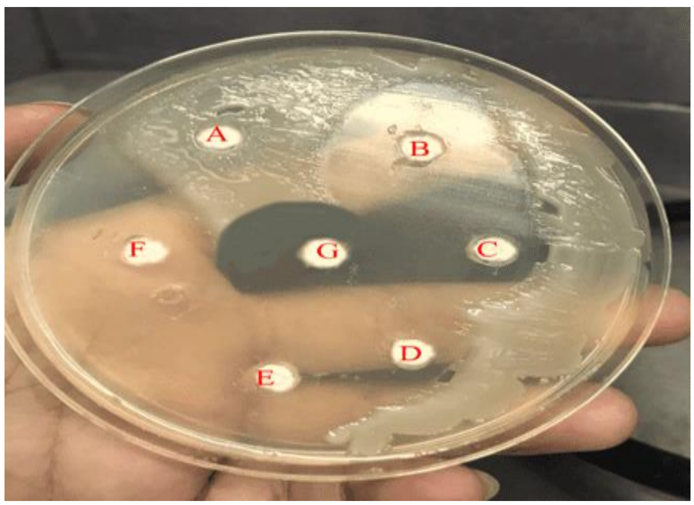

Figure 8. The antibacterial activity of Calcite ($CaCO_3$) Nanoparticles concentrations against *Escherichia coli* (No.5). A-3.12 µg/ml, B=6.25 µg/ml, C=12.5, D=25 µg/ml, E=50 µg/ml, F=100 200 µg/ml and G=200 µg/ml.

The results showed $CaCO_3$NPs have antibiofilm activity against ten types of bacteria six belong to Gram negative bacteria *A. baumannii*, *E. coli*, *B. cepacia*, *K. pneumonia*, *P. mirabilis* and *P. aeruginosa*. And four bacteria belong to Gram positive bacteria *Staphylococcus aureus* No.1, 2, 3 and 4 at 0.1 and 0.2 mg/ml $CaCO_3$NPs concentrations. The suppression of biofilm-forming was 70% for Gram negative bacteria and for Gram positive bacteria were 60% when using $CaCO_3$NPs at 0.1 concentrations. While the suppression of biofilm-forming was 80% for Gram-negative bacteria and for Gram-positive bacteria was 70% when using $CaCO_3$NPs at 0.2 concentration (**Fi gures 9**).

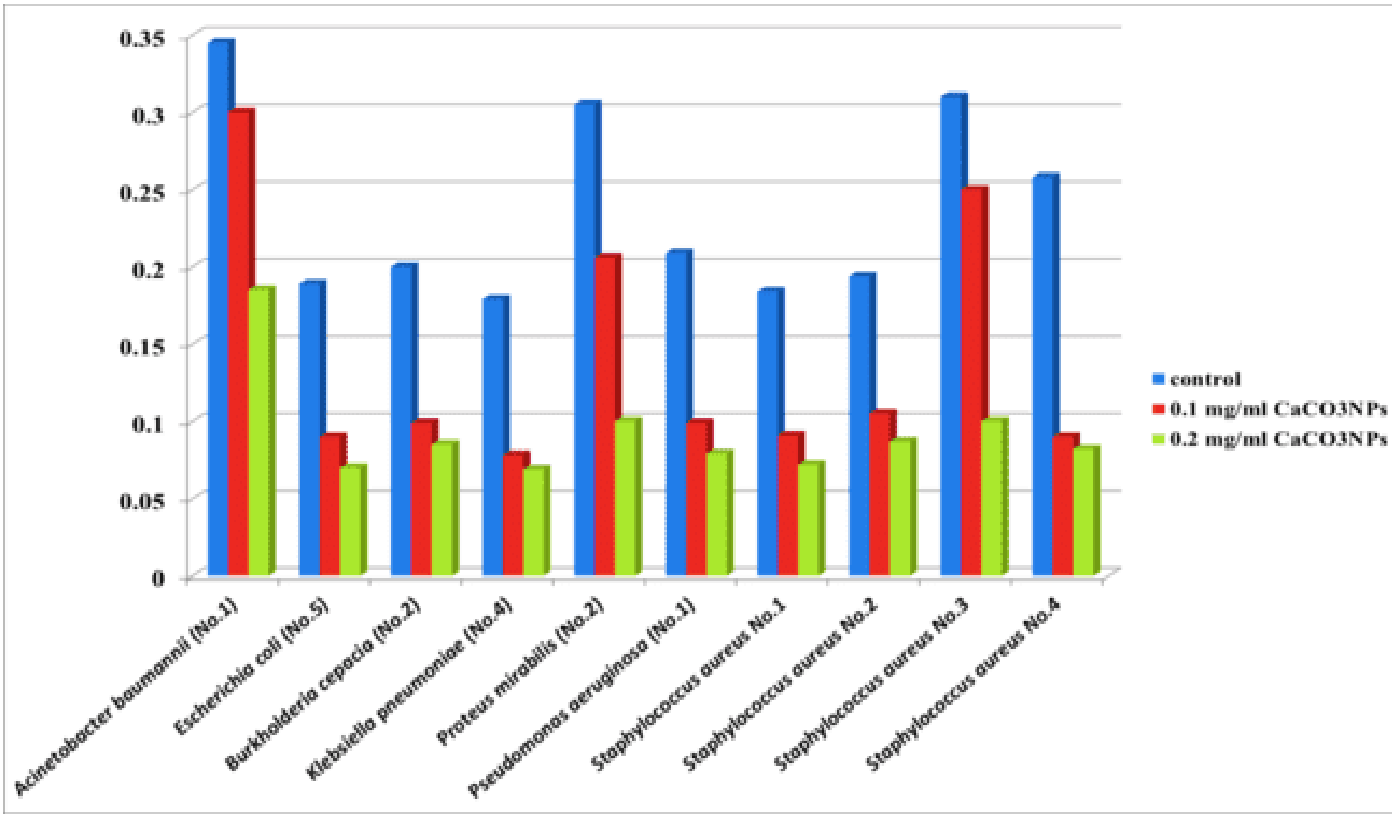

Figure 9. The suppression of biofilm-forming of Pathogenic bacteria isolates using $CaCO_3$NPs.

DISCUSSION

The major goal of the current study was the possibility of exploiting the eggshell (ES), which is estimated the amount of ES that are thrown into landfills annually in the millions of tons, and it is thrown without any pretreatment, which constitutes an additional burden on the environment.

Throwing ES without any pretreatment increases waste and attracts rodents and insects due to its strong odor. It also leads to the growth of bacteria and fungi.

When thrown into lakes and rivers, it causes the downfall and poisons the sea population. The CES powder can be employed as a food additive, antimicrobial factor, and fertilizer. The present study converts this eggshell to $CaCO_3$NPs. The organic matter in the eggshells, the membrane, and the egg whites were heated and removed to maximize the yield. From previous studies, there are different techniques listed for the synthesis of $CaCO_3$NPs from the dry eggshell selected for our project due to synthesizing $CaCO_3$NPs from the dry eggshell yields the maximum amount of $CaCO_3$NPs. These finding are in agreement with [10, 16]. in which noted that CaCO3 was the great ingredient in eggshell powder. Eggshell contained 87.5 (SD 0.5) wt. % CaCO3, which increased to 95 (SD 0.5) wt. % by purified eggshells after the heat treatment. This enhancement was due to the burning of protein and organic membranes in the eggshell [17].

Calcium carbonate nanoparticles synthesized from chicken eggshells were confirmed by UV-Vis analysis. The strong absorbance peak obtained was 301 nm correspond to $CaCO_3$ nanoparticles, indicating that the $CaCO_3$NPs were sparse in the aqueous solution with no guide of accumulation. These results are in agreement with [18,19,20] in which revealed that the $CaCO_3$ has peaks in the wavelength between (200-400) nm. FTIR results obtained the absorbance spectrum of calcite ($CaCO_3$) nanoparticles presented bands at 2501 cm^{-1}, 1790 cm^{-1}, 1408 cm^{-1}, 1080 cm^{-1}, 850-cm^{-1} and 710-cm^{-1} for chicken eggshells due to carbonate (CO_3). These results are in agreement with [21,22]. The XRD data revealed the crystalline nature and phase composition of the prepared calcium carbonate nanoparticles. The sharp peaks of the diffractogram indicating high crystallinity of the prepared powder and crystal shape was rhombohedral cubic. These finding are in agreement with [23, 24]. SEM image of Calcite $CaCO_3$NPs synthesized from chicken eggshell. It shows that $CaCO_3$NPs appear cubic and particles form clusters. The sizes of particles range 31-34 nm. These results are in agreement with [25,26]. The stability assay of calcite ($CaCO_3$) nanoparticles made from chicken eggshell gives a value of -30mv which is an indication of the stability of $CaCO_3$NPs. Zeta potential analysis gives values between (+30 mV and -30 mV) this indicated the adherent is considered constant. These results are in agreement with several previous studies [27,28].

Sixty bacterial isolates were obtained from the burn samples. The predominant bacteria isolated were *Staphylococcus aureus* (25.0%), followed by *Acinetobacter baumannii* (20.0%), *Pseudomonas aeruginosa* (16.7%), *Klebsiella pneumoniae* (13.3%), *Escherichia coli* (11.7%), *Proteus mirabilis* (10.0%) and Burkholderia cepacia (3.3%). These results are in agreement with numerous studies [9,29,30] which confirmed the same bacterial isolates from burn wounds, but in close proportions.

The results of the antimicrobial activity of $CaCO_3$NPs show the inhibition zone was 12 mm for *Acinetobacter baumannii*, *Proteus mirabilis* and *Staphylococcus aureus*, 13 mm for *Escherichia coli*, 11 mm for *Klebsiella pneumoniae*, 14 mm for *Burkholderia cepacia* and *Pseudomonas aeruginosa*. The antimicrobial activity of eggshell powder had the least effectiveness against the tested isolates, as it ranged between 7-8 mm. The deionized distilled water had no influence against the bacteria. The results agreed with some studies [31,32] which found $CaCO_3$NPs derived from eggshells have antibacterial activity.

Seven concentrations of calcium carbonate nanoparticles (3.125, 6.35, 12.5, 25, 50, 100, and 200) µg/ml were used to determine the minimum inhibitory concentration. The results showed that the MIC for *Acinetobacter baumannii* was 25µg/ml. The same MIC values were for *Klebsiella pneumoniae* and *Staphylococcus aureus*, while *Burkholderia cepacia*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* have the MIC was 6.25µg/ml. These results are in agreement with [32].

Results showed $CaCO_3$NPs have antibiofilm activity against ten types of bacteria six belong to Gram-negative bacteria *A. baumannii*, *E. coli*, *B. cepacia*, *K. pneumonia*, *P. mirabilis* and *P. aeruginosa*, and 4 bacteria belong to Gram- positive bacteria *Staphylococcus aureus* No.1, 2, 3 and 4 at 0.1 and 0.2 mg/ml $CaCO_3$NPs concentrations. The suppression of biofilm-forming was 70% for Gram negative bacteria and for Gram positive bacteria were 60% when using $CaCO_3$NPs at 0.1 concentrations. While the suppression of biofilm production was 80% for Gram negative bacteria and for Gram positive bacteria was 70% when using $CaCO_3$NPs at 0.2 concentrations. These results are in agreement with [33,34].

CONCLUSION

We concluded that synthesizing calcium carbonate nanoparticles from chicken eggshell waste is an easy and inexpensive process, and it has two characteristics. First, it reduces the environmental pollution caused by eggshells without preliminary treatment. Second, the production of a material has unique properties and is used in many medical and industrial applications.

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