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Investigations of Antibacterial and Antioxidant Properties of Calcite ($CaCO_3$) Nanoparticles Synthesized from Eggshell Extract for Biomedical Applications

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Abstract: Green nanotechnology aims to create nanomaterials and products that are ecologically safe and non-hazardous to human health, while simultaneously tackling environmental issues through creative nanoproducts. The instability of leftovers from non-synthetically created compounds needs the improvement of green chemistry in nanoparticle manufacturing. The aim of this study was to synthesize and characterize calcium carbonate nanoparticles ($CaCO_3$NPs) from methanol extracts of eggshells and assess their antibacterial effectiveness against *Staphylococcus aureus* and other Gram-positive coagulase-positive cocci. The optical properties, crystallinity, diameters, and shape of the biosynthesized nanoparticles were assessed using UV-Vis spectroscopy, FTIR (Fourier Transform Infrared Spectroscopy), and SEM (Scanning Electron Microscopy). The synthesis and characterization of nanoparticles were examined in an environmental study, which indicated that a more sustainable method could be discerned even at the laboratory size. This study determined that $CaCO_3$ nanoparticles may be synthesized using extracts from egg shells. This technology offers environmentally advantageous alternatives to hazardous operations, while promoting pollution prevention through the synthesis of nanoparticles in their natural settings. Recent research suggests that the generated calcium carbonate nanoparticles may function as an antioxidant and antibacterial agent against many harmful microorganisms.

Keywords: Pathogenic bacteria, Chicken eggshells, Nanoparticles, $CaCO_3$NPs, Antibacterial activity, DMSO

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Introduction

Chicken eggs constitute an essential component of the global human diet, providing an economical and high-quality source of nutrition. An eggshell is the outer covering of a chicken egg, primarily composed of calcium carbonate ($CaCO_3$), along with various proteins, minerals, and organic membranes (Ahmed *et al*., 2019). Egg production increased from 61.7 million tonnes in 2008 to 76.7 million tonnes in 2018, according to FAO data. Egg production in Iraq has been increasing annually, with a total of 55,920 tonnes recorded in 2020. ES waste is utilized solely for scientific research, while the remaining quantities are disposed of in landfills without pre-treatment (FAO, 2022).

The objective of the multidisciplinary field of nanotechnology is to create materials with enhanced atomic, molecular, and supramolecular properties. Abdelrahman *et al*. (2019) assert that nanomaterials are employed in microelectronics, glucose detection, DNA and RNA analysis, heavy metal detection, disease diagnosis, and disease management. Calcite nanoparticles can be synthesized using several physical, chemical, and biological methods (Daghir, 2008). The synthesis of nanoparticles using bacteria, fungi, algae, yeast, and plants is referred to as the "biological method". The narrow size distribution of nanoparticles is a benefit of physical and chemical methods; nevertheless, the main disadvantages are high energy, time, and cost requirements, along with the usage of dangerous substances (Ain *et al*., 2023). Biological technologies provide numerous advantages over conventional methods, including high yields, low prices, absence of downstream processing, and environmental sustainability. Additionally, an alternative term for the biological processes is "green synthesis." Consequently, the synthesis of nanoparticles by plant extracts offers a simple, single-step reduction process suitable for large-scale production (Owuamanam and Cree, 2020). Numerous research indicate that plant extracts exhibiting significant biological activity can be utilized to synthesize nanoparticles.

Managing the substantial quantities of electronic waste generated annually worldwide poses a challenge, as this material is typically disposed of in landfills, resulting in odor emissions and microbiological proliferation. Environmental damage caused by the disposal of electronic waste in landfills, including the emission of ammonia (NH_3) and hydrogen sulfide (H_2S), as well as noxious aromas that attract rats and insects (Kiranda *et al*., 2018). Calcium carbonate, being an unaltered natural mineral, has been utilized extensively across several industries, including medicine, manufacturing, and nanotechnology. Calcium carbonate ($CaCO_3$) from eggshells is mostly utilized as a diluent in solid dosage forms within pharmaceutical excipients. Calcium carbonate ($CaCO_3$) is utilized in the pharmaceutical and dental industries as a filler, a buffering agent, and a disintegration aid in effervescent tablets, in addition to serving as a food additive and calcium supplement (Murakami *et al*., 2007).

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

Materials and Methods

Preparation of chicken eggshell (*CES*) powder

The CES powder was mechanically converted into nanomaterials using a hot air oven set at 50°C for 12 h. This process took place in a Laboratory Ball Mill (IndiaMART, India) for 3 h. The powder was then stored at 50°C in a sterile bottle before being used. To create calcium carbonate nanoparticles, the eggshell powder was calcined for 1 h at 850°C in a furnace (NaberthermTM Muffle Furnace, Denmark) (Rasheed *et al*., 2019).

Synthesis of Calcium carbonate Nanoparticles

Using a propane sonicator (Cole-Parmer Ultrasonic Processor Stainless Steel Temperature Probe), $CaCO_3$NPs powder was dissolved in deionized distilled water. The suspension was then used to test the further activity such as antioxidant activity and, antibacterial activity and character of the $CaCO_3$NPs (Alkalifawi and Esam, 2014). Formation of calcite nanoparticles is depicted in Figure 1.

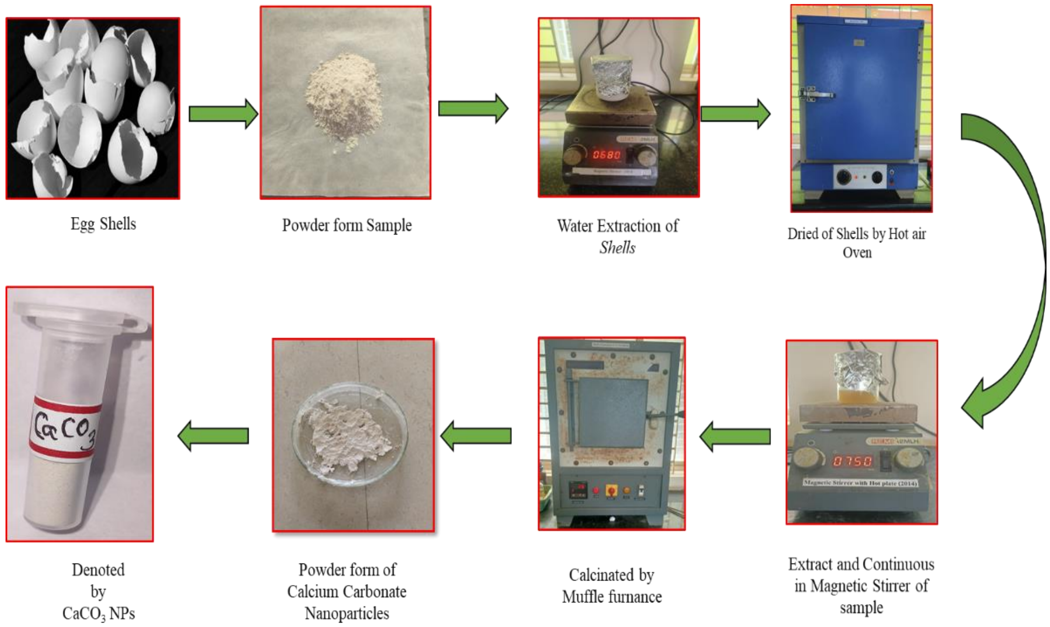


Fig. 1: Schematic illustration for the synthesis of $CaCO_3$NPs.

Characterization of Calcite Nanoparticles

UV-Vis Spectrophotometer

A UV-visible spectrophotometer with an identical beam was employed to measure calcium carbonate nanoparticles with wavelengths ranging from 300 nm to 700 nm. The current investigation was conducted using a Perkin Elmer Spectrophotometer with a resolution of 1 nanometer.

Fourier Transform Infrared Spectroscopy

The FT-IR (Shimadzu) was employed to ascertain the functional groups of Calcium carbonate nanoparticles that were prepared from egg shells. The KBr particles were used to grind the materials, and the FT-IR spectrum was analyzed from 4000 to 400 cm^{-1}.

Scanning Electron Microscopy

A scanning electron microscope (SEM) was used to ascertain the morphologies of the $CaCO_3$NPs sample. Additionally, each sample's grain size was estimated using quantitative analysis using the imaging program.

Antibacterial activity by Agar Well Diffusion

Method

The antibacterial efficacy of calcium carbonate nanoparticles derived from methanol extracts of egg shells was evaluated against pathogenic bacterial strains, specifically *Pseudomonas*, *Escherichia coli*, and *Salmonella*. The bacterial cultures were cultivated in Mueller Hinton Agar and Broth (Himedia). Antibacterial activity was assessed using diffusion disc plates on agar, with approximately 0.1 ml of each bacterial culture spread across the agar plate surfaces. For the antibacterial assay, all bacterial strains were incubated in Mueller Hinton Broth (Himedia) for 24 h at 37°C and subsequently plated on Mueller Hinton Agar (Himedia) for agar diffusion experiments (Ade *et al.*, 2019). Paper discs (6 mm in diameter) were positioned on the agar medium to absorb calcite nanoparticles from 100 µl of methanol extracts of egg shells. Inhibition diameters were assessed following incubation for 24 to 48 h at 37°C. In this study, Gentamycin (100 µg/ml) served as the positive control, while DMSO was utilized as the negative control to assess the sensitivity of the bacterial culture. Different amounts of methanol extracts (400 µg/ml, 500 µg/ml, and 600 µg/ml), prepared identically, from selected samples are utilized to assess antibacterial activity.

Antioxidant activity by DPPH assay

The free radical scavenging potential of calcium carbonate nanoparticles derived from egg shell extract was assessed. A DPPH solution (0.006% w/v) was prepared using 95% methanol. Various concentrations of the methanolic extract of nanoparticles (10, 20, 30, 40, and 50 µg/ml) were prepared in methanol. Three milliliters of various concentrations of prepared nanoparticles were combined with one milliliter of DPPH solution in the dark. Ascorbic acid, a potent antioxidant, is utilized as a standard and prepared in various concentrations using methanol (10, 20, 30, 40, and 50 µg/ml). Three milliliters of various concentrations of a standard ascorbic acid solution were combined with one milliliter of DPPH solution in a dark environment (Reig *et al*., 2002). The solution of ascorbic acid and plant extract samples was incubated for 30 min, after which absorbance was measured at 517 nm using a UV spectrophotometer. Methanol acted as a control, and the experiment quantified the inhibition percentage of free radicals by the sample, calculated using the following formula:

DPPH radical scavenging activity (%) = (Control OD-Sample OD) / Control OD x 100

Results and Discussion

Over the course of the past few years, a great number of major research have been carried out in the subject of nanobiotechnology with the purpose of boosting the activity that is associated with this field. In order to analyze the $CaCO_3$NPs that were produced from egg shell extract, a series of analyses were carried out. These analyses included scanning electron microscopy, as well as ultraviolet and Fourier transform infrared spectroscopy. The reducing and capping agent that was utilized was comprised of these nanoparticles (Moulin and Roques, 2003). The information obtained from the scanning electron microscope (SEM) is useful for determining the architecture and dimensions of the nanoparticles that have been produced. The antioxidant, anti-inflammatory, and antibacterial activity of the $CaCO_3$NPs was examined during the process of assessing the effectiveness of the $CaCO_3$NPs. In light of the facts, it is possible to draw the conclusion that both the process and the results were substantially more effective than anyone had anticipated (Venkateswarulu, 2019).

UV-Visible spectroscopy analysis

Calcium carbonate ($CaCO_3$) nanoparticles synthesized from chicken eggshells were examined by UV-Vis analysis, and the results of that analysis are displayed in Figure 2. An absorbance peak was detected at 205 nm, which validated the presence of $CaCO_3$NPs. This peak indicated that the $CaCO_3$NPs were present in the aqueous solution in a sparse manner, with no signs of buildup (Haney *et al*., 2021).

Fourier Transform Infrared (FT-IR) Spectroscopic Analysis

The FTIR spectra of the synthesized Calcite ($CaCO_3$) nanoparticles was derived from chicken eggshells. Figure 3 illustrates the diverse absorption bands observed at multiple locations within the FTIR spectra, attributable to the distinct vibrational modes of various chemical groups, including C-O, C=O, C=C, and Ca-CO, present on the surface of the particles (Makuchowska-Fryc, 2019). The absorbance spectrum of calcite ($CaCO_3$) nanoparticles exhibited six distinct bands at different peaks.

The following absorption peaks were found in the FT-IR analysis: 3405cm^{-1}, 2926cm^{-1}, 2875cm^{-1}, 2514 cm^{-1}, 1798 cm^{-1}, 1417 cm^{-1}, 1083 cm^{-1}, 874cm^{-1}, 848cm^{-1}, and 708 cm^{-1}, characteristic of the calcite phase. Alcohol's O-H stretching is indicated by the peaks at 3405 cm^{-1}. The band located at 2926 cm^{-1} signifies the aldehyde's H-C=O stretching (Bonardo *et al*., 2021). The conjugated alkene's C=C stretching is represented by the peak at 2875 cm^{-1}, The presence of alkene C-H bending is shown by a

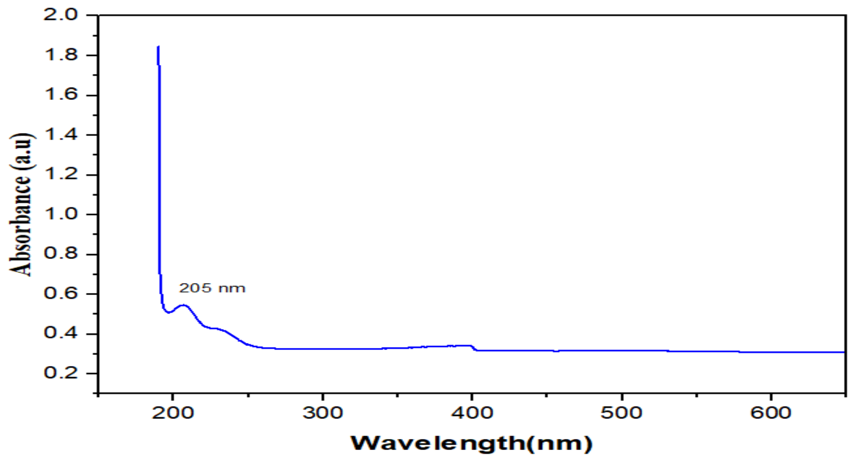


Fig. 2: UV-Vis spectrum $CaCO_3$NPs from chicken eggshells.

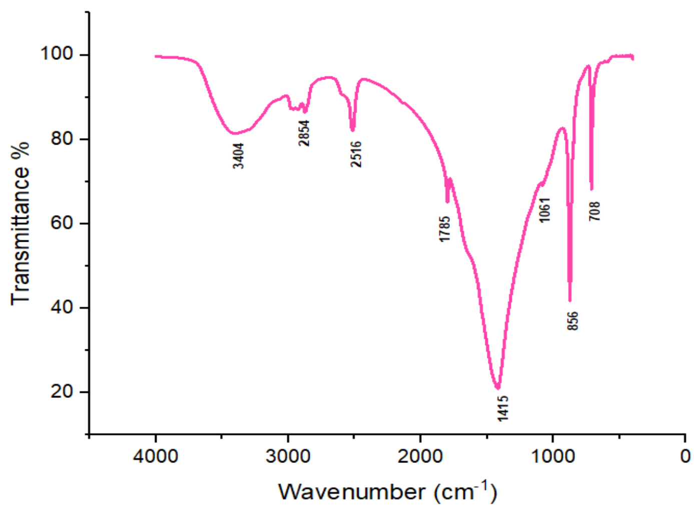


Fig. 3: FTIR spectrum of Calcite nanoparticles from TIFs.

peak at 2514 cm^{-1} and 1798 cm^{-1}. Additionally one more peaks obtained in the same bending at 1083 cm^{-1}. The existence of aromatic amine C-N stretching is indicated by the band seen at 1798 cm^{-1}. The C-O stretching of aliphatic ether is represented by the peak level of 874 cm^{-1}. Sharp bands were seen at 848, and 708 cm^{-1}; they were shown to be potent nitro compounds in the asymmetric stretch, which played a significant role in the synthesis of calcium carbonate nano-particles and presented in Table 1.

SEM Analysis

Figure 4 displays the SEM picture of Calcite $CaCO_3$ nanoparticles generated from chicken eggshells (Sronsri *et al.*, 2020). It identifies that $CaCO_3$NPs exhibit a rhombohedral cubic structure and that the particles aggregate into clusters. Particle diameters vary from 31 to 34 nanometres.

Antibacterial activity

Throughout the beginning of human civilization, people have employed plants as medicine. The use of nanoparticles from egg shells to treat diseases was inevitable, as is clear from the problems with

Table 1: FT-IR spectrum $CaCO_3$ NPs from chicken eggshells

S. No.	Abs cm^{-1}	Appearance	Group	Compound
1	3405.14	Strong, broad	O-H Stretching	Alcohol
2	2926.27	Medium	C-H Stretching	Alkane
3	2875.26	Strong, broad	N-H Stretching	Amine salt
4	2514.53	Strong, broad	O-H Stretching	Carboxylic Acids
5	1798.78	Strong	C-O Stretching	Conjugated Acid halide
6	1417.62	medium	O-H Bending	Carboxylic Acids
7	1083.34	medium	C-N Stretching	Amine
8	874.55	Strong	C-H Bending	1,2,4 trisubstituted
9	848.04	Strong	C-C1 Stretching	Halo compound
10	712.58	strong	C-C1 Stretching	Halo compound

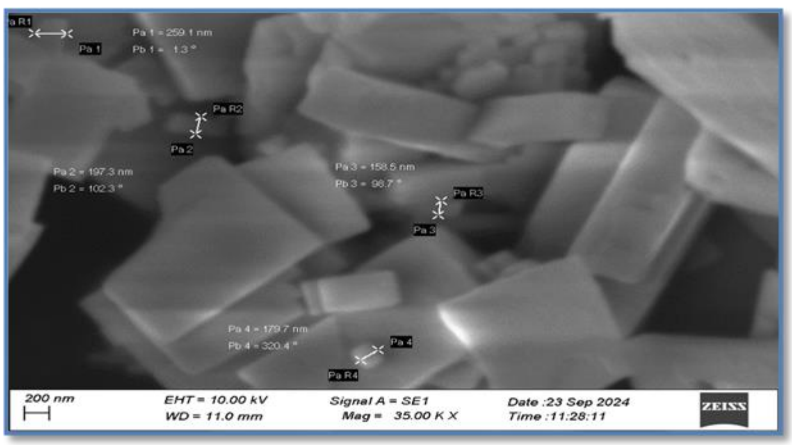


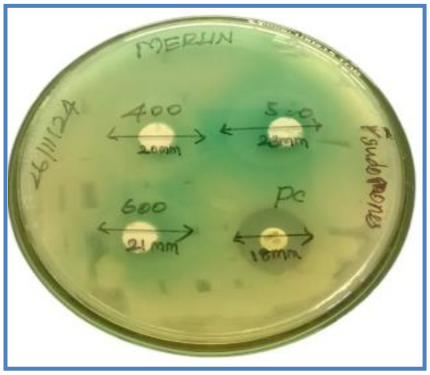
Fig. 4: SEM $CaCO_3$NPs from chicken eggshells.

synthetic antibiotics. The antibacterial activity of the calcium carbonate nanoparticles against the bacterial strains *Pseudomonas sp.*, *Escherichia coli*, and *Salmonella sp.* is illustrated in Table 2. The observed zones for Gram Negative bacteria *Escherichia coli* and Gram Positive bacteria *Pseudomonas sp.*, and *Salmonella sp.* at different concentrations of 400 µg/ml, 500 µg/ml, and 600 µg/ml. The comparison with Gentamicin highlights the test compound's potential as an alternative or supplementary antibacterial agent. While Gentamicin produced consistent inhibition zones across all strains, the test compound exhibited superior activity against *E. coli* and comparable activity against *Salmonella sp.* at higher concentrations. At 400 µg/ml, the zone of inhibition was 20 mm, which increased to 23 mm at 500 µg/ml (Zou and Zhu, 2018).

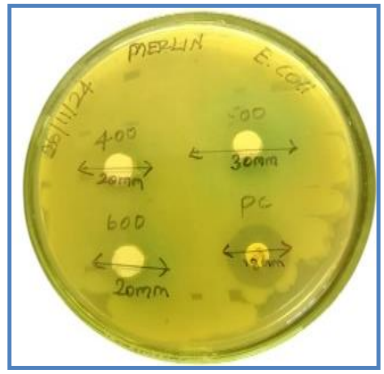
However, a slight reduction to 21 mm was observed at 600 µg/ml, suggesting a potential

Table 2: Antibacterial activity of $CaCO_3$NPs

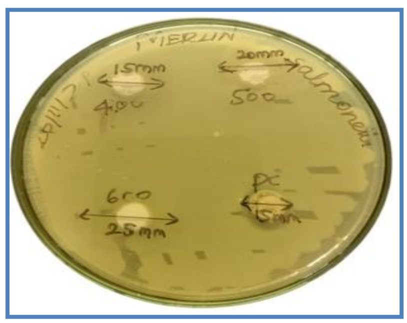
S. No.	Organisms	NC (DMSO)	PC (Gen)	Zone of Inhibition		
				400 µg/ml	500 µg/ml	600 µg/ml
1.	*Pseudomonas*	_	18 mm	20 mm	23 mm	21 mm
2.	*Escherichia coli*	_	18 mm	20 mm	30 mm	20 mm
3.	*Salmonella*	_	15 mm	20 mm	15 mm	25 mm



(a)



(b)



(c)

Fig. 5: Antibacterial activity of $CaCO_3$NPs (a) *Pseudomonas sp.*, (b) *Escherichia coli* and (c) *Salmonella sp.*

saturation or inhibitory effect at higher concentrations. The compound demonstrated strong antibacterial activity, especially at 500 µg/ml, where the zone of inhibition reached 30 mm, the highest among the tested concentrations (Tabish *et al.*, 2018). However, at 600 µg/ml, the zone decreased to 20 mm, indicating possible cytotoxic effects or reduced efficacy at higher concentrations. The activity was variable, with a moderate inhibition zone of 15 mm at 400 µg/ml and 20 mm at 500 µg/ml. Interestingly, the inhibition increased significantly to 25 mm at 600 µg/ml, suggesting a concentration-dependent effect (Fig. 5). The greatest inhibitory zone was against *Pseudomonas sp.*, *Escherichia coli*, and *Salmonella sp.* were 21 mm, 20 mm, and 25 mm, respectively when methanol extracts were compared to the well diffusion method (Sembiring *et al.*, 2021). Calcium carbonate nanoparticles from egg shells that had been dissolved in DMSO had its antibacterial efficacy against the pathogens tested (Fig. 6).

Antioxidant activity

Recently, it was found that produced nano-

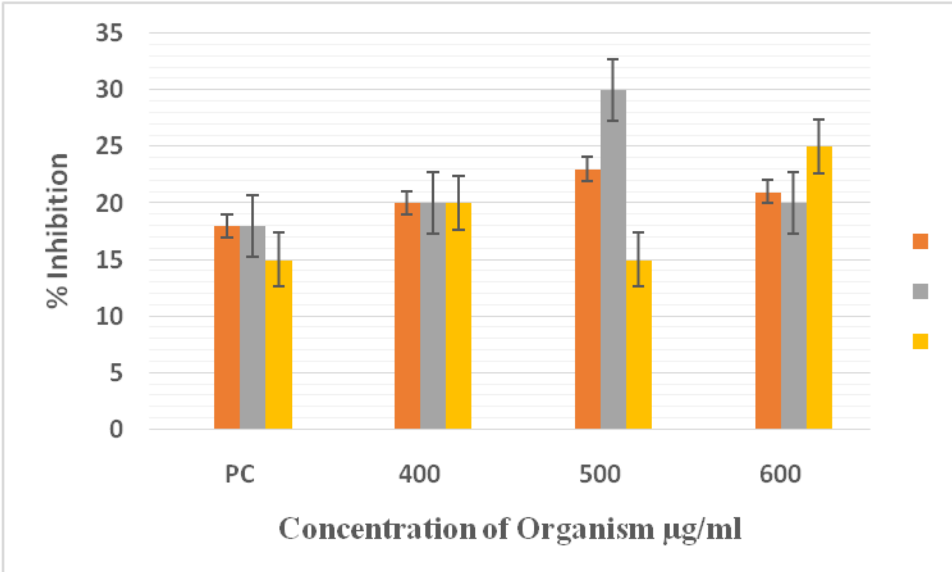


Fig. 6: Antibacterial activity of $CaCO_3$Nanoparticles.

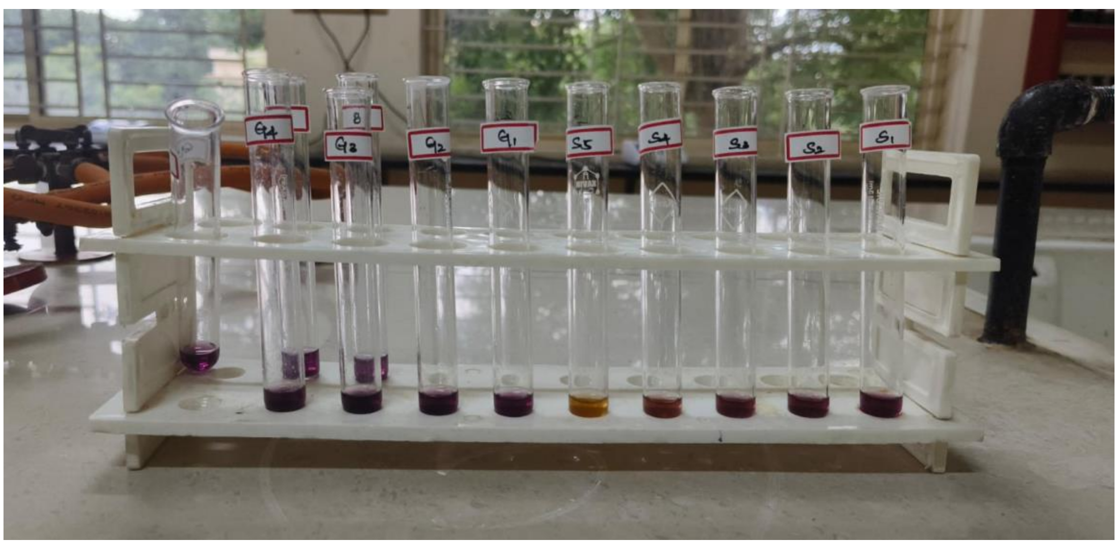


Fig. 7: Anti –oxidant activity of Calcite Nanoparticles.

particles had free radical scavenging activity, which provides defense. We successfully isolated and characterized calcium carbonate NPs that exhibited improved scavenging activity. The DPPH reaction is commonly used to evaluate scavenging activity due to its simplicity and clarity (Chen and Xiang, 2009). The DPPH radical absorption activity test seems to have shown that the nanoparticles derived from egg shells are more active than the positive control, ascorbic acid. Figure 7 shows that at varying doses, the antioxidant property of $CaCO_3$ NPs becomes increasingly noticeable, enabling them to remove DPPH free radicals (Wang *et al.*, 2006; Guo *et al.*, 2007; Islam *et al.*, 2011). The results also show that the combination of standard (ascorbic acid) and calcium carbonate

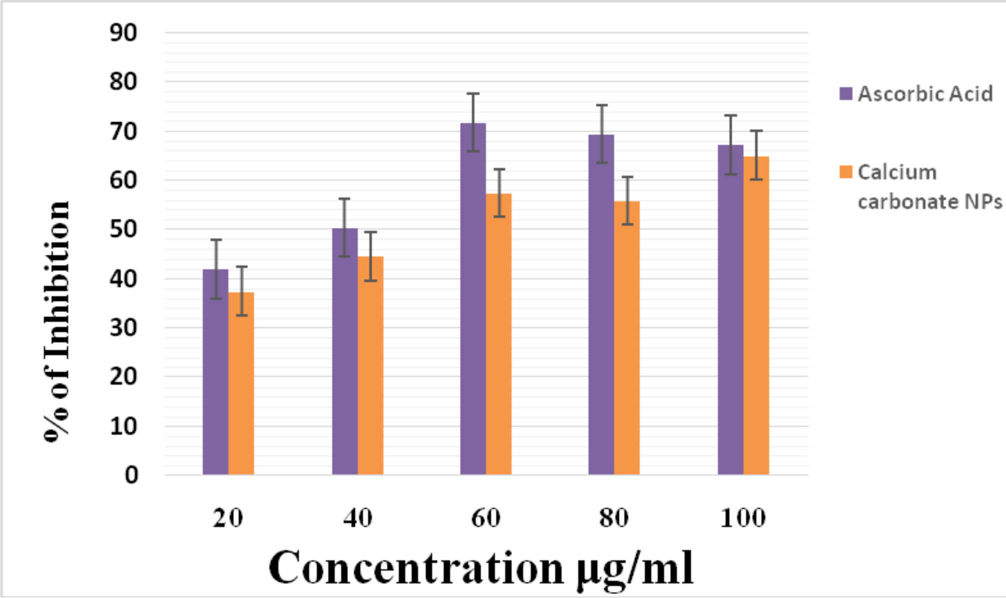


Fig. 8: Antioxidant activity of $CaCO_3$Nanoparticles.

NPs increases the effectiveness of the solution. Visual representation (Fig. 8) reveals a 9.8 percentage point ($p<0.05$) significant difference. The DPPH assay is systematic, well-planned, and regenerative since the coefficient of difference is smaller in all harvests (Xu *et al.*, 2006).

Conclusion

Synthetic calcite has recently been recognized as a major class of nanomaterials with numerous potential uses in medicine and industry. Effective, cost-effective, ecologically friendly, and agreeable to large-scale production, the extract of egg shell used for calcium carbonate synthesis is a great choice. Calcium carbonate nanoparticles show a clear absorption peak at 205 nm in the UV-Vis spectra. Absorption bands related to the main functional groups in the natural shells extract of nanoparticles were shown by the FTIR analysis. Crucial to the biosynthetic strategy is the significant improvement in reaction time compared to previous nanoparticle manufacturing methods, from the utilization of Calcium Carbonate Nanoparticles derived from egg shells. According to scanning electron microscopy, the generated nanoparticles have a spherical shape and a diameter of about 31-34 nm. Furthermore, it shows effectiveness against Gram-positive and Gram-negative bacteria that have developed resistance to many drugs in laboratory settings. It reduced the minimum inhibitory concentrations (MICs) of conventional antibiotics by acting synergistically with them. With IC_{50} values of 20 µg for ascorbic acid and 42.5 µg for the aqueous extract, respectively, The DPPH experiment proved that calcium carbonate nanoparticles could scavenge free radicals. Calcium Carbonate Nanoparticle derived from the shell of the chicken egg have recently been suggested as a possible environmental and medicinal agent.

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the Department of Biochemistry, Sacred Heart College, Tirupathur, Tamil Nadu, India.

Author Contributions

Merlinreshma M.: Investigation, Methodology, Writing-Original draft, Supervision, Acquisition; *Sivaranjani D.*: Conceptualization, Investigation; *Sheela K.*: Methodology, Writing review and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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