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Eco-friendly synthesis of calcium carbonate nanoparticles using *Azadirachta indica* extract and evaluation of their antibacterial and anti-inflammatory properties

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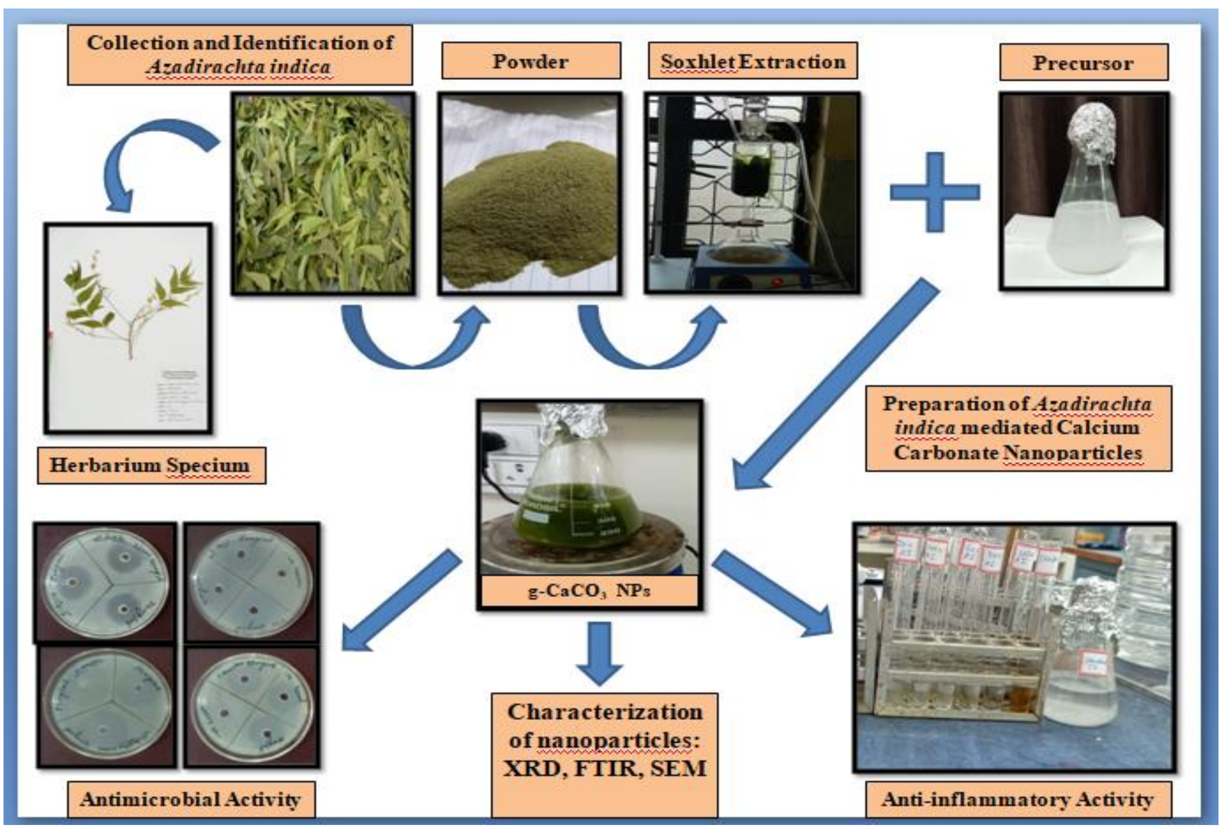
ABSTRACT

The present study aims to explore the green synthesis of calcium carbonate ($CaCO_3$) nanoparticles using *A. indica* leaf extract and their anti-inflammatory and antibacterial properties. Green nanotechnology, applying plant-based resources, is a sustainable and eco-friendly alternative for nanoparticle construction. Calcium carbonate, known for its biocompatibility and bioresorbable activities, is mainly useful in biomedical uses like as bone repair and treatments for dental care. In this research, ethyl acetate extracts of *A. indica* were used to create $CaCO_3$ NPs through a precipitation technique involving calcium chloride and sodium carbonate.



The production of the nanoparticles was identified using XRD, FE-SEM, and FTIR. The antibacterial efficiency of the nanoparticles was tested against *Staphylococcus aureus* and *Streptococcus mutans* using the well diffusion method and the minimum inhibitory concentration method, representing substantial zones of inhibition at different concentrations, closely comparable to standard antibiotics. The result demonstrates that the calcium carbonate inhibits the ZOI against both bacterial strains. The zone for *S. aureus* from 28.80-33.50 mm, whereas for *S. mutans*, it ranged from 30.83-35.53 mm. These results demonstrate that calcium carbonate nanoparticles showed good antimicrobial activity against both bacterial strains. Additionally, *in vitro* protein denaturation assays showed substantial anti-inflammatory properties, with increasing inhibition percentages detected at higher nanoparticle concentrations. The percentage inhibition of protein denaturation by nanoparticles across different concentrations ranging from 200 to 1000µg/ml. calcium carbonate nanoparticles show the percentage of inhibition from 20.84% to 92.34% over the same concentration range.

This study highlights the potential of *A. indica*-mediated $CaCO_3$ nanoparticles as promising agents for treating dental infections and inflammation-related conditions in a biocompatible and eco-friendly manner.

Keywords: *Azadirachta indica,* Antibacterial activity, Anti-inflammatory, Calcium carbonate nanoparticles, Dental bacteria.

INTRODUCTION

Green nanotechnology is a developing field that uses biological processes to produce nanoparticles. This area is crucial in different industries, such as pharmaceuticals, nuclear energy, electronics, fuel production, and biotechnology. Green synthesis methods are mainly advantageous for producing nanoparticles in the range of 1-100 nm. As they are safer as compared to other methods, this method is more eco-friendly, non-toxic, and cost-effective. Metal nanoparticles can be produced through both top-down and bottom-up methods, with techniques alike as chemical, physical, and biological methods. Characterizing these nanoparticles is important, and their properties are confirmed by numerous systematic methods, with UV–Vis spectrophotometry, FT-IR, SEM, and XRD [1].

Calcium derivatives are the key natural components of teeth and bones. The main tissue of bone, known as osseous tissue, is primarily composed of a composite material that includes inorganic minerals, particularly calcium-based compounds [2]. Tooth enamel is one of the four tissues that make up a tooth and contains the highest concentration of minerals, primarily calcium. As a result, various calcium derivatives have demonstrated significant potential for treating bone and teeth-related disorders, owing to their excellent biocompatibility with natural bone and teeth structures and their biodegradability [3]. Diverse types of calcium derivatives have shown promising applications in filling dental caries, treating initial dental caries lesions, and promoting new bone tissue [4]. Calcium carbonate ($CaCO_3$) nanoparticles offer unique assistance due to their excellent biocompatibility and potential as a drug delivery for numerous types of drugs. With advantages such as availability, low budget, protection, pH sensitivity, osteoconductivity, and sluggish biodegradability, $CaCO_3$ nanoparticles are well-suited for use as drug delivery carriers.

Staphylococcus aureus is a common microorganism which found in the normal oral and nasal flora, and is a gram-positive bacterium. It can invade endothelial, epithelial, and osteoblastic cells. *Streptococcus mutans,* a group of bacteria in the oral flora, is the main cause of dental decay. The bacteria produce acidic byproducts produced by erode the enamel surface, breaking down its components. Dental decay can then progress from the enamel to the dentin and eventually reach the pulp [3].

Hence, the present research purposes to estimate the antimicrobial and anti-inflammatory properties of *Azadirachta indica* mediated calcium carbonate nanoparticles for the treatment of dental infection-causing bacteria, i.e., *Staphylococcus aureus* MTCC 744 and *Streptococcus mutans* MTCC-497.

MATERIALS AND METHODS

Collection and identification

Azadirachta indica fresh leaves were obtained from the Gurukula Kangri University, Haridwar, Uttarakhand. The plant was identified from the BSI, Dehradun; the identification number is 1672.

Preparation of *Azadirachta indica* leaf extract in ethyl acetate

Leaves were thoroughly washed through tap water and distilled water, air-dried, and then crushed into a powder. 150 grams of this plant powder were loaded in a Soxhlet apparatus and extracted with 1500 mL of ethyl acetate. The solvents were heated, causing them to vaporize and then condense, which allowed the extract to be collected back into the flask.

Formation of $CaCO_3$ nanoparticles

Formation of nanoparticles was conducted by adding plant extract and a mixture of $CaCl_2$ (50 mM), and subsequently by the addition of Na_2CO_3 (50 mM). This process was carried out at a 1:4:5 ratio. The formation of the green synthesis of calcium carbonate nanoparticles was systematically performed. In 1ml of extract, 4ml of $CaCl_2$ solution and 5ml of Na_2CO_3 solution were added. The colour change of the solution indicates the formation of nanoparticles of calcium carbonate, and by centrifugation, the precipitates obtained were separated. The precipitates were rinsed twice with distilled water, dried, kept in the furnace at 600˚C for 4-5 hrs., and used for further characterization [5].

Characterization of calcium carbonate nanoparticles

X-ray diffraction

The samples form and size were identified by using XRD analysis to consider and examine the information about size and shape. It provides detailed data on the different properties of the compounds, such as their structure, preferred crystal phase, orientations, and several parameters, which include their average grain size, crystal defects, crystallinity, strain, etc. For the present study, the size, crystallite properties, and structure of the samples were recorded by "PAN Analytic diffractometer with Cu-Kα radiation (λ= 1.540498 Å) in the 2Θ range 20 ̊- 80̊."

Fourier transform infrared spectroscopy

The FT-IR offers evidence about the present functional groups in the compound by the measurement of infrared radiation absorption by the compound in a particular wavelength range. Data on the functional groups existing in the samples prepared in the present study were recorded by using JASCO FTIR, model name FT/IR-4X 1 type A.

Scanning electron microscopy

The FESEM micrographs of the calcium carbonate nanoparticle were taken using a FEI Nova NanoSEM450 FE-SEM. The beam energy of 10 kV is used for SEM.

Antibacterial activity of *Azadirachta indica* calcium carbonate nanoparticles

The *Staphylococcus aureus* MTCC-7443 and *Streptococcus mutans* MTCC-497 were tested for antibacterial activity by calcium carbonate nanoparticles mediated from *Azadirachta indica* ethyl acetate extract. The well diffusion method was used, with the cultures of the bacteria grown in nutrient broth and spread on Mueller–Hinton agar plates [6]. Wells of 6 mm were filled with 50 µl of nanoparticles at concentrations of 30 mg/ml, 40 mg/ml, and 50 mg/ml, and DMSO was used as negative control and Streptomycin were used as positive control. The plates of the samples were incubated at 37 ºC for 24 hours, and the zones of inhibition were measured in millimeters.

MIC of calcium carbonate nanoparticles

Minimum inhibitory concentration was determined by the microdilution assay. The nanoparticle concentrations were 25, 12.5, 6.35, 3.125, and 1.56 mg/ml were prepared in DMSO. The lowest concentration of nanoparticles that prevents the growth of the bacteria. For the confirmation of the percentage of inhibition of the bacteria, the inoculum was inoculated onto freshly ready agar plates [7].

***In-vitro* anti-inflammatory effects of nanoparticles**

The protein denaturation experiment was done by using the procedure defined by [8] with slight modifications. The reaction mixture (5ml) comprised 2ml of the sample, 2.80 ml of phosphate-buffered saline (PBS, pH 6.4), and 0.2 ml of 1% bovine albumin. After mixing the nanoparticles and desired chemicals, the solution was incubated for 15 min. at 37°C in a water bath before being heated for 5 minutes at 70°C. After cooling, a UV/VIS spectrometer (Shimadzu UV-1800) was used to measure the turbidity at 660 nm. The control was 2.80 ml of phosphate-buffered saline and 0.2 ml of 1% bovine albumin without nanoparticles, and the standard was Ibuprofen. the percentage inhibition of protein denaturation was determined by using the following formula:

$$\% \text{ inhibition of denaturation} = 100 \times (A1 - A2/A1)$$

Where A1 = absorption of the control sample, and A2 = absorption of the test sample.

RESULT

Characterization of calcium carbonate nanoparticles

X-ray diffraction

Figure 1 is showing XRD of $CaCO_3$ NPs by plant material extract exhibiting the following diffraction peaks (2θ) at 23.46, 29.88, 36.57, 39.85, 43.56, 47.93, and 48.92, which are interrelated to the (hkl) indices as (012), (104), (110), (113), (202), (018), and (116), respectively. The calcium carbonate's calcite phase was given to the diffraction peaks. The nanoparticle size obtained from XRD was 59.57nm. According to the [5] displays XRD formation of CaCO3 NPs by using peel extract of orange showing the following bending peaks (2θ) at 23.07, 29.15, 36.03, 39.41, 43.19, 47.13, and 48.38, which are associated to the (hkl) indices as (012), (1 0 4), (11 0), (113), (2 0 2), (0 18), and (116). The diffraction peaks were given to the calcite, a calcium carbonate phase [9]. The XRD examination of the crystal structure indicates that the nano material synthesized from cockle shells mainly consists of calcite, a $CaCO_3$. This demonstration is one of the three calcium carbonate crystalline forms, distinct from aragonite and vaterite. The position of 2θ ranges from 20 to 60 [11].

Fourier Transform Infrared Spectroscopy (FTIR)

Figure 2, showing the FTIR spectrum of *Azadirachta indica mediated* calcium carbonate nanoparticles, discloses main functional groups that confirm the successful synthesis and possible biofunctionalization of the nanoparticles. The diverse peak at 1383 cm^{-1} links to the symmetric widening vibration of the carbonate ion (CO_3^{2-}), whereas the peaks at 874 cm^{-1} and 706 cm^{-1} are official to the out-of-plane and in-plane bending vibrations of carbonate, respectively. These bands strongly demonstrate the existence of calcium carbonate ($CaCO_3$), possibly in the calcite or aragonite polymorphic forms. The peak at 2357 cm^{-1} is usually associated with atmospheric CO_2 adsorption, which is a frequent occurrence in FTIR spectra of carbonate-based materials. The FT-IR spectrum showed vibrational bands at 1456.391 cm^{-1}, 876.72 cm^{-1}, 712.7281 cm^{-1}, and 409.891 cm^{-1}, indicating plane bending. FT-IR analysis confirmed that the presence of CaCO3 nano powder derived from cockle shell had a distinguishing peak, which is a group of carbonates. A spectrum at 876.72 cm-1 confirmed that the CaCO3 nano powder was calcite, gained from the cockle shell. FT-IR analysis also confirmed that the powder contained $CaCO_3$ [10]. Additionally, the IR spectrum of $CaCO_3$ represents the particles peaks at 488 cm^{-1}, 814 cm^{-1}, and 1142 cm^{-1}, respectively [12]. The infrared spectra of the prepared nanoparticles revealed the absorption peaks ranging from 600 to 3000 cm^{-1}. It represents the characteristic carbonate peaks at 1480-1410 cm^{-1} and between 880-860 cm^{-1} [11].

Figure 1: Showing the XRD of calcium carbonate nanoparticles

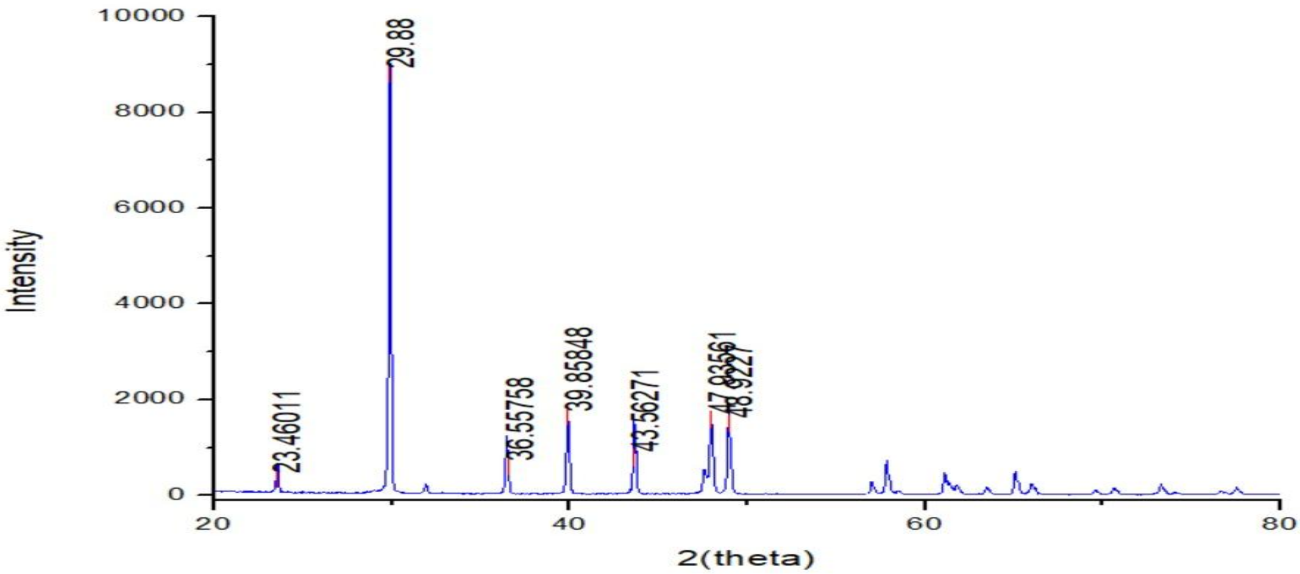


Figure 2: FTIR of calcium carbonate nanoparticles

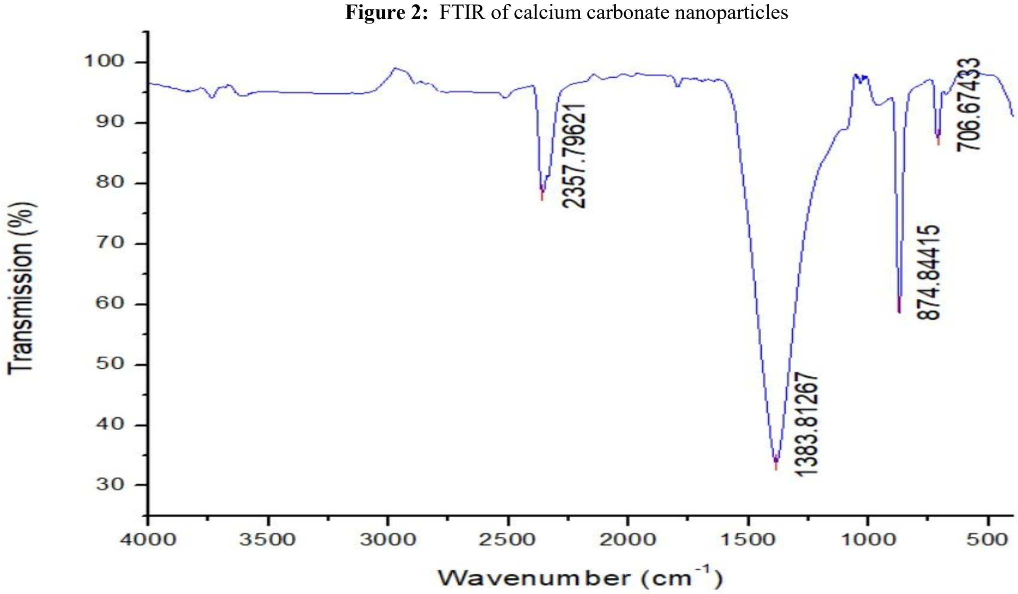


Figure 3: FESEM of calcium carbonate nanoparticles

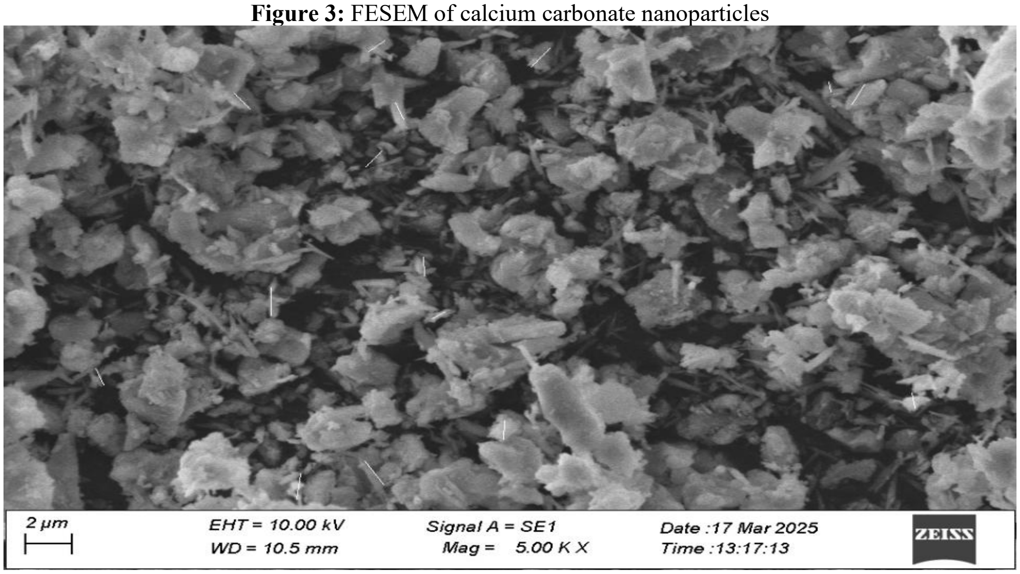


Figure 3 shows the size, shape, and morphological features at different magnifications of synthesized nanoparticles. It has been detected that spherical cluster nanostructures were formed, the size of NPs 62.747 nm. The micrographs also demonstrated the presence of larger particles due to agglomeration, with a more or less consistent size, shape, and morphology of NPs. It has been detected that spherical cluster nanostructures were formed, with a mean size of 62.747nm. The micrographs also verified the presence of larger particles due to agglomeration, with a more or less consistent shape, size, and morphology. The $CaCO_3$ nanoparticles found by orange peel extract showed rhombohedral morphological structure and a diameter in the range from 80–120 nm [5]. The shape and size of $CaCO_3$-NPs were examined using the FE-SEM. The NPs have a cubic shape, showing the size of the NPs was about 78nm [13]. The size dispersal was carried out by determining the size of 120 spherical calcium carbonate nanoparticles over five diverse SEM micrographs. The size dispersal revealed an average value of the particles of 155±50 nm [14].

Antibacterial activity of *Azadirachta indica* calcium carbonate nanoparticles

Table 1 presents the antibacterial properties of calcium carbonate nanoparticles synthesized using *Azadirachta indica* against *Staphylococcus aureus* and *Streptococcus mutans* at different concentrations (30, 40, and 50mg/ml), compared with the standard antibiotic Streptomycin. For *S. aureus*, the nanoparticles displayed zones of inhibition 28.80±0.60 to 33.50±0.43 mm at 30, 40, and 50 mg/ml, respectively, whereas Streptomycin showed zones of 30.66±0.55 to 36.53±0.41 mm at the same concentrations. Also, for *S. mutans*, the nanoparticles exhibit inhibition zones of 30.83±0.56 to 35.53±0.41 mm, while Streptomycin produced zones of 28.80±0.65 to 32.13±0.24 mm. Calcium carbonate nanoparticles are powerful opposed to Gram-negative and Gram-positive bacteria, including *Agrobacterium tumefaciens* and *Staphylococcus aureus* [15]. Calcite ($CaCO_3$) Nanoparticles of the Chicken Eggshell have effective antibacterial activity against different bacteria. The ZOI was 12 mm against *Acinetobacter baumannii*, Proteus mirabilis, and *Staphylococcus aureus*. The inhibition zone against *Escherichia coli* was 13mm, 11mm for *Klebsiella pneumoniae*, 14mm for *Burkholderia cepacia* and *Pseudomonas aeruginosa,* respectively [16].

Table 1: ZOI of antibacterial activity of calcium carbonate NPs

Microorganism	Conc. Mg/ml	Zone of inhibition (mm)	
		A. indica Nano	Streptomycin
S. aureus	50	33.50±0.43	36.53±0.41
	40	30.06±0.90	34.66±0.37
	30	28.80±0.60	30.66±0.55
S. mutans	50	35.53±0.41	32.13±0.24
	40	33.60±0.65	30.26±0.44
	30	30.83±0.56	28.80±0.65

Figure 4: Showing antimicrobial activity of calcium carbonate nanoparticles and positive control against A. *Staphylococcus aureus* and B. *Streptococcus mutans*

A. *Staphylococcus aureus* MTCC-7443

B. *Streptococcus mutans* MTCC-497

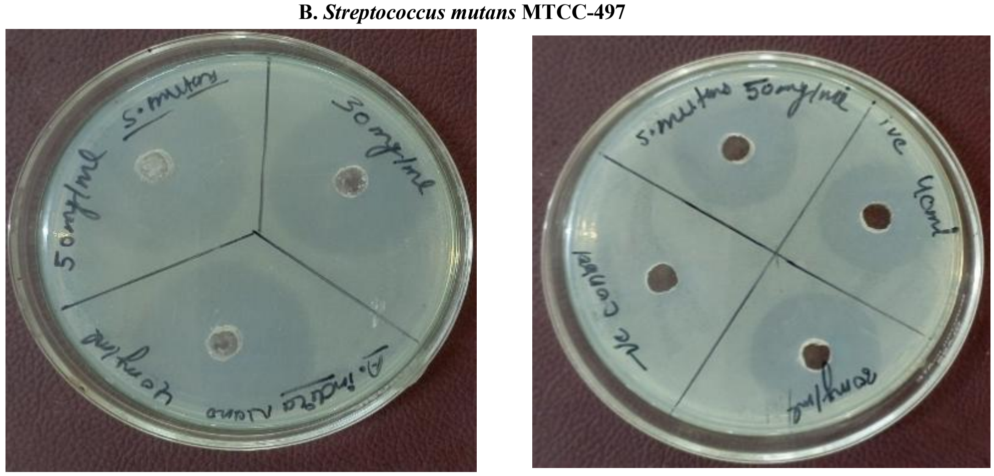


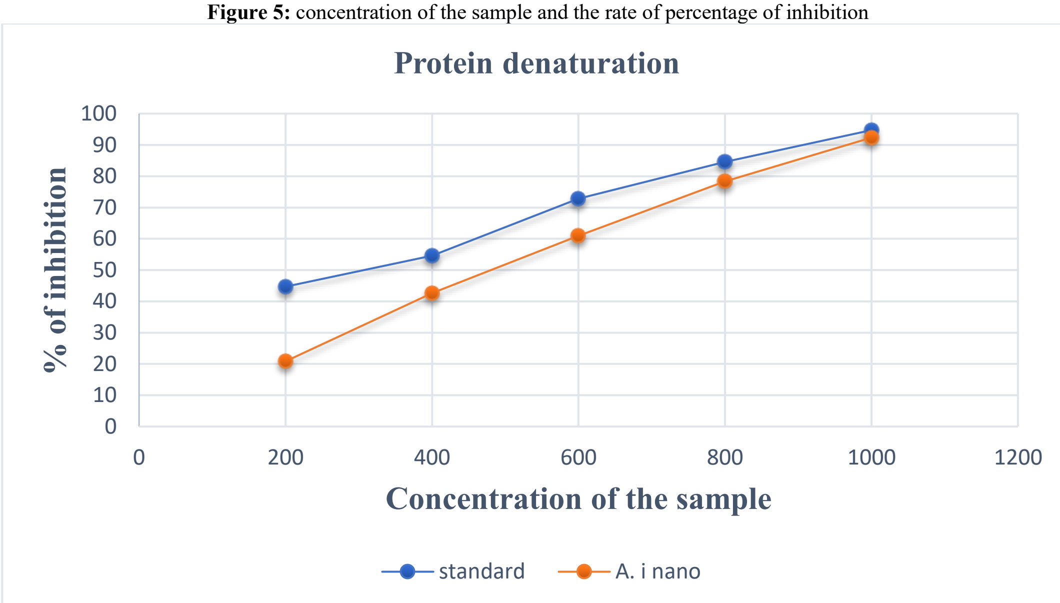
Table 2: showing the MIC of calcium carbonate nanoparticles

Microorganism	MIC (mg/ml)
Staphylococcus aureus	8.35
Streptococcus mutans	4.125

MIC of calcium carbonate nanoparticles

The calcium carbonate nanoparticles' minimum inhibitory concentration (MIC) was evaluated against two bacteria, *Staphylococcus aureus* and *Streptococcus mutans*. The MIC value for *S. aureus* was 8.35mg/ml, whereas for *S. mutans*, at 4.125mg/ml (Table 2).

Figure 5: concentration of the sample and the rate of percentage of inhibition



Protein denaturation

Table 3: Percentage of inhibition rate of protein denaturation

Concentration Conc.µg/ml	% of inhibition	
	Ibuprofen	**Nanoparticles**
200	44.70±0.14	20.84±0.54
400	54.66±0.30	42.63±0.52
600	72.83±0.27	61.00±0.00
800	84.53±0.42	78.32±0.56
1000	94.73±0.35	92.34±0.43
IC_{50} µg/ml	287.52	499.22

Table 3 shows the inhibition percentage of protein denaturation via Ibuprofen and *nanoparticles* across different concentrations from 200 to 1000 µg/ml. Ibuprofen demonstrates the inhibition percentage from 44.70% at 200µg/ml to 94.73% at 1000 µg/ml. Similarly, calcium carbonate nanoparticles show the inhibition percentage from 20.84% to 92.34% over the same concentration range. The IC_{50} value, which indicates the concentration needed to inhibit 50% of protein denaturation, for Ibuprofen was 287.52µg/ml and for nanoparticles was 499.22 µg/ml.

CONCLUSION

The present research effectively synthesized calcium carbonate nanoparticles by using *Azadirachta indica* (neem) plant extract, highlighting their structural, morphological, and functional appearances done by XRD, FTIR, and FESEM analyses. XRD analysis established the crystalline nature of the nanoparticles and their phase of calcite, with a regular crystallite size of 59.57nm. FTIR spectra confirmed carbonate functional groups, approving the formation of $CaCO_3$ and suggesting probable biofunctionalization from plant-derived biomolecules. FTIR spectra confirmed carbonate functional groups, approving the formation of $CaCO_3$ and suggesting probable biofunctionalization from plant-derived biomolecules. FESEM analysis showed spherical clustered nanoparticles with the particle size of 62.75 nm and some agglomeration. The calcium carbonate nanoparticles showed good antibacterial motion against *Staphylococcus aureus* and *Streptococcus mutans*, with ZOI equivalent to the standard antibiotic Streptomycin. Particularly, the MIC values showed a stronger antibacterial effect against *S. mutans* than *S. aureus*. Moreover, nanoparticles of calcium carbonate revealed promising anti-inflammatory potential, Overall, the results suggest that calcium carbonate nanoparticles created by using *Azadirachta indica* extract are effective bioactive materials with substantial antibacterial and anti-inflammatory properties, supporting their potential application in pharmaceutical and biomedical fields.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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