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Original Article

SYNTHESIS, CHARACTERIZATION AND *IN VITRO* EVALUATION OF VATERITE MICROPARTICLES

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ABSTRACT

Objective: The aim of this research is to synthesize submicron-sized calcium carbonate vaterite particles of elliptical shape with different reaction durations. We also aim to assess their antioxidant and anti-inflammatory properties, which may be beneficial for treating diseases such as asthma and rheumatoid arthritis.

Methods: Calcium carbonate vaterite particles were prepared using the co-precipitation method with varying reaction times, characterized by Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM), Fourier Transformation Infrared Spectroscopy (FTIR), and Poly-Dispersity Index, with antioxidant activity assessed by the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) method and anti-inflammatory activity by the protein denaturation method.

Results: SEM and TEM analysis revealed that the synthesized vaterite particles had an elliptical shape with nano-crystalline particles of around 50 nm size. FTIR verified the production of vaterite particles. Research on antioxidants and anti-inflammatory agents revealed that the crystalline particles exhibited DPPH scavenging action, with an IC_{50} of 12.6 µg/ml, and a noteworthy reduction in protein denaturation in the albumin protein denaturation test, with an IC_{50} of 222.49 µg/ml, in comparison to the reference value.

Conclusion: The results highlight the potential of elliptical vaterite submicron micro-particles as versatile platforms with anti-inflammatory and antioxidant properties, paving the way for future advancements in drug delivery systems, food additives, and natural supplements by efficiently encapsulating drugs and proteins.

Keywords: Calcium carbonate, Vaterite particles, Anti-oxidant activity, Anti-inflammatory activity

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INTRODUCTION

Calcium carbonate particles exhibit extensive applicability in industries like pharmaceuticals, textiles, and beyond, serving as a versatile component in various manufacturing processes. In medicine and healthcare, calcium carbonate serves as a phosphate binder to manage hyperphosphatemia and as an inexpensive source of dietary calcium in renal failure patients while also serving as an inert filler in pharmaceutical preparations [1]. Calcium carbonate in its polycrystalline vaterite phase remains relatively understudied, despite its considerable therapeutic potential. For instance, while Calcium carbonate maintains structural integrity at neutral physiological pH, it rapidly dissolves and releases drugs in the acidic tumor microenvironment [2, 3]. Furthermore, the size, shape, content, and structure of Calcium carbonate can be easily tailored to optimize the efficiency of drug or protein delivery [4]. Three anhydrous polymorphic forms that are known to exist, which includes calcite, aragonite, and Vaterite forms. Multiple spherical nano-crystallines combine to form a highly developed mesoporous structure with an average pore size of 10 to 60 nm, which makes up the structural components of Vaterite crystals. The size of this hole is perfect for trapping big macromolecules of comparable diameters from nm to mm. The particles' shapes can also be adjusted, whereas organic solvents and polymer additives are frequently utilized in the manufacturing of Calcium carbonate, and a variety of stimuli may be employed to alter its pore size [5]. According to recent findings, aqueous solutions with a pH of neutral and an ionic strength close to physiological values may be able to produce vaterite Calcium carbonate crystals. Furthermore, Calcium carbonate crystals are fully hydrolyzable at a slightly acidic pH and in the presence of Ca^{2+}-binding agents. Moreover, they might recrystallize into more stable calcite polymorphs, which would liberate the medication from its capsule and transform the porous structure into a non-porous one [6, 7].

However, most of the Calcium carbonate studies used particles that were only a few micrometers in size as creating Nano containers proved to be somewhat challenging. The most promising applications, such as drug delivery and active coating, require sub-micrometer-sized containers. This is because more uniform and efficient dispersion and access to micrometer-scale structures like cells or tissue are made possible by smaller sizes [8, 9]. The physical characteristics are related to its cellular absorption efficiency. According to a research study, human intestinal epithelial cellular absorption behavior of Calcium carbonate microparticles differed markedly from that of bulk Calcium carbonate. Calcium (Ca^{2+}) ions showed that the Calcium carbonate nanoparticles had effective cellular internalization and energy-dependent endocytic pathways through the migration of microfold cells [10].

The physiological process of free radicals or Reactive Oxygen Species (ROS) formed during cellular metabolism is referred to as oxidative stress, where an imbalance between oxidants and antioxidants induces a variety of diseases, including cancer, diabetes, and inflammatory disorders. ROS attacks cell membranes and proteins, resulting in impairment of cellular functions. The antioxidant activity can suppress the morbidity and mortality associated with the onset of diseases. Human inflammatory reactions triggered by invading pathogens or chemicals by inflammatory cells such as monocytes, macrophages (antigen-presenting cells), or polymorph nuclear leucocytes (white blood cells) result in the production of mediators such as Nitric Oxide (NO), prostaglandins, and other inflammatory factors. NO content increases during the progression of inflammation and mediates lethal physiological cytotoxicity Referred to as enzymes belonging to the anti-inflammatory group of Lipoxygenases (LOXs), these signaling molecules regulate inflammatory responses such as pro-inflammatory leukotrienes and anti-inflammatory lipoxins, modulating rheumatoid arthritis and asthma [11].

In this work, we describe the synthesis of elliptical porous submicron vaterite containers and provide a detailed characterization and evaluation of their antioxidant and anti-inflammatory capabilities. While calcium carbonate and its various

forms have been extensively studied, there is a notable gap in research regarding the antioxidant and anti-inflammatory evaluation of elliptical submicron vaterite containers. Prior studies have indicated that cancer cells exhibit a greater tendency to internalize ellipsoidal particles [4]. Consequently, our investigation centers on submicron vaterite particles and their potential to mitigate diseases like Asthma and Arthritis.

MATERIALS AND METHODS

All chemicals used in this experiment were of analytical grade and included calcium carbonate and sodium carbonate (Sigma-Aldrich), 2,2-diphenyl-1-picrylhydrazyl DPPH and Bovine serum albumin. Solvents include ethanol and distilled water.

Synthesis of calcium carbonate particles

Calcium carbonate vaterite particles were created utilizing the technique outlined in previous publications [12]. In summary, ethylene glycol (1:5, v/v) was combined with 0.33 M solutions of $CaCl_2{\cdot}2H_2O$ and Na_2CO_3, and the mixture was magnetically stirred for 30 min. Using ethanol, the resultant nanoparticles were centrifuged for five minutes at 6,000 rpm to remove any leftover co-solvent molecules and unreacted ions. After that, they were dried at 60 °C for an hour. Another synthesis was carried out in reaction time 60 and 120 min also.

Characterization procedure

FEI Tecnai TF30 HR-TEM was used for Transmission Electron Microscopic analysis. Two microliters of the sample were placed on an Electrotech K100X (400-mesh carbon-coated copper grid; Ted Pella, Inc.) that had been negatively glowing and discharged before usage to prepare the samples. The sample was allowed to adsorb on the grid for two minutes before any excess material was whipped off with some filter paper. The grid was then allowed to air dry. The FEI Nova Nano SEM 450 (FEG type) was used for FE-SEM. To prepare the sample for SEM analysis, two microliters of the sample to be analyzed were drop-cast onto a newly split mica surface.

Using a Malvern Zetasizer Nano ZS (655 nm laser), the polydispersity index was calculated. The compositional properties of the prepared samples were assessed using an FTIR spectrometer from Frontier. FTIR investigations were conducted in the 4000–400 cm^{-1} range using a Perkin Elmer FTIR, with the powders mixed with KBr dried at room temperature.

Anti-oxidant activity by the DPPH method

Antioxidant studies were conducted using the following method reported in previous research [12, 33]. A stock solution containing varying concentrations of the material (12.5µg/ml to 200µg/ml) was created, with a final volume of 20µl. DPPH (0.1 mmol) solution was then added in 1.48 ml. A control was made using the same volume of distilled water but without the test component. The reaction mixture was well mixed and then incubated for 20 min at room temperature in the dark. The primary cause of the observed purple-to-yellow color shift in DPPH was the antioxidant agent's absorption of hydrogen. After 20 min, the mixture's absorbance was measured at 517 nm. The control included three milliliters of DPPH. To function, antioxidants convert DPPH to DPPH-H, which lowers absorption. The extent of the discoloration reveals how effectively the antioxidant chemicals or extracts can donate energy to scavenge hydrogen. Radical scavenging activity or percent inhibition is calculated as follows:

$$\%\ \text{Inhibition} = \frac{\text{Control absorbance} - \text{Sample absorption}}{\text{Control absorbance}} \times 100$$

Anti-inflammatory activity by protein denaturation method

The protein denaturation analysis to examine the anti-inflammatory efficacy was performed with few modifications from the literature [22]. 10 mg/ml was used as a stock solution, which was diluted to provide a range of sample values from 62.5µg/ml to 500µg/ml. Bovine serum albumin was mixed with 0.05 ml of distilled water to create the test control. Two milliliters of bovine serum albumin and different amounts of samples made up the test mixture. Various sample concentrations were combined with 0.45 ml of distilled water to create the product control sample. The reference solution was diclofenac sodium. Each solution's pH was raised to 6.3 using 1N hydrochloric acid. After incubating at 37 °C for 20 min, the samples were heated to 57 °C for three minutes. After chilling, 2.5 ml of phosphate buffer was added to each solution. To sum up, an Ultraviolet-Visible Spectrophotometer (UV – 1900i, SHIMADZU) was utilized to measure absorbance at 416 nm. The percentage inhibition can be calculated is calculated as follows.

$$\text{Percentage inhibition} = \frac{\text{Control absorbance} - \text{Sample absorption}}{\text{Control absorbance}} \times 100$$

RESULTS AND DISCUSSION

Several researches on the vaterite form of Calcium carbonate have examined the impact of additive-directed crystallization on crystal development. The final Calcium carbonate particles' size and polymorph form are greatly impacted by several other factors, including pH, temperature, reaction time, mixing speed, and concentrations of Ca^{2+} and CO_3 [13]. Among these variables, reaction time, defined as the duration for which the reactants are allowed to interact under specific conditions, has been recognized as a key determinant in shaping the characteristics of vaterite particles. They are produced by precipitating concentrated $CaCl_2$ and Na_2CO_3 solutions, which crystallize into spherical, polycrystalline vaterite particles [14, 15]. The nucleation and development rate of the vaterite spheres is determined by the super-saturation level of the dissolved amorphous Calcium carbonate [16, 17]. Factors like reagent concentration, salt solubility, reaction time, and mixing rotation all have an impact on the final size of the vaterite particles. Studies show that increasing the salt concentration to 1 m, the rotation speed to 1500 rpm, and the reaction duration to 2 min can reduce the vaterite particle size to 3 mm [18]. Hence, the duration of the reaction profoundly impacts crystal size, morphology, phase stability, surface properties, and the overall suitability of vaterite for biological applications. Here we have followed the reaction conditions mentioned in previous literature and the reaction was carried out in 30, 60, 120, and 180 min to optimize the reaction conditions. In this study, we followed the methodology outlined by Parakhonskiy *et al.*, investigating how the average size of vaterite particles changes over time under varying concentrations of Ethylene Glycol (EG), in which they noticed that the smallest vaterite particle size emerged at an 83% EG concentration after 2 h of mixing, leading to the formation of stable vaterite spheres with a specific average diameter.

The observations through TEM revealed that the $CaCO_3$ spheres were made up of tiny building blocks consisting of Calcium carbonate nanocrystals. These nanocrystals then came together to create the intricate pore structures seen in fig. 1. The lighter areas between the different $CaCO_3$ building blocks indicate the possibility of pores inside the $CaCO_3$ particles. The Nano/microspheres have a three-dimensional network and the aggregation of these nanoparticles into a porous structure. The material's hierarchical porosity enhances its specific surface area and facilitates mass transfer between its interior and outside. The results confirm the development of calcium carbonate nanocrystals into microparticles. The SEM image (fig. 2) magnified to a high magnification clearly illustrates the $CaCO_3$ particles' porous nature, which is similar to the unique pores seen in coral. The porous nature of CaCO3 particle are evident in fig. 4c, where the black nanoparticles and lighter edges contrast sharply [19].

As seen in fig. 1 and 2 of the SEM and TEM images, the $CaCO_3$ particles are composed of equally sized primary particles with an average size of roughly 50 nm. The average size of the vaterite particles was approximately 1 µm. As already reported in previous studies [20], The TEM and SEM photos clearly show the existence of Nano-crystalline submicron particles, which combine to form extremely mesoporous structures.

Polydispersity index analysis (PDI) was conducted to examine the particle size distributions and intensity of the calcium carbonate particles synthesized at various reaction durations of 30, 60, and 120 min. The results indicate a noteworthy variation in particle size

among the samples prepared at different reaction times. Specifically, the calcium carbonate particles synthesized at 30 min exhibited a size of approximately 100 nm, whereas those prepared at 60 and 120 min showed sizes of around 500 nm and 1000 nm, respectively.

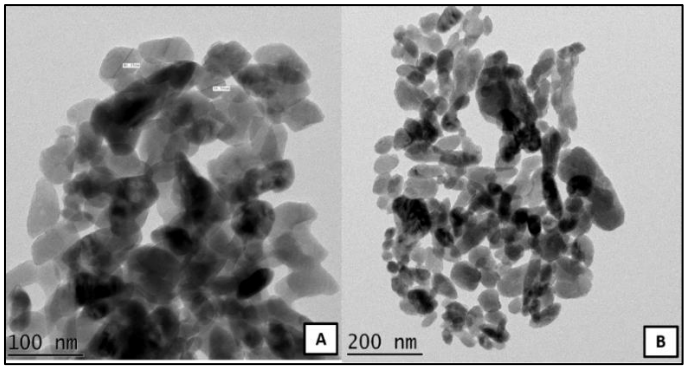

Fig. 1: Transmission electron microscopy images of calcium carbonate particles at 100 nm scale bar (A) and 200 nm scale bar (B)

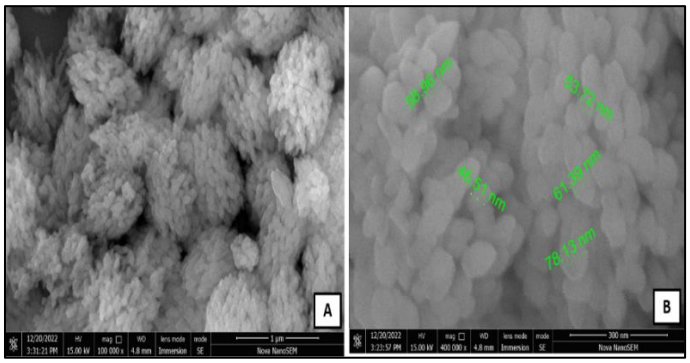

Fig. 2: Scanning electron microscopy images of vaterite particles (A), Nano-crystalline particles (B) at 1µm scale bar (magnified at 1, 00000x) and 300 nm scale bar (magnified at 4, 00000x), respectively

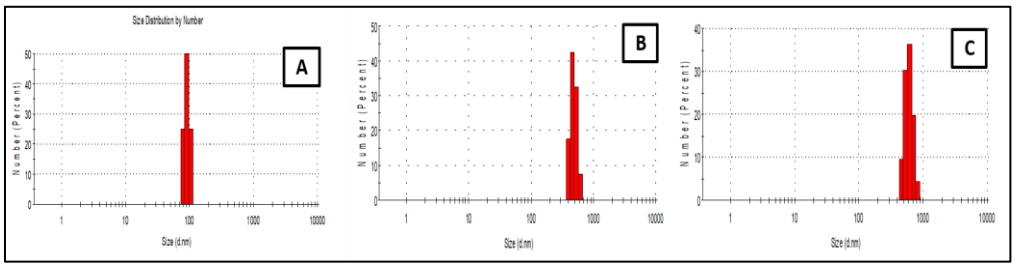

Fig. 3: Polydispersity index (PDI) analysis using dynamic light scattering by size of the vaterite particles at varying reaction times. (A) 30 min, (B) 60 min (C) 120 min

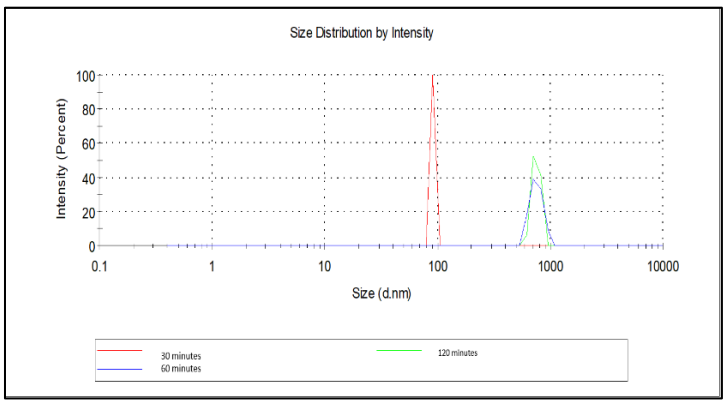

Fig. 4: Particle size distribution by intensity of the vaterite particles at different reaction times (30 min, 60 min, 120 min)

The Poly-Dispersity Index and particle size analysis of the sample were performed (fig. 3 and 4), and the findings showed that the DLS of the synthesized submicron particles differed from the observed size as determined by SEM/TEM. According to results similar to those of other researchers for drug-loaded nanoparticles, it might have happened as a result of a mistake in the Stokes-Einstein equation for viscosity magnitudes in the DLS approach [21-24]. While particles prepared on 30 min reaction time showed lesser diameter than 60 and 120 min.

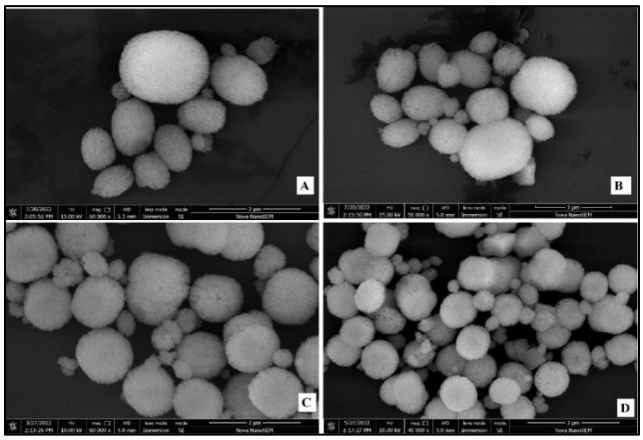

Fig. 5: Scanning electron microscopy images of vaterite particles at varying reaction times of 30 min (A), 60 min (B), 120 min (C), at 2µm scale bar and 180 min (D) at 3µm scale bar respectively. Images were magnified at 40,000x (D), 50,000x (B) and 60,000x (A, C) respectively.

Since the duration of reactions significantly influences the shape and size of calcium carbonate polymorphs, we experimented with varying reaction durations of 30, 50, and 120 min. Analysis of SEM images (fig. 5) revealed a slight decrease in particle size at 30 min, with sizes below one micron. However, at 60, 120, and 180 min, a slower but noticeable increase in size to around one micron or larger was observed with increasing reaction time. Another method for determining the polymorph makeup of Calcium carbonate particles is FTIR. Pure vaterite often exhibits vibrational bands at 1480, 1070, 1087, 877, 848, and 745 cm^{-1} [25]. The formation of vaterite calcium carbonate particles is confirmed by these characteristic Fourier transformation infrared spectroscopy peaks (fig. 6).

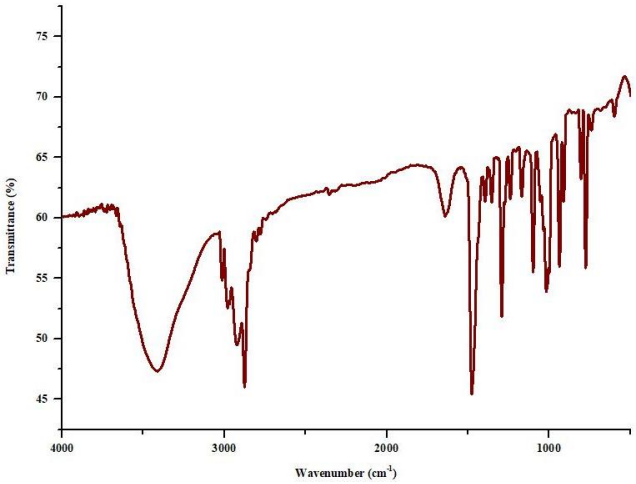

Fig. 6: Fourier transformation infrared spectroscopy of vaterite particles

Inflammation, a complex biological process, involves orchestrated interactions among various immune cells and a wide array of signaling molecules within cells. It happens as a result of chemical, physical, or biological stimuli that damage healthy tissues. Because protein denaturation causes inflammation, it is known to be a hallmark of inflammatory diseases. Pro-inflammatory cytokines like TNF-α, IL-1β, and mitogen-activated protein kinase (MAPK) are released from stimulated and activated immune cells such as neutrophils and macrophages. TNF-α is necessary to initiate several inflammatory processes. Interleukin 1β can promote signal transmission through nuclear factor κB (NF-κB) and MAPK activation. Certain cytokines, such as TNF-α and IL-1β, are created when NF-κB is activated, which triggers an inflammatory response These factors play a major role in inflammation [26, 31]. When proteins undergo denaturation, they lose their complex structures, including quaternary, tertiary, and secondary formations, which affects their ability to function properly. External factors like heat or chemical exposure, such as organic solvents, can cause this alteration. Therefore, to explore the anti-inflammatory properties, we assessed how well protein extract and peptide hydrolysates prevented heat-induced albumin denaturation. Several studies have reported based on anti-inflammatory activity of these particles [27, 28, 32]. The results of this investigation show that calcium carbonate particles significantly inhibit the denaturation of proteins, especially albumin. The analysis shown in fig. 8 shows that the inhibition rate reaches 79% at 500µg/ml, while the known anti-inflammatory drug diclofenac sodium shows 89% inhibition (fig. 7). The standard Diclofenac sodium and bio-synthesized calcium carbonate particles was determined to have IC_{50} values of 100.76µg/ml and 222.49µg/ml, respectively. These findings highlight that the calcium carbonate submicron particles possess strong anti-inflammatory properties.

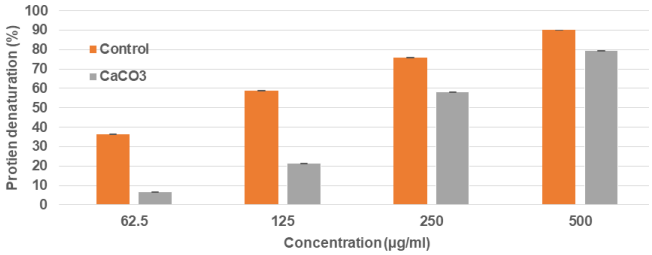

Fig. 7: *In vitro* anti-inflammatory activity of calcium carbonate particles, data shown above are measured in mean±SD, (n = 3)

Antioxidants combat oxidative bursts using multiple methods, including the deactivation of peroxynitrite and lipoxygenases, the creation of chelate complexes with pro-oxidant metals, the quenching of singlet oxygen, the scavenging of free radicals, and the activity of photosensitizers. Since oxidative damage is known to be caused by reactive oxygen species (ROS), these substances are crucial in preventing it.

Calcium carbonate submicron particles were tested for antioxidant capacity using the DPPH test. The IC_{50} value was determined to gauge the extent of antioxidant activity, indicating the concentration of antioxidants required to reduce free radicals by 50%. Lower IC_{50} values correspond to higher scavenging activity of antioxidants as reported in various studies [27, 29]. The antioxidant potential of different concentrations of Calcium carbonate (12.5–200µg/ml) is depicted in fig. 9. As a standard reference, ascorbic acid exhibited 94% antioxidant activity, whereas $CaCO_3$ demonstrated 91% DPPH radical scavenging ability at its highest concentration of 200µg/ml (fig. 8).

The DPPH scavenging potential of both standard ascorbic acid and bio-synthesized calcium carbonate particles was determined to have IC_{50} values of 19.9µg/ml and 12.6µg/ml, respectively. Prior research indicates the remarkable antioxidant properties of $CaCO_3$ [28, 29]. Hence, it has been proposed that Calcium carbonate scavenges free radicals by transferring hydrogen atoms or electrons to DPPH radicals. This action enables it to intercept the oxidation process, thereby safeguarding lipids, proteins, nucleic acids, and carbohydrates from oxidative harm [28]. Along with its potential antioxidant activity, Calcium carbonate holds promise as a potential anticancer agent for addressing multiple diseases.

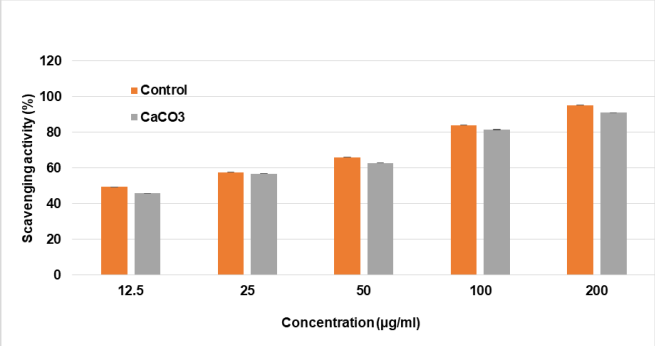

Fig. 8: *In vitro* antioxidant activity of calcium carbonate particles, all values are expressed as mean±SD (n=3)

CONCLUSION

Calcium carbonate has emerged as a compelling inorganic biomaterial, notable for its remarkable chemical stability, bioactivity, and biocompatibility. The elliptical vaterite polymorph stands out due to its distinctive physical and chemical properties, biodegradability, and enhanced cell engulfment. In this study, we synthesized vaterite particles of calcium carbonate, an area under active investigation for its diverse potential applications. Our findings underscore the significant impact of reaction time on submicron particle production, with optimal results observed at thirty minutes, corroborated through poly-dispersity index, fourier transformation infrared spectroscopy, scanning electron microscopy, and transmission electron microscopic analyses, confirming particle size and morphology. TEM and SEM results further elucidated the formation of nano-crystalline particles and a mesoporous structure. Biological assessment via DPPH assay and protein denaturation assay revealed outstanding antioxidant and anti-inflammatory activity of the vaterite particles, demonstrated by comparing IC_{50} values. These findings suggest a potential synergy between antioxidants and anti-inflammatory agents in modulating immune responses in inflammatory diseases like arthritis.

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ABBREVIATION

NO: Nitric Oxide, ROS: Reactive Oxygen Species, DPPH: 2,2-Diphenyl-1-Picryl Hydrazyl, PDI: Poly Dispersity Index, $CaCO_3$: Calcium Carbonate, SD: Standard Deviation, SEM: Scanning Electron Microscopy, TEM: Transmission Electron Microscopy, FTIR: Fourier Transformation Infrared Spectroscopy, IC_{50}: Half Maximal Inhibitory Concentration.

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Nil

AUTHORS CONTRIBUTIONS

The contributions of authors in the preparation of manuscript are as follows: Deena Jose did the literature review, method selection, and writing of original draft; D. Kumudha did data compilation, editing, and supervision. All authors have reviewed the results and approved the final version.

CONFLICTS OF INTERESTS

There are no conflicts of interest

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