

ANTI-INFLAMMATORY POTENTIAL EVALUATION AND MORPHOLOGICAL CHARACTERISATION OF NANOHYDROXYAPATITE INCORPORATED GREEN SYNTHESIZED NANOCOMPOSITE- AN *IN-VITRO* STUDY

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ABSTRACT

Objective: This research intended to investigate the anti-inflammatory capabilities of nano-hydroxyapatite (nHAP) based nanocomposites developed through green synthesis from citrus fruit peels.

Methods: The experimental design involved the green synthesis of nHAP-mediated chitosan-based nanocomposites from *Citrus reticulata* and *Citrus limonum* peel extract. The anti-inflammatory potential was assessed using the Bovine serum albumin (BSA) denaturation assay, membrane stabilization assay and Egg albumin denaturation assays. The developed nanocomposite was also subjected to characterization using X-ray diffraction (XRD) and Fourier transform infrared (FTIR) spectroscopy analysis.

Results: The successful generation of the nanocomposite based on nHAP was carried out. The generated nanocomposite exhibited anti-inflammatory behavior. The characterization using XRD and FTIR analysis also confirmed the presence of nHAP particles from the green precursors.

Conclusion: The *C. reticulata* and *C. limonum* peel extract mediated nHAP-based nanocomposite exhibited potential anti-inflammatory effects, its suggested use in biomedical applications

Keywords: Anti-inflammatory, Hydroxyapatite, Green chemistry, Nanocomposite, Nanoparticle.

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INTRODUCTION

Inflammation is a condition characterized as a complex immune response of the human body against any infection or trauma. It is manifested in the form of rubor, calor, dolor, tumor and functio laesa. The complex interactions between proinflammatory and anti-inflammatory mediators make the mechanism more complicated. The management of inflammation ranges from local cold pack application to conventional anti-inflammatory drugs [1]. However, these conventional Non steroidal anti-inflammatory drugs (NSAIDs) possess certain adverse effects pertaining to the gastrointestinal and cardiovascular systems due to its Cyclooxygenase inhibitors (COX)-dependent prostanoid inhibition [2]. Development of drug resistance is also one of the global concerning issues with conventional therapies. This demands an efficient alternative anti-inflammatory strategy.

The creation of innovative anti-inflammatory drugs through nanotechnology that can treat chronic inflammation, a prevalent underlying cause of many diseases, is one area of great attention. Revolutionization on nanoparticles and nanoscience has widely increased due to its potential benefits, such as bioavailability and bioactivity. The scientific community has witnessed its significant advances from nanorobotics to revolutionized therapeutic strategies, including chemotherapy, phototherapy, gene therapy, immune therapy, etc. [3].

An array of materials, including nanoparticles, is also under investigation for anti-inflammatory properties. Among them, nano hydroxyapatite (nHAP) has emerged as a viable option due to its biocompatibility, anti-

inflammatory and osteoconductive properties. The anti-inflammatory property is due to the suppression of proinflammatory cytokines such as tumor necrosis factor-beta, interleukin (IL)-1, 17 and also by increasing the anti-inflammatory cytokines like IL-4 [4-6]. In addition, they also serve as potential nano-carriers for the polymeric drugs [7].

However, the efficacy of nHAP in mitigating inflammation has been limited by its inherent properties and the need for enhanced delivery and bioactivity. To overcome these challenges, researchers have explored the incorporation of nHAP into various nanocomposites. Nanocomposites comprise of nanoscale particles at two or more distinct phases. The nanocomposite exhibits more potent bioactivity and biocompatibility, combined with superior mechanical characteristics like increased surface area to volume ratios, targeted application of drugs [8]. It has been witnessed that particular nanocomposites demonstrated anti-inflammatory qualities, like in a rat model of induced arthritis, nano-HAP-Vit B₁₂ composition has displayed substantial anti-arthritis, anti-inflammatory and anti-oxidant benefits [5]. Galactose nanoencapsulated triptolide exhibited enhanced anti-arthritis activity in a mice rodent model [9]. Remarkable anti-inflammatory, antioxidant, and anti-angiogenic activity was shown by metformin-loaded gold-poly catechin core-shell nanocomposite [10]. Nanocomposites are also used as carriers for drug delivery in inflammatory conditions. Poly(lactic acid)-based nanocomposite showed 95.8% efficiency in ibuprofen drug delivery [11].

Nanocomposites with HAP and other bioactive compounds may influence the production of proinflammatory cytokines, inhibit the activation of nuclear factor kappa B, or modulate the activity of other

key inflammatory mediators. Nanocomposites can lessen the intensity of inflammation and encourage tissue healing by focusing on the routes.

Coupling nHAP into nanocomposites is a noteworthy strategy that might bring about synergistic effect. A notable approach involves the use of green synthesis methods to integrate nHAP with natural compounds, aiming to harness their additive effects and maximize therapeutic benefits. The origin of green synthesis or green chemistry is dated back to early 2000s by Anastas and Warner. This process basically involves the use of micro-organisms as substrates, particularly algae, yeast, fungi, bacteria, and even some plant species and natural polymers for the synthesis of nanoparticles. It offers advantages of economy, safety, and nature friendliness with natural reducing and stabilizing attributes [12-14].

Citrus fruits and their peels, seeds, and extracts exhibit significant anti-inflammatory activities due to their composition of flavanones. By inhibiting crucial enzymes that generate and transduce inflammatory stimuli, citrus flavanones have been identified to modulate inflammatory responses [15]. *Citrus reticulata* and *Citrus limonum* are utilized in this study for the green synthesis of nHAP nanocomposite as they contain tetranortriterpenoids compounds, which are Citrus limonoids reported to demonstrate anti-inflammatory and antioxidant properties in rat model with liver ischemic condition [16].

This study focuses on the anti-inflammatory potential of an nHAP-incorporated green-synthesized nanocomposite. By leveraging eco-friendly synthesis techniques and the inherent properties of nHAP, this research aims to evaluate the effectiveness of the developed nanocomposite in reducing inflammation through *in vitro* experimentation. The goal is to provide insights into the mechanistic pathways by which this nanocomposite operates and its potential application in treating inflammatory conditions in safer ways. This study also involves the characterization of citrus fruit-mediated nHAP nanocomposites using X-ray diffraction (XRD) and Fourier transform infrared (FTIR) Spectroscopy analysis.

METHODS

Sample preparation

Preparation of C. reticulata and C. limonum mediated nHAP nanocomposites

Citrus fruits *C. reticulata* and *C. limonum* were procured, and peels were separated, and extracts were prepared based on standard practice. Green synthesized HAP nanoparticles were then subsequently generated by the addition of HAP powder particles to the citrus fruit peel extract. The mixture is then centrifuged (Lark) at 8000 rpm for 10 min [6]. 1 mL of the resultant HAP nanoparticle solution was mixed with 4 mL of chitosan solution.

The chitosan solution was prepared by mixing 500 mg of medium molecular weight chitosan and 1 mL glacial acetic acid, followed by an incorporation of 49 mL distilled water. The solution was kept in a magnetic stirrer (REMI) for 24 h at 650 rpm. The chitosan HAP nanoparticle mixture solution was then kept at a magnetic stirrer at 450 rpm for 3-h duration. The obtained HAP chitosan nanocomposites solution was stored for further assessments.

The prepared nanocomposite was then subjected to various assays for assessment of anti-inflammatory activity. Various concentrations of the green synthesized nanocomposite were evaluated to observe the crystalline nature, phase composition of the nanocomposite using XRD analysis, and its functional groups were clarified using FTIR analysis. Crystallite size calculation in XRD analysis was done using Scherrer's equation.

Anti-inflammatory activity assessment of the nanocomposite

Bovine serum albumin (BSA) denaturation assay, membrane stabilization assay (MSA) and Egg albumin (EA) denaturation assays were done to analyze anti-inflammatory activity of nHAP incorporated

C. reticulata and *C. limonum* mediated green synthesized nanocomposite.

BSA denaturation assay

About 1% BSA was used for the procedure. The assay was carried out in three groups, namely, the test group, control group and standard group.

Test group

Five test tubes each containing 0.05 mL of 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL concentration of *C. reticulata* and *C. limonum*-mediated nHAP nanocomposite. To this, 2 mL BSA was added, and the test tubes were incubated in normal room temperature and heated water bath at 55°C for 10 min each [17].

Control group

The control group consisted of dimethyl sulphoxide, on to which 2 mL BSA of 1% concentration was added.

Standard group

Diclofenac sodium was used as the standard. Followed by the addition of BSA, the group was subjected to incubation and heating similar to the test group. All three groups were made to undergo spectrophotometric (Elico, India) evaluation to determine the denaturation of proteins at 660 nm.

The percentage of protein denaturation was calculated with the given formula:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (\text{Eqn 1})$$

Membrane stabilization assay

This assay was performed to evaluate the ability of the green synthesized chitosan-based nHAP nanocomposite to stabilize cell membranes and henceforth preventing hemolysis. The materials used and procedure done was based on existing standard protocols [18].

The percentage of hemolytic inhibition was calculated with the given formula:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (\text{Eqn 2})$$

EA denaturation assay

The EA denaturation assay was carried out similar to BSA denaturation assay in three groups namely; test group, control group and standard group.

Test group

Five test tubes each containing 0.05 mL of 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL and 50 µg/mL concentration of *C. reticulata* and *C. limonum*-mediated nHAP nanocomposite. Followed by the addition of 0.2 mL EA, and 2.8 mL phosphate buffer mixture was done. The test tubes were incubated in normal room temperature and heated water bath at 55°C for 30 min each [17].

Control group

Control group consisted of dimethyl sulphoxide, on to which 0.2 mL EA and 2.8 mL phosphate buffer mixture was added.

Standard group

Diclofenac sodium was used as the standard. Followed by the addition of EA-phosphate buffer mixture, the group was subjected to incubation and heating similar to the test group. All three groups were made to undergo spectrophotometric evaluation to determine the denaturation

of proteins at 660 nm.

The percentage of protein denaturation was calculated with the given formula:

$$\% \text{ of inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \quad (\text{Eqn 3})$$

Statistical analysis is done using Levene's Test, T-test.

RESULTS

Anti-inflammatory property was evaluated by assessing the inhibition of protein denaturation by BSA denaturation, EA denaturation assay, and stabilization of cell membrane by MSA.

C. reticulata-mediated nHAP nanocomposites demonstrated a dose-dependent percentage of inhibition in the BSA denaturation assay of 79% at 50 µg/mL concentration, which is comparable to the standard used. Similarly, for *C. limonum* mediated HAP nanocomposites, the maximum percentage of inhibition was also observed at 50 µg/mL concentration, reaching 81% (Figs. 1 and 2).

Denaturation inhibition percentage of EA was proportional to the standard used for both *C. reticulata* and *C. limonum* mediated nHAP nanocomposites. The maximum inhibition was observed at 50 µg/mL concentration. (79% and 80%) (Figs. 3 and 4)

In MSA, *C. reticulata* and *C. limonum* mediated nHAP nanocomposites showcased varying hemolytic inhibition percentage. At a 10 µg/mL concentration 54% and 55% inhibition was demonstrated by *C. reticulata* and *C. limonum* compared to the 58% inhibition by the standard Diclofenac sodium. When the concentration was increased to 20 µg/mL the percentage of inhibition became 68% and 67% for test groups and 70% for standard, at 30 µg/mL and 40 µg/mL concentration, *C. reticulata* exhibited 76% and 79% of inhibition and

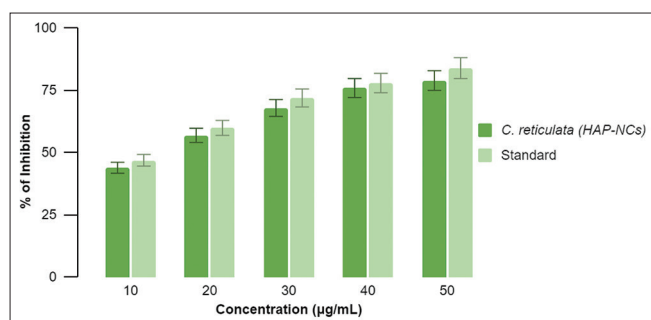


Fig. 1: Bovine serum albumin assay of *Citrus reticulata*-mediated nHAP nanocomposites

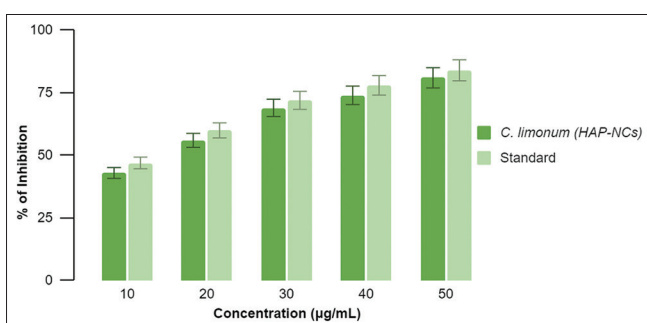


Fig. 2: Bovine serum albumin assay of *Citrus limonum*-mediated nHAP nanocomposites

for *C. limonum* 75% and 78%, respectively, whereas standard exhibited 77% and 82%. At the maximum experimented 50 µg/mL concentration 84% and 85% inhibition shown by the test groups and 89% inhibition by the standard diclofenac sodium. The standard showed significant anti-inflammatory property, which was in a comparable range to that of the *C. reticulata* and *C. limonum* mediated nHAP nanocomposites at varying concentrations (Figs. 5 and 6).

XRD analysis of *C. reticulata* mediated nHAP nano composites exhibited a crystalline content of 31.2% and amorphous content of 68.8% whereas *C. limonum* mediated nHAP nanocomposites had 73.9% amorphous and 26.1% crystalline content. The strong characteristic peaks were observed at around 25.7°, 31.8°, 32.3°, and 34.1° and minor peaks at higher angle i.e. 50°, 60° in *C. reticulata* mediated nanocomposite and for *C. limonum* mediated nHAP nanocomposites the strong peaks were observed at around 17.065°, 22.320°, 25.947°, 26.687°, 31.794°, 34.384°, 35.495°. The average crystallite size was 25.6 nm and 16.52 nm for *C. reticulata* and *C. limonum* mediated nHAP

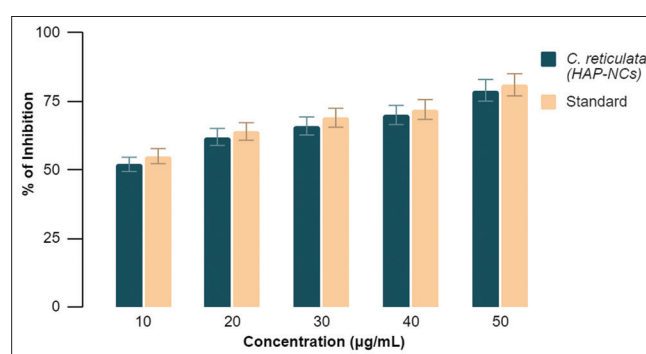


Fig. 3: Egg albumin assay of *Citrus reticulata*-mediated nHAP nanocomposites

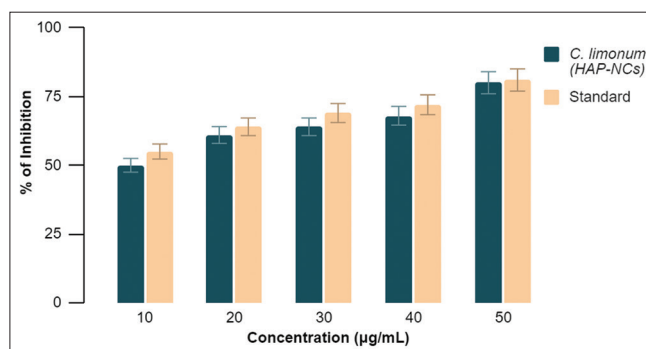


Fig. 4: Egg albumin assay of *Citrus limonum*-mediated nHAP nanocomposites

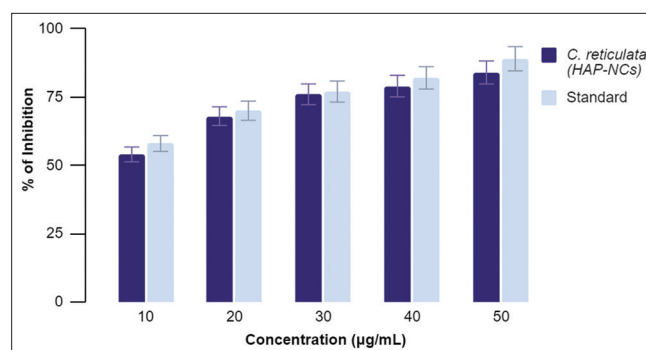


Fig. 5: Membrane stabilisation assay of *Citrus reticulata*-mediated nHAP nanocomposites

nanocomposites (Figs. 7 and 8).

FTIR analysis revealed the confirmed production of nHAP in both samples by typical absorption bands recorded in the spectra, which corresponds to the hydroxyl (OH⁻), phosphate (PO₄³⁻), and carbonate (CO₃²⁻) groups. The O-H stretching vibration of water molecules and surface hydroxyl is demonstrated by the wide absorption bands at 3274.8 cm⁻¹ for *C. reticulata* and 3308.6 cm⁻¹ for *C. limonum*. The bands at 1383.8 cm⁻¹ of *C. reticulata* and 1411.1 cm⁻¹ of for *C. limonum* are caused by the CO₃²⁻ vibrations. The peaks at 1023.8 cm⁻¹ in both samples confirm the existence of phosphate functional groups. The presence of C=O stretching is seen in *C. reticulata* mediated nHAP nanocomposite, but is absent in *C. limonum* group (Figs. 9 and 10).

DISCUSSION

This research focused on the anti-inflammatory property of *C. reticulata* and *C. limonum* mediated chitosan-based nHAP nanocomposites for developing a novel composite material for treatment against inflammation. Inflammation is a complex biologic response to harmful stimulus, injuries, and pathogens. A potential strategy that has made it possible to create new therapeutic medicines for such conditions with improved bioavailability and lower side effects, and toxicity is

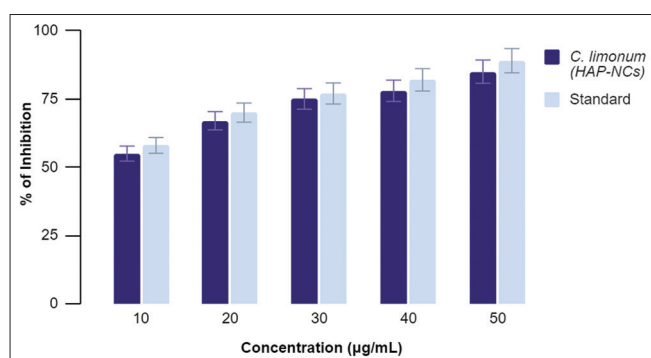


Fig. 6: Membrane stabilisation assay of *Citrus limonum*-mediated nHAP nanocomposites

nanotechnology. A nanocomposite formulation was developed as the distinctive characteristics of nanocomposites have generated interest, specifically when they are functionalized with components or substances which are biologically relevant. Nanocomposites of different forms have proven to have improved mechanical properties and control, bioactivity, biocompatibility, antibacterial qualities, angiogenesis, bioavailability, and much more [8].

The structural and functional characteristics of the nanocomposite synthesized from nHAP were assessed using XRD and FTIR analysis. The XRD analysis of *C. reticulata* mediated nHAP nanocomposites revealed 31.2% of crystalline phase and 68.8% of amorphous phase. The higher amorphous state can improve the strength and plasticity of nanocomposites [19]. The higher amorphous content suggests the significant amorphous calcium phosphate (ACP). ACP under biological conditions is known to convert into crystalline HAP over time [20]. The crystallinity is the result of organic or polymeric components from the *C. reticulata* peel extract. The peaks at 25.8°, 31.8° confirm the presence of HAP nanoparticles. The broadness of peaks indicates nanocrystalline HAP, which is beneficial for bioactivity and osseointegration. Some peak shifts or intensity variations were observed, which are due to the biogenic source of origin, the green synthesis. The estimated crystalline size of the HAP nanocomposite was 25.6 nm using the Full Width at Half Maximum (FWHM) of 0.5°.

In the XRD patterns of *C. limonum* mediated nHAP nanocomposites, there were sharp peaks, which indicate crystalline phases and a broad hump, which suggestive of an amorphous material. The green synthesized nanocomposite from *C. limonum* was primarily amorphous (73.9%), and the crystallinity percentage was 26.1%. This was in accordance with the *C. reticulata* peel extract outcomes. Similarly, the characteristic peaks at 25.9°, 31.8°, 32.9°, 34.3°, and 49.5° suggested that nHAP is the crucial component of the nanocomposite. A wide hump that ranges from 15° to 35° indicates the presence of amorphous silica generated from plants. Additional broad peaks at 26° could be a sign of graphitic carbon, from the organic leftovers that have been pyrolyzed. The crystalline size of the nHAP nanocomposite was 16.52 nm using FWHM of 0.5°.

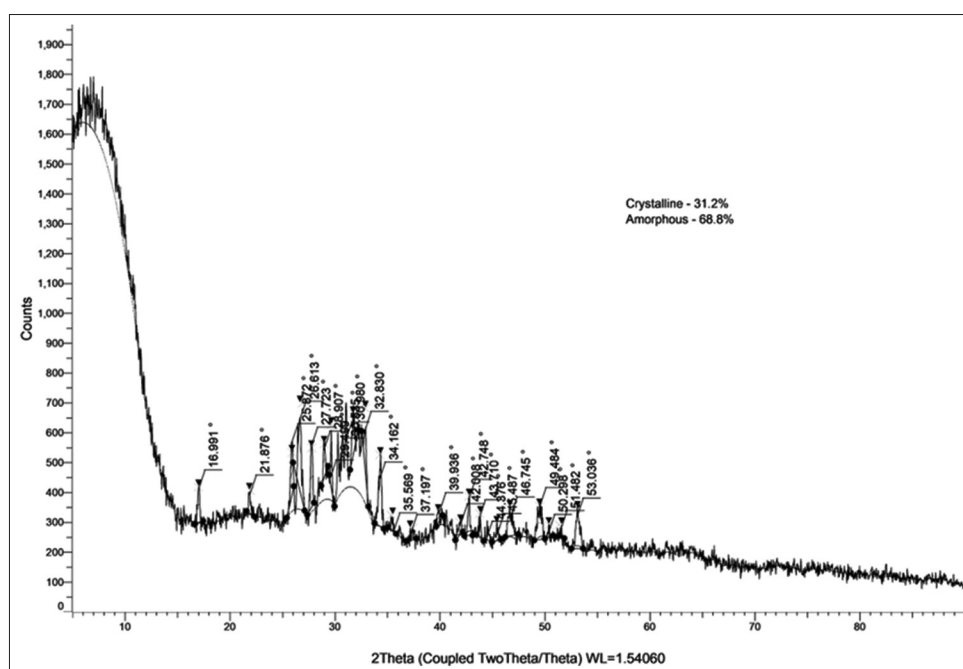


Fig. 7: X-ray diffraction image of *Citrus reticulata*-mediated nHAP nanocomposites

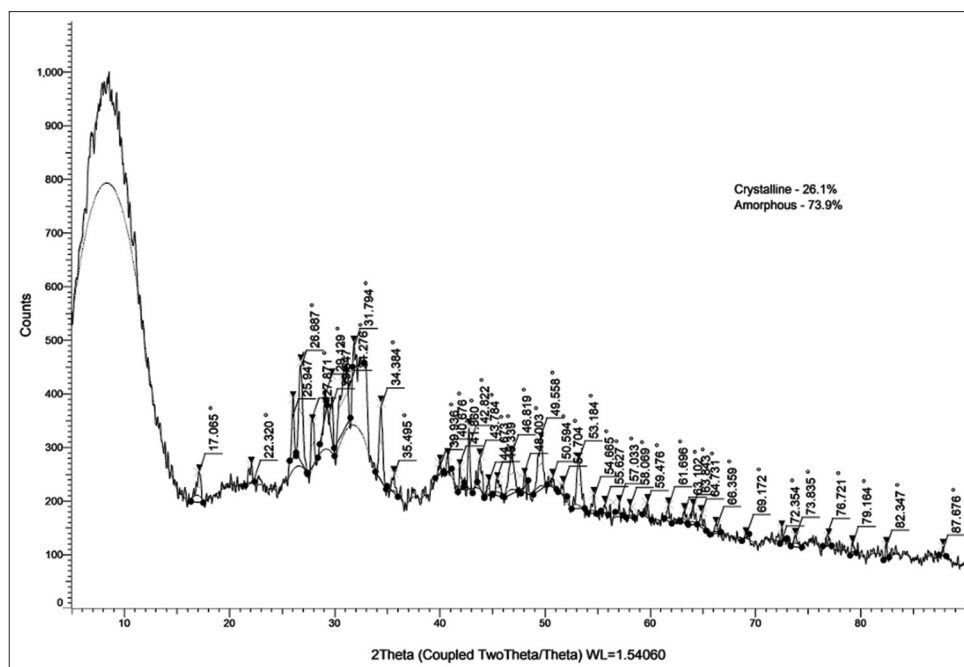


Fig. 8: X-ray diffraction image of *Citrus limonum*- mediated nHAP nanocomposites

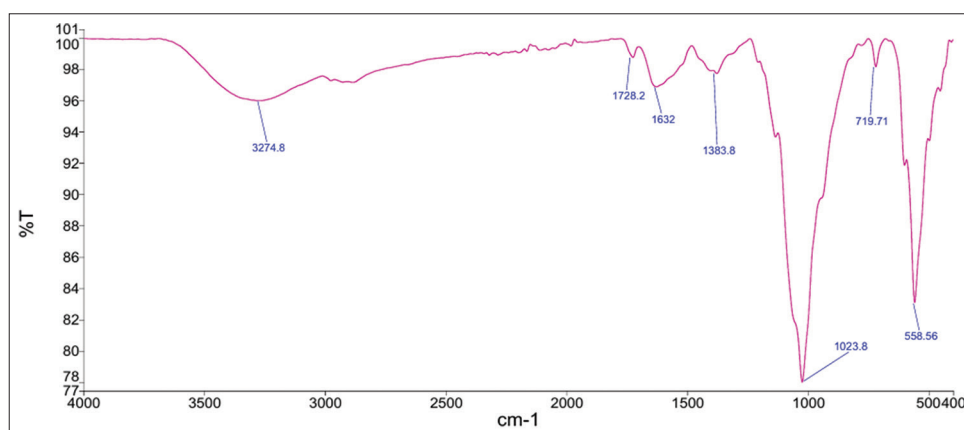


Fig. 9: Fourier transform infrared image of *Citrus reticulata*-mediated nHAP nanocomposites

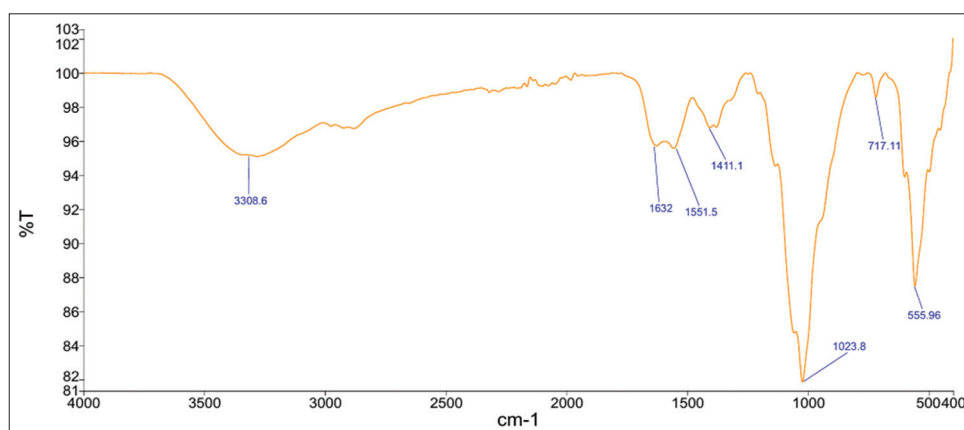


Fig. 10: Fourier transform infrared image of *Citrus limonum*-mediated nHAP nanocomposites

The high amorphous content of the *C. reticulata* and *limonum* mediated nHAP nanocomposites is suggestive of its potential biodegradability, which could be helpful for biomedical applications. The strong peaks

correspond to the nHAP presence, whereas minor peaks suggest secondary phases. The nanometer range of crystallinity makes the material ideal for use in bone repair and regeneration due to the

improved bioactivity and surface area. The presence of nano-sized crystallites also suggests the potential enhanced mechanical and bioactive qualities. This could be made useful in biomedical implants and its coatings.

FTIR evaluated the functional groups present in the synthesized nanocomposite and thereby offered relevant information about the chemical interactions seen between the nanocomposites constituent parts, particularly when the green precursors and nHAP are combined. Both groups, *C. reticulata* and *C. limonum*, mediated nHAP nanocomposites reported to exhibit the characteristics of nHAP particles confirmed by the presence of hydroxyl (OH⁻), phosphate (PO₄³⁻), and carbonate (CO₃²⁻) groups. This confirms the ability of the nanocomposite to modulate inflammatory responses, as nHAP has anti-inflammatory potential. The carbonate substitution in both groups makes them closer to biogenic bone minerals. The presence of C=O stretching in *C. reticulata* group and its absence in *C. limonum* group suggests the greater residual organic content in the group obtained from the precursor.

The anti-inflammatory property assessment of the *C. reticulata* and *C. limonum*-mediated nHAP nanocomposites was done using three conventional assays, namely, BSA denaturation assay, MSA, and EA denaturation assays. All three assays exhibited similar dose-dependent results comparable to the standard (Diclofenac sodium) used, which indicates the inhibition of protein denaturation and stabilisation of cell membranes. Denatured proteins trigger an inflammatory response. The inhibition of the protein denaturation clearly demonstrates the anti-inflammatory property. This result was in accordance with Dathan *et al.* [21]. The maximum inhibition percentage was showcased at 50 µg/mL concentration in both groups. In the comparative evaluation among *C. reticulata* and *C. limonum*-mediated nHAP nanocomposites, slightly higher percentage of inhibition of protein denaturation was exhibited by *C. limonum*-mediated nHAP nanocomposites, thereby the greater anti-inflammatory capability.

The anti-inflammatory capability of the nanocomposite is thought to stem from the additive effects of nHAP and green-synthesized citrus fruit peel matrix. HAP is recognized for its immunomodulatory characteristics, probably due to its biocompatibility, increased surface characteristics, its ability to engage with immune cells, such as macrophages, and controlled release of the bioactive elements. The addition of citrus fruit peel mediated green synthesized components contain bioactive elements like polyphenols, glycosides, alkaloids, carotenoids, flavonoids, or terpenoids, likely played a role in modulating inflammatory pathways by neutralizing free radicals and lowering oxidative stress, a condition closely associated with inflammation [22]. Hence, green synthesis can be considered as a reliable option for the development of nanoparticles and nanocomposites for clinical and other applications due to its sustainable eco-friendly approach with elevated biological activities and therapeutic efficacy due to synergistic effects and minimal environmental hazards due to natural pre-cursors [23].

Various authors have reported potential anti-inflammatory activity of nanoparticle and nanocomposites derived through green synthesis, similar to the current study [24-26]. The results of this study imply that the green synthesized nanocomposite with nHAP has potential for the treatment of inflammatory diseases. The coupled effect of green synthesized nanocomposite against inflammation, nHAP's osteoconductive nature, and biocompatibility make it a desirable option for bone regeneration and as a possible drug delivery system for the localized treatment of inflammation. Moreover, the development of nanocomposites with controlled release properties could offer a promising approach for sustained delivery of anti-inflammatory agents at the site of inflammation, improving the therapeutic outcome and reducing systemic side effects. Furthermore, it may be less likely to induce adverse immune reactions when compared to synthetic compounds. Moreover, the interaction between the green synthesized component and nHAP could influence the release kinetics of these bioactive compounds, potentially offering controlled or sustained release at the site of inflammation. This could further enhance the

Group statistics for *Citrus reticulata*

| Conc | Group | BSA assay | | MSA assay | | EA assay | |
|----------|----------------------|-----------|--------------------|-----------|--------------------|----------|--------------------|
| | | Mean | Standard deviation | Mean | Standard deviation | Mean | Standard deviation |
| 10 µg/mL | Standard | 47.0000 | 2.74874 | 58.0000 | 4.32049 | 55.0000 | 4.10961 |
| | <i>C. reticulata</i> | 44.0000 | 1.63299 | 54.0000 | 2.82843 | 52.0000 | 4.05518 |
| 20 µg/mL | Standard | 60.0000 | 2.58199 | 70.0000 | 5.22813 | 65.0000 | 3.74166 |
| | <i>C. reticulata</i> | 57.0000 | 3.33333 | 68.0000 | 4.61880 | 62.0000 | 3.82971 |
| 30 µg/mL | Standard | 72.0000 | 3.82971 | 77.0000 | 5.55778 | 69.0000 | 7.07107 |
| | <i>C. reticulata</i> | 68.0000 | 4.61880 | 76.0000 | 2.90593 | 66.0000 | 5.47723 |
| 40 µg/mL | Standard | 78.0000 | 2.78887 | 82.0000 | 4.94413 | 72.0000 | 3.82971 |
| | <i>C. reticulata</i> | 76.0000 | 2.90593 | 79.0000 | 4.37163 | 70.0000 | 5.22813 |
| 50 µg/mL | Standard | 84.0000 | 4.87625 | 89.0000 | 5.73488 | 81.0000 | 5.37484 |
| | <i>C. reticulata</i> | 79.0000 | 4.37163 | 84.0000 | 4.87625 | 79.0000 | 4.37163 |

C. limonum: *Citrus limonum*

Group statistics for *Citrus limonum*

| Conc | Group | BSA assay | | MSA assay | | EA assay | |
|----------|----------------------|-----------|--------------------|-----------|--------------------|----------|--------------------|
| | | Mean | Standard deviation | Mean | Standard deviation | Mean | Standard deviation |
| 10 µg/mL | Standard | 47.0000 | 2.74874 | 58.0000 | 4.32049 | 55.0000 | 4.10961 |
| | <i>C. reticulata</i> | 43.0000 | 5.71548 | 55.0000 | 7.49815 | 50.0000 | 2.94392 |
| 20 µg/mL | Standard | 60.0000 | 2.58199 | 70.0000 | 5.22813 | 65.0000 | 3.74166 |
| | <i>C. reticulata</i> | 56.0000 | 3.33333 | 65.0000 | 3.68179 | 61.0000 | 4.37163 |
| 30 µg/mL | Standard | 72.0000 | 3.82971 | 77.0000 | 5.55778 | 69.0000 | 7.07107 |
| | <i>C. reticulata</i> | 69.0000 | 4.47214 | 75.0000 | 3.33333 | 64.0000 | 5.41603 |
| 40 µg/mL | Standard | 78.0000 | 2.78887 | 82.0000 | 4.94413 | 72.0000 | 3.82971 |
| | <i>C. reticulata</i> | 74.0000 | 7.43864 | 78.0000 | 0.66667 | 68.0000 | 4.98888 |
| 50 µg/mL | Standard | 84.0000 | 4.87625 | 89.0000 | 5.73488 | 81.0000 | 5.37484 |
| | <i>C. reticulata</i> | 81.0000 | 3.26599 | 85.0000 | 5.20683 | 80.0000 | 3.71184 |

C. limonum: *Citrus limonum*

therapeutic potential of the nanocomposite in treating chronic inflammatory conditions.

FDA has approved small trials on nanotechnology-based analgesic and anti-inflammatory drugs and demonstrated superiority over a placebo or an active comparator at a dose below the clinically effective threshold [27]. However, large-scale trials or long-term effects are not available. Although the study's findings are encouraging, it is important to recognize a number of limitations. Our capacity to completely forecast the clinical result of utilizing these nanocomposites in human patients is limited by the study's *in vitro* design. More research using animal models is necessary to fully understand the intricate relationships between the nanocomposite and the immune system, as well as the possibility of long-term adverse consequences. To guarantee the safety and effectiveness of the nanocomposite in clinical applications, it is also necessary to carefully evaluate its behavior under various physiological situations and stability over long periods of time.

CONCLUSION

The present study demonstrated that *C. reticulata* and *C. limonum*-mediated nHAP nanocomposites exhibit significant anti-inflammatory potential. The synergistic effects of the nHAP and the green synthesis components contribute to its efficacy in modulating inflammatory pathways. The promising *in vitro* results, coupled with the material's biocompatibility and sustainability, position the nanocomposite as a potential candidate for treating inflammatory diseases, particularly in the field of bone regeneration. Further research is required to confirm these findings *in vivo* and to explore the broader therapeutic applications and safety of these nanocomposites in clinical settings.

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AUTHOR CONTRIBUTIONS

Lakshmi Ajithan S carried out the study. Dhanraj Ganapathy, Rajeshkumar Shanmugam, Revathy CV, and Arya Nair R participated in the design and coordination of the study.

CONFLICTS OF INTEREST STATEMENT

Nil.

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