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In vitro anti-inflammatory activity and cytotoxic effect of *Citrus reticulata*- and *Citrus limonum*-incorporated hydroxyapatite nanoparticles

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Abstract

Hydroxyapatite (HAP) is an excellent biocompatible material with osteoconductive potential. Numerous studies have reported the potential role of hydroxyapatite nanoparticles in bone tissue engineering because of their bone cell adhesion, proliferation and differentiation. Likewise**,** citrus fruits possess anti-oxidant properties. Anti-oxidants are found to reduce oxidative stress, which in turn is found to be effective in bone remodelling. Also, the ease, cheap availability and potential benefits make citrus fruits a material of choice. So, this study aimed to green synthesize *Citrus reticulata*- and *Citrus limonum*-mediated HAP nanoparticles. The green synthesis of *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles were conducted and the anti-inflammatory properties of the nanoparticles were assessed using the membrane stabilization assay, the bovine serum albumin denaturation assay and the egg albumin denaturation assay. The cytotoxicity of the nanoparticles was also assessed and the assay used for evaluation was brine shrimp lethality. The successful green synthesis of *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles was done. Also, the results revealed that the anti-inflammatory actions of the green synthesized nanoparticle are comparable with the standard. Based on the study results, it was revealed that the green synthesized *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles are non-cytotoxic and possess anti-inflammatory activity.

Keywords

hydroxyapatite; green synthesis; anti-inflammatory activity; product development; sustainable

Introduction

Hydroxyapatite is a naturally occurring $Ca_3(PO_4)_2$-based mineral with the molecular formula $Ca_{10}(PO_4)_6(OH)_2$ (1). Due to its excellent biocompatibility, non-toxic, osteoconductive nature and similarity to bone and teeth, hydroxyapatite can be used as a filler material in bone defects. Numerous materials showed improvements in mechanical properties when converted to nanoscale. Similarly, hydroxyapatite particles, when converted into nanoparticles, exhibited enhanced mechanical properties, thereby increasing biological activities. Its chemical composition and structure were identical to those of natural bone apatite. Hence, hydroxyapatite nanoparticles can be used as a material of choice for bone repair (2, 3). In actuality, hydroxyapatite crystals encased in a collagen matrix make up the nanoscale structural makeup of bone tissue (4).

However, the presence of hydroxyapatite particles is not just confined to the bone tissues. The other natural sources of hydroxyapatite particles are fish scales, animal bones, shells of eggs and snails, teeth, etc. There are numerous commercial methods for the synthesis of nanoscale hydroxyapatite particles. It includes sol-gel, wet chemical precipitation, hydrothermal and microwave methods. Researchers have used numerous materials and techniques for the synthesis of hydroxyapatite crystals. The sol-gel method offers the generation of hydroxyapatite particles with greater homogeneity. Experiments on morphology-enhanced low-temperature sintering have been conducted for the synthesis of hydroxyapatite. The use of a wet chemical precipitation reaction between calcium nitrate and diammonium phosphate as precursors resulted in the synthesis of dense nanocrystalline hydroxyapatite (5, 6). Hydroxyapatite nanorods with diverse sizes and morphologies were developed using ammonia and calcium nitrate solutions by the hydrothermal method. This method offers the generation of an end product suitable for medical applications (7).

The synthesis of hydroxyapatite can be done by dry methods, wet methods, high-temperature processing, combination processing or even synthesis from biological sources. One of the commonly used methods for the synthesis of hydroxyapatite nanoparticles is green synthesis (8). Green synthesis is the production of nanoparticles by using biological routes such as microorganisms, enzymes or plants. This method is more advantageous due to its efficiency, ease of production, eco-friendliness and less toxicity. The green synthesis of hydroxyapatite nanorods using xanthan and its strontium-substituted counterpart was carried out (9). Another green template technique for the manufacture of hydroxyapatite nanorods employing extracts from three separate naturally occurring sources that include tartaric acid was developed (10).

In one of the studies, the effect of sugarcane juice on the stabilized synthesis of hydroxyapatite nanoparticles was evaluated and it was concluded that for the synthesis of hydroxyapatite nanoparticles, sugarcane juice can act as both a stabilizing agent and an organic modifier (11). The green synthesis of hydroxyapatite nanoparticles was carried out by the chemical precipitation method using piperine (12).

A fascinating and rapidly evolving aspect of nanotechnology by the development of nanoparticles by green synthesis using plants will help promote nanoscience in the future and protect the environment. From the green approach to their manufacturing, applications for nanoparticles are expected to develop exponentially. However, there are concerns about the long-term impacts of these particles on humans and animals. Also, the build-up of these particles in the environment needs to be taken into account in the future (13).

Citrus fruits like oranges, lemons, grapes, etc., have the potential to be used for the green synthesis of hydroxyapatite nanoparticles because of their anti-inflammatory, anti-tumour and anti-oxidant activities. Bioactive substances including polyphenols, flavonoids, carotenoids and ascorbic acid are responsible for these qualities (14). Citrus fruits are rich in flavonoids. Flavonoids demonstrate an exceptional ability to scavenge free radicals and reflect their antioxidant activity. Numerous studies also point towards the anti-inflammatory, anti-viral, anti-cancer and neuroprotective effects of flavonoids (15). The anti-inflammatory activity is attributed to the flavonoids, coumarin and volatile oils in citrus fruits (16). Not only the fruit but also the peels and oils generated from citrus fruits have an anti-inflammatory effect. Another component found in citrus fruits that is responsible for the anti-inflammatory effect is α-terpineol (17).

Malleshappa *et al*. conducted a study to evaluate the anti-inflammatory and anti-nociceptive potential of citrus fruits. They used the peel of five citrus fruits, namely *Citrus aurantifolia, Citrus reticulata, Citrus aurantium, Citrus grandis* and *Citrus medica*. Study results conclude that the citrus fruit peel extract has anti-inflammatory and anti-nociceptive properties and is attributed to the presence of flavonoids, terpenoids, steroids, glycosides, alkaloids, carotenoids and phenolic compounds found by phytochemical analysis (18).

The flavonoids found in citrus groups are of four types: flavanones, flavones, flavanols and anthocyanins. Among these components, flavanones are found in greater proportions. The concentration of these components depends on the age of the plant (19). Flavonoids have the potential to alter the enzymatic activity of the body. They also are powerful radical scavengers (20).

Apart from flavonoids, another component was discovered that could also be responsible for the anti-inflammatory activity of citrus fruits, which is citrusin XI, a cyclic peptide (21). Henceforth, citrus fruits exhibit the potential to be used as an anti-inflammatory agent and combining citrus fruits with any other materials can be beneficial and might have a synergistic effect on anti-inflammatory action.

Numerous studies are being conducted for the green synthesis of hydroxyapatite nanoparticles. However, not much data is available regarding the green synthesis of hydroxyapatite nanoparticles from citrus fruits. So, the rationale of this study is to prepare the citrus fruit peel extract-mediated HAP nanoparticles and assess their anti-inflammatory and cytotoxic activity. The null hypothesis is that citrus fruit peel extract-mediated HAP nanoparticles will not have anti-inflammatory activity and will be cytotoxic. The alternate hypothesis is that the citrus fruit peel extract-mediated HAP nanoparticles will exhibit anti-inflammatory activity and will not be cytotoxic.

Materials and Methods

Sample preparation

Preparation of Citrus reticulata- and Citrus limonum-mediated HAP nanoparticles

The citrus fruits, *C. reticulata* and *C. limonum* were procured and the fruits' peels were removed, air-dried and coarsely ground. Coarsely ground *C. reticulata* and *C. limonum* powder were weighed out at 2 g and dissolved in 100 mL of deionized water. The mixture was then subjected to additional heating by employing a heating mantle at 50-60 °C for 20 min. The obtained extract was further subjected to a filtration process with muslin cloth. A quantity of 200 mg of HAP powder was carefully measured and combined with 50 mL of deionized water. Subsequently, 50 mL of HAP solution was poured into a mixture of 50 mL of *C. reticulata* and *C. limonum* extract. The resulting mixture was kept on a magnetic stirrer without any disturbance for duration of 48 h. After 24 h, a change in colour was observed in the HAP solution. The solution was then centrifuged at a rate of 8000 rotations for 10 min, after which a collection of pellets was fetched and stored for future use (Fig. 1-4).

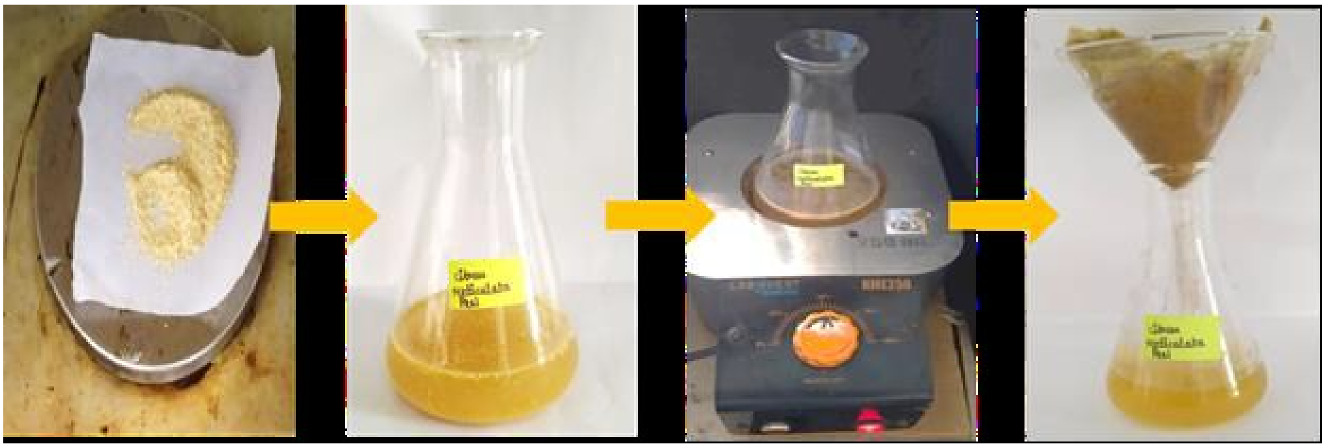

Fig. 1. Preparation of *C. reticulata* extract.

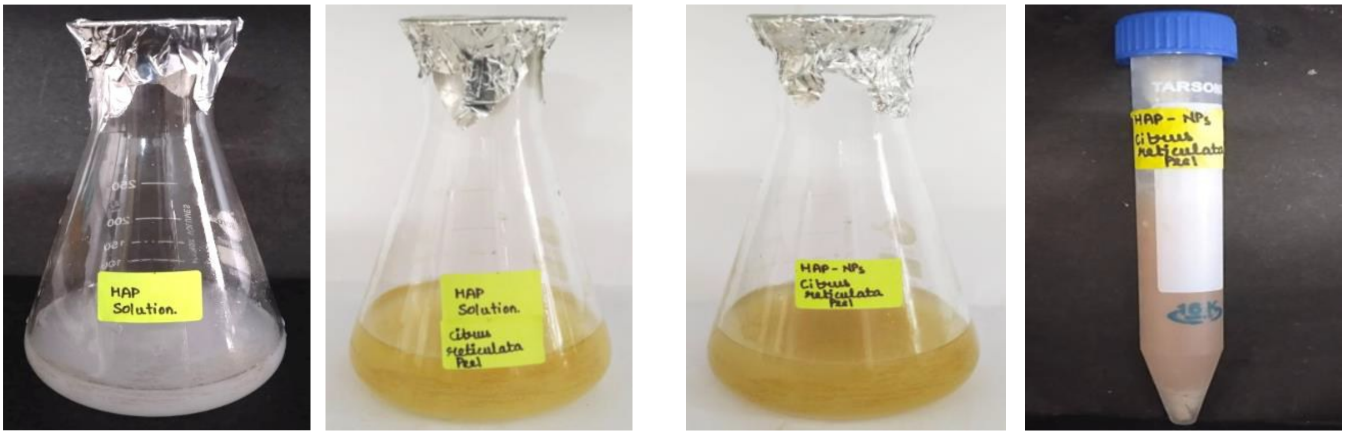

Fig. 2. Green synthesis of *C. reticulata*-mediated HAP nanoparticles.

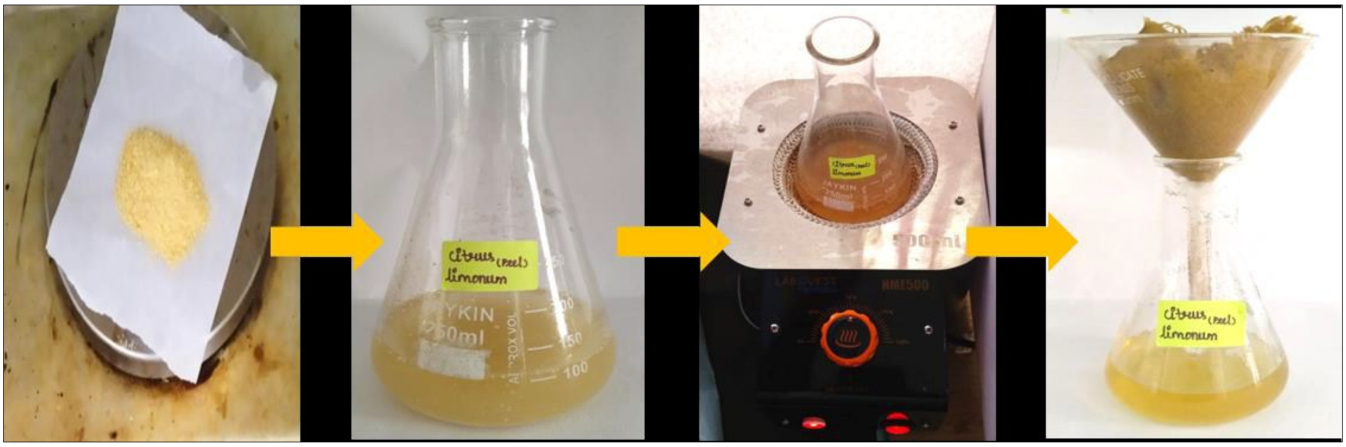

Fig. 3. Preparation of *C. limonum* extract.

Fig. 4. Green synthesis of *C. limonum*-mediated HAP nanoparticles.

Assessment of anti-inflammatory activity of C. reticulata- and C. limonum-mediated HAP nanoparticles

Three assays, a bovine serum albumin denaturation, an egg albumin denaturation and membrane stabilization, were conducted on green synthesized *C. reticulata-* and *C. limonum-*mediated HAP nanoparticles to assess their anti-inflammatory activity.

Bovine serum albumin denaturation assay

In this assay, 0.05 mL of varied concentrations (10 to 50 µg/mL) of *C. reticulata-* and *C. limonum-*mediated HAP nanoparticles were used. The assay was carried out on the basis of a standardized protocol (22). The standard group was comprised of diclofenac sodium, while the control group consisted of dimethyl sulphoxide. The samples were assessed using a spectrophotometer at a wavelength of 660 nm to ascertain the extent of protein denaturation. The following equation was used to calculate the percentage of protein denaturation:

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

Egg albumin denaturation assay

The amount of protein denaturation of HAP nanoparticles mediated by *C. reticulata* and *C. limonum* was calculated by evaluating an egg albumin denaturation assay. For the procedure, varying concentrations ranging from 10 to 50 µg/mL of HAP nanoparticles mediated by *C. reticulata* and *C. limonum* were used. A standard protocol for the assay was carried out (22). Diclofenac sodium was the standard group, whereas dimethyl sulphoxide was the control group. The samples were analysed using a spectrophotometer at 660 nm to calculate the protein denaturation percentage. The following equation is used to calculate the percentage of protein denaturation:

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

Membrane stabilization assay

The *in vitro* stability maintenance of the cell membrane by HAP nanoparticles mediated by *C. reticulata* and *C. limonum* was assessed by the membrane stabilization test (MST). This assay was used to assess the HAP nanoparticles mediated by *C. reticulata* and *C. limonum* compounds' capacity to prevent cell membrane rupture and the resultant exudation of intracellular substances. The standardized protocol of the assay was carried out and analysed using a spectrophotometer (22).

RBC suspension preparation

RBC suspension was prepared as per standard protocol (22). After that, varying amounts of *C. reticulata-* and *C. limonum-*mediated HAP nanoparticles were carefully combined with the suspension. Further, the tubes were incubated at 37 °C for 30 min. In order to give the RBCs time to pellet, the tubes were centrifuged for 10 min at room temperature. To test the absorbance at 540 nm, a UV-Vis spectrophotometer was employed. The following equation is used to calculate the percentage of haemolysis:

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

Where, the absorbance of the RBC suspension in the absence of the test compound(s) is the OD of the control and the absorbance of the RBC suspension in the presence of the test compound is the OD of the sample.

Evaluation of the cytotoxic activity of C. reticulata- and C. limonum-mediated HAP nanoparticles

Brine shrimp lethality assay

A solution of salt was prepared by weighing 2 g of salt, free of iodine and dissolving it in 200 mL of deionized water. Subsequently, 10–12 mL of the saline solution was added to each of the six-well ELISA plates. Gradually 10 nauplii were introduced into each well, adding them in increments of 20 µL, 40 µL, 60 µL, 80 µL and 100 µL. Next, the nanoparticles were introduced in the appropriate concentrations. Plates were incubated for 24 h. Following the incubation period, the ELISA plates were counted and inspected. The following formula was used to estimate the number of living nauplii present (Fig. 5):

$$\text{No. of living nauplii} = \frac{\text{No. of dead nauplii}}{\text{No. of dead nauplii + No of live nauplii}} \times 100 \quad \text{(Eqn.4)}$$

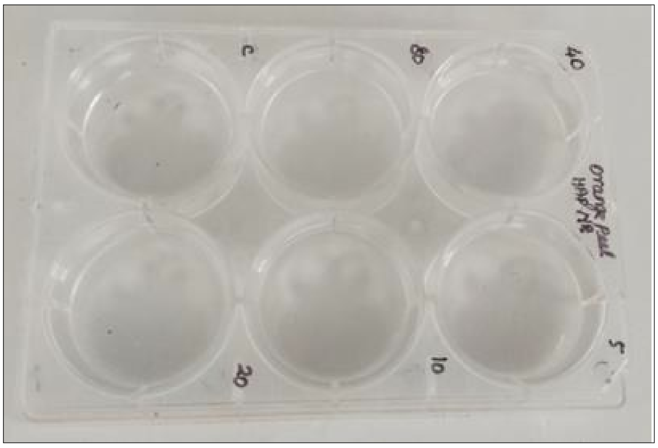

Fig. 5. Cytotoxic effect assessment of *C. reticulata-* and *C. limonum-*mediated HAP nanoparticles.

Results

Using the following assays, bovine serum albumin denaturation, egg albumin denaturation, membrane stabilization and the anti-inflammatory activity of *C. reticulata-* and *C. limonum-*mediated HAP nanoparticles were evaluated. In the bovine serum albumin denaturation assay, different concentrations (10-50 µg/mL) of *C. reticulata-* and *C. limonum-*mediated HAP nanoparticles were mixed with bovine serum albumin and the percentage of inhibition was calculated. The percentage of inhibition of both *C. reticulata-* and *C. limonum-*mediated HAP nanoparticles was proportionate to the standard used. Also, the maximum percentage of inhibition (80% and 78%) was seen at 50 µg/mL concentration in both *C. reticulata-* and *C. limonum-*mediated HAP nanoparticles (Fig. 6 and 7).

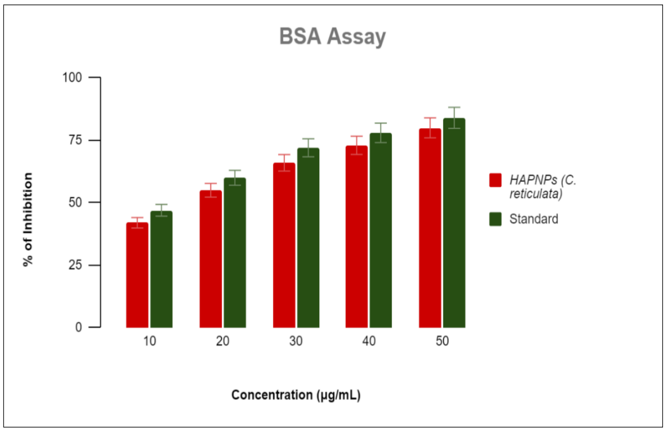

Fig. 6. BSA assay of *C. reticulata*-mediated HAP nanoparticles.

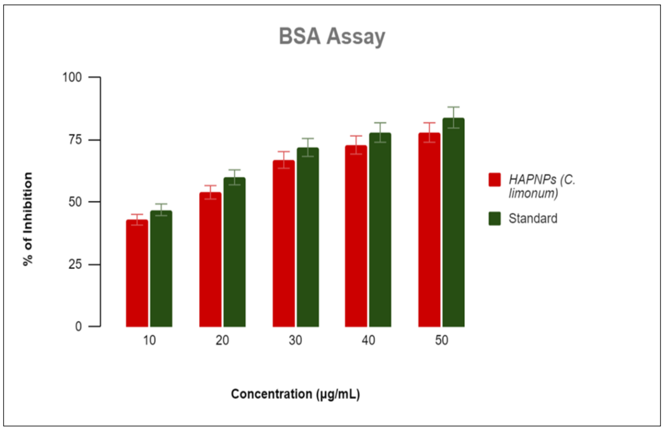

Fig. 7. BSA assay of *C. limonum*-mediated HAP nanoparticles.

In egg albumin denaturation and membrane stabilization assays, both *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles exhibited a percentage of inhibition proportionate to that of the standard. The concentrations at which the maximum percentage of inhibition was shown at 50 µg/mL were 77% and 75% for the egg albumin denaturation assay and for the membrane stabilization assay, it was 84% and 83%, respectively (Fig. 8-11).

Cytotoxicity evaluation revealed 100% cell viability for *C. reticulata*-mediated HAP nanoparticles, whereas cell viability was reduced to 70% for an 80 µg/mL concentration on day 2 for *C. limonum*-mediated HAP nanoparticles, still a non-lethal percentage (Fig.12 and 13).

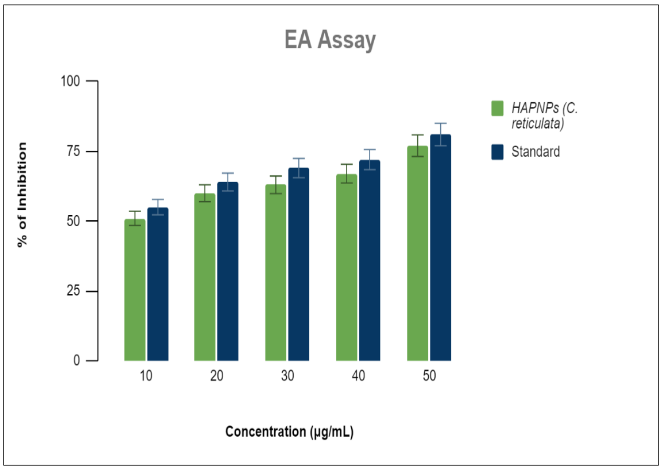

Fig. 8. EA assay of *C. reticulata*-mediated HAP nanoparticles.

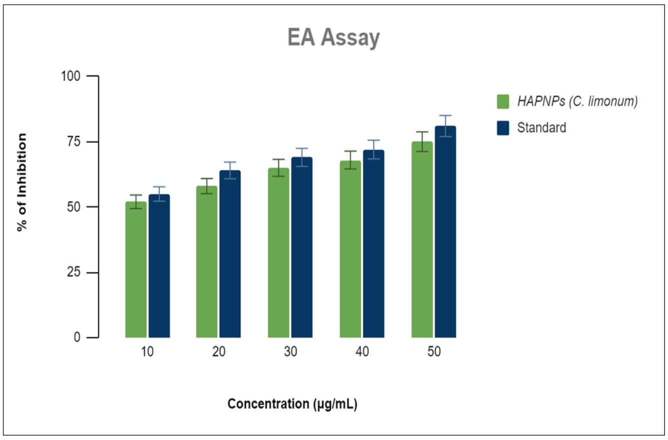

Fig. 9. EA assay of *C. limonum*-mediated HAP nanoparticles.

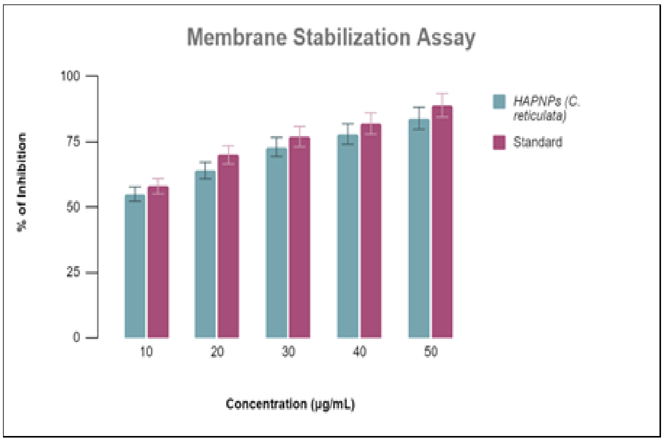

Fig. 10. MSA assay of *C. reticulata*-mediated HAP nanoparticles.

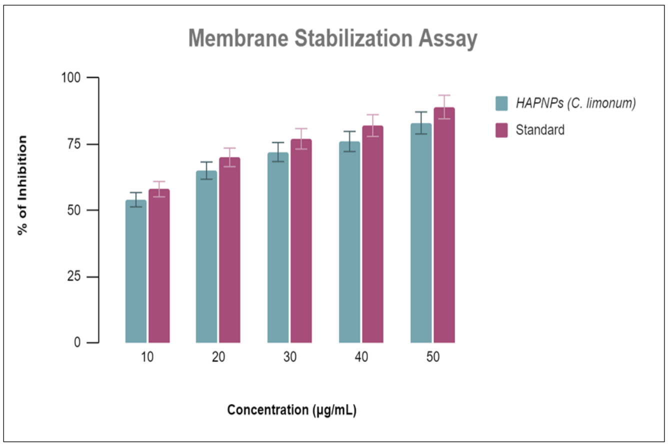

Fig. 11. MSA assay of *C. limonum*-mediated HAP nanoparticles.

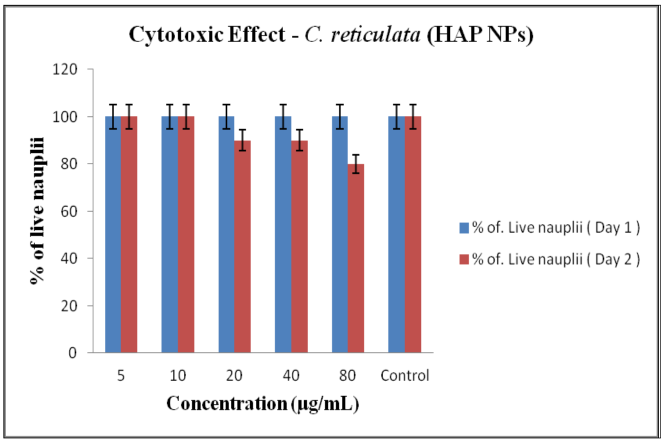

Fig. 12. Cytotoxic effect of *C. reticulata*-mediated HAP nanoparticles.

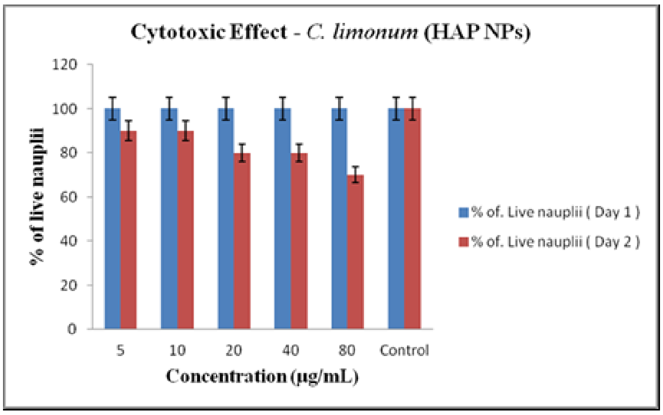

Fig. 13. Cytotoxic effect of *C. limonum*-mediated HAP nanoparticles.

Discussion

Fruits and vegetables we consume on a day-to-day basis have many medicinal properties. However, the medicinal properties are least explored and made use of. Citrus fruits are one such category. Citrus fruits like oranges, lemons and grapes possess anti-oxidant, inflammatory and tumour activities. The presence of different bioactive compounds is the reason for this (23).

The green synthesis from these fruits can be utilized for the preparation of nanoparticles. Chatterjee *et al.* green-synthesized zinc oxide nanoparticles using green tea and chamomile tea extract and demonstrated that green tea- and chamomile tea-mediated zinc oxide nanoparticles have better anti-inflammatory and anti-oxidant activity. Similar to the present study, they carried out a bovine serum assay and an egg albumin assay to assess the anti-inflammatory activity. Also, the concentration at which maximum anti-inflammatory activity was expressed is the same, i.e., 50 µg/mL (24). VT *et al.* suggest the therapeutic potential of green synthesized nanoparticles. They green-synthesized titanium oxide nanoparticles using rosemary and ginger extract and concluded that they have antibacterial potential (25).

The green synthesis of HAP nanoparticles using various biological components has been evaluated. Ganta *et al.* used an aqueous extract of *Monoon longifolium* leaves for the preparation of hydroxyapatite nanoparticles as an adsorbent for fluoride ion removal from an aqueous solution (26). Padmanabhan *et al.* conducted the green synthesis of hydroxyapatite nanorods using *Camellia sinesis* (white tea extract) (27). By using Indian nettle (*Acalypha indica*) leaf extract and papaya (*Carica papaya*) leaf extract as solvents, Devi *et al.* produced hydroxyapatite nanoparticles by sol-gel green synthesis (28). The hydroxyapatite nanorods were synthesized by Kalaiselvi *et al.* using the water-based extract of *Moringa oleifera* flowers (29). Kumar *et al.* green synthesized hydroxyapatite nanorods and evaluated their antibacterial activity and their usage for orthopaedic purposes (30). The green synthesis of hydroxyapatite nanoplates from the extract *of M. oleifera* flowers and the ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate was carried out by Sundrarajan *et al.* (31).

Various researches proved numerous biological extracts to be effective anti-inflammatory agents. Venkatesh *et al.* evaluated the anti-oxidant and anti-inflammatory actions of the marigold flower tea formulation and concluded that the newly developed composite exhibited better properties than the controls they used (32). The anti-inflammatory activity of a mouthwash containing a formulation of *Syzygium aromaticum* and *Zingiber officinale*-mediated by zinc oxide nanoparticles (ZnO NPs) was assessed by Selvaraj *et al.* (33). Navya *et al.* green synthesized red sandal-mediated gold nanoparticles and reported that the composite exhibited good antioxidant and anti-inflammatory properties (34).

In the current study, we attempted the green synthesis of hydroxyapatite nanoparticles using *C. reticulata* and *C. limonum.* The formation of HAP nanoparticles was confirmed by spectrophotometric results. Then, evaluations of the anti-inflammatory and cytotoxic activity of *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles were carried out. *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles revealed less protein denaturation and comparable anti-inflammatory activity to that of the standard used in the study. The percentage of inhibition increased with increased concentrations of the nanoparticle. This result was in accordance with the result of Devi *et al.*, where green-synthesized HAP nanoparticles from *C. papaya* and *A. indica* exhibited excellent antimicrobial activity and a rise in concentration of HAP nanoparticles increased inhibitory activity (28). The study outcome of Kalaiselvi *et al.* exhibits similar results; hydroxyapatite nanorods produced by the green synthesis of *M. oleifera* show good antibacterial activity (29). Padmanabhan *et al.* also demonstrated similar results of higher antioxidant activity when hydroxyapatite nanorods were green-synthesized using *C. sinesis* (white tea extract) (27). All these study results are evidence of therapeutic effects in the form of anti-inflammatory, anti-microbial and antioxidant activities of green-synthesized nanoparticles.

Various concentrations of *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles exhibited dose-dependent anti-inflammatory activity. The highest concentration of 50 µg/mL showed the highest percentage of inhibition. This proves that *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles exhibit good anti-inflammatory activity. Hence, the null hypothesis was rejected.

Regarding the cytotoxicity evaluation, numerous concerns have been raised regarding the safety of nanoparticles and nanocomposites (35). The properties of nanoparticles, like their smaller size, greater absorbability and ability to cross the blood-brain barrier, have been found advantageous. However, it could raise a potential threat of toxicity as well. There is no guaranteed evidence of the non-toxicity of nanoparticles, as it lacks long-term study results. Hence, we decided to investigate the cytotoxic effects of *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles.

A brine shrimp lethality assay was used for the evaluation of the cytotoxicity of *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles. A 100% cell viability was shown on day 1 for both *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles. On day 2, the viability percentage of *C. limonum*-mediated HAP nanoparticles was reduced, but the values were still below the lethal percentages. Thus, the non-cytotoxicity of our composite, *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles, was observed. Annu *et al.* also demonstrated similar results with the citrus fruit peel-mediated green synthesis of silver nanoparticles (36). Similar, non-cytotoxic copper nanoparticles were reported to be water-based green synthesized using *C. sinensis* by Jahan *et* *al*. (37). The significant benefits of nanoparticles over conventional materials necessitate the employment of nanoparticles. So, toxicity evaluation of all newly discovered materials should be carried out before their clinical application to prevent any sort of safety concern.

Conclusion

The green synthesis process was successful in creating the citrus fruit peel-mediated HAP nanoparticles with ease, suggesting the use of green synthesis as an eco-friendly and cost-effective method for the synthesis of nanoparticles. The evaluation of the anti-inflammatory efficacy of HAP nanoparticles mediated by *C. reticulata* and *C. limonum* exhibits similar anti-inflammatory activity to that of the standard. These nanoparticles hold potential for future commercial applications. The result of this study emphasizes the interdisciplinary connection of nanotechnology with natural and oriental medicine and its synergistic effects in the management of complex health issues. Further research is to be conducted to understand the molecular mechanisms involved in the anti-inflammatory activity. In addition, the cytotoxicity evaluation revealed the green synthesised *C. reticulata*- and *C. limonum*-mediated HAP nanoparticles to be non-cytotoxic, which suggests their potential use in living tissues.

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Authors' contributions

LA carried out the study. DG and RS participated in the design and coordination of the study. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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