

Assessment of Antioxidant Property and Characterisation of Citrus Fruit Mediated Green-synthesised Hydroxyapatite Nanoparticles: An In-vitro Study

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ABSTRACT

Introduction: Citrus fruits and their peels are recognised as excellent naturally available antioxidants. Antioxidants play a crucial role in preventing oxidative damage, Deoxyribonucleic Acid (DNA) damage, and protein modification. Therefore, developing compounds from these citrus fruit peels would be highly beneficial. Synthesising compounds of plant origin falls under green synthesis. The rationale behind this study is to create a novel class of biomaterials with improved biological properties, offering significant potential for therapeutic uses.

Aim: To assess the antioxidant activity of green-synthesised hydroxyapatite nanoparticles using citrus fruit peel extract.

Materials and Methods: This in-vitro study was conducted at the Nanomedicine Biolab, Department of Pharmacology, Saveetha Dental College, Chennai, Tamil Nadu, India, from March 2024 to June 2024. Hydroxyapatite nanoparticles were green-synthesised from *Citrus reticulata* (orange) and *Citrus limonum* (lemon). Only the peels of these fruits were included in the green synthesis, which was the primary inclusion criterion. Green-synthesised nanoparticles at different concentrations (10-50 µg/mL) were evaluated for antioxidant properties using

the Hydrogen Peroxide assay, the Ferric Reducing Ability of Plasma assay, the 2,2-Diphenyl-1-Picrylhydrazyl assay, the 2,2-Azino-Bis-3-Ethylbenzothiazoline-6-Sulphonic Acid assay, and the Nitric Oxide Radical Inhibition assay. The results were tabulated and statistically analysed using independent t-tests, ANOVA analysis, and post-hoc tests. A p-value of less than 0.05 was considered statistically significant. Characterisation of the generated nanoparticle samples was performed using UV-Vis spectroscopy and Scanning Electron Microscopy (SEM).

Results: The successful synthesis of *C. reticulata* and *C. limonum*-mediated hydroxyapatite nanoparticles and their comparable antioxidant activity with the standard (ascorbic acid) were observed. The absorbance spectrum ranged from 340 nm to 360 nm in UV-Vis spectroscopy, and distinct morphologies were observed in the SEM analysis.

Conclusion: Green-synthesised *C. reticulata* and *C. limonum*-mediated hydroxyapatite nanoparticles exhibited potential antioxidant activity. These green-synthesised nanoparticles possess antioxidant properties.

Keywords: Free radical scavenging, Scanning electron microscopy, Spectroscopy

INTRODUCTION

Antioxidants are compounds that inhibit oxidation and can trap free radicals. Free radicals, or Reactive Oxygen Species (ROS), are produced during metabolic and functional activities. However, excessive amounts of ROS are deleterious to the body and result in oxidative stress. This may lead to pathological conditions affecting the Central Nervous System (CNS), Cardiovascular System (CVS), eyes, bones, endocrine system, and more. Antioxidants prevent oxidative damage, lipid peroxidation, DNA damage, and protein modification [1]. They are available in both natural and synthetic forms. Physiological antioxidants can be classified into two forms: hydrophilic and lipophilic. Some common antioxidants include polyphenols, flavonoids, phenolic compounds, tocopherols, and ascorbic acid.

The enzymatic antioxidants include Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Peroxidase (GPX), Glutathione Reductase (GR), Glutathione Transferase (GST), heme oxygenase, biliverdin reductase, and lipophilic radical scavengers. The non-enzymatic antioxidants consist of vitamins, minerals, nitrogen compounds, etc. [2]. Natural antioxidants are primarily derived from plants, with some originating from microorganisms and animals. Red, orange, and purple-colored fruits and vegetables are rich sources of antioxidants. Oranges, lemons, red bell peppers, strawberries, plums, and prunes exhibit high antioxidant activity [3].

Notably, one-third of dietary phenols are found in plants in the form of flavonoids and carotenoids. Flavonoids possess redox potential and help prevent oxidative cell damage. Citrus fruits are particularly rich in flavonoids and possess numerous biological properties, including anti-inflammatory, anticarcinogenic, anti-ulcer, antioxidant, anti-aging, and lipolytic properties [4]. Polyphenols are responsible for these biological properties, with flavonoids being major components of polyphenols. Flavonoids are abundantly found in fruit peels [5].

Citrus flavonoids are involved in scavenging oxygen radicals by transferring hydrogen or electrons. The common forms of citrus flavonoids are flavanones, flavones, and flavanols. Among these, flavanones are the primary form, followed by flavones in terms of antioxidant activity. Citrus fruits containing flavonoids include *Citrus sinensis*, *Citrus reticulata*, *Citrus aurantium*, *Citrus clementina*, *Citrus limonum*, and *Citrus paradisi*. Hesperidin is the flavonoid found in *C. sinensis*, *C. reticulata*, *C. clementina*, and *C. limonum*, whereas naringin is abundantly found in *C. aurantium* and *C. paradisi* [6].

The antioxidant activity of citrus flavonoids is also favorable for bone health. ROS contribute to the apoptosis of bone regenerative cells like osteoblasts and osteocytes, resulting in bone loss or a lack of new bone formation [7]. Increased oxidative stress also affects RANKL (Receptor Activator of Nuclear Factor Kappa Beta) and Osteoprotegerin (OPG), which leads to osteocalcin formation and

subsequently enhances bone resorption. Antioxidants neutralise this oxidative stress, thereby preventing bone loss [8].

Green synthesis is the procedure of producing materials from bioactive substances such as plants, biowastes, and microbes. This process is gaining popularity nowadays as it does not generate harmful byproducts. It is widely used for synthesising metal and metal oxide nanoparticles. The advantages of green synthesis include its effectiveness in creating non-toxic products, cost-effectiveness, ease of procedure, compatibility with the environment, potential for large-scale production, and reduced time consumption [9].

There are two common processes for the synthesis of nanoparticles: the top-down approach and the bottom-up approach. Green synthesis falls under the category of the bottom-up approach, which involves building nanoparticles from simple molecules. The synthesis of nanoparticles is commonly performed using plant materials due to the presence of numerous beneficial components, such as phytochemicals, amino acids, and proteins. Plant-based green synthesis occurs by mixing the plant extract with precursors of metals and metal oxides [10]. Different parts of a plant can be used for the green synthesis of metal nanoparticles, including leaf extracts, fruit peel extracts, and seed extracts. Jha et al., reported that for the green production of metal and metal oxide nanoparticles, leaf extract is the best choice [11].

Numerous studies have been conducted in which various metal-based nanoparticles were synthesised. For example, zinc oxide nanoparticles were synthesised from *Mangifera indica* leaves [12], while gold and silver nanoparticles were synthesised from clove extract [13]. Platinum nanoparticles were synthesised from *Ocimum sanctum* [14], titanium dioxide from *Echinacea purpurea* [15], and calcium oxide nanoparticles from *Moringa oleifera* [16]. All these plants possess biological potentials. *M. indica* has several beneficial properties, including antioxidant, anti-diabetic, anti-degenerative activity, and wound healing [17]. *O. sanctum* exhibits antimicrobial, anti malarial, and antioxidant properties [18]. *M. oleifera* has bactericidal, fungicidal, antiviral, antioxidant, anti-hyperglycemic, anti-parasitic, anti-inflammatory properties, and wound healing abilities [19].

Hydroxyapatite nanoparticles are materials that aid in bone regeneration [20]. The chemical production of hydroxyapatite nanoparticles can be achieved through dry methods, wet methods, and high-temperature processing. However, Ganta et al., green-synthesised hydroxyapatite nanoparticles using *Monoon longifolium* leaf extract [21]. Green synthesising hydroxyapatite nanoparticles using citrus fruit extract could be useful in generating compounds with antioxidant potential and additive bone regenerative ability. Numerous researchers have developed and evaluated synthetic antioxidants, such as butylhydroxyanisole and butylhydroxytoluene [22-24].

Some studies indicate that high doses of synthetic phenolic antioxidants can have toxic effects on the reproductive system, as well as carcinogenic and teratogenic effects [25]. Therefore, synthesising antioxidants from natural sources may have fewer or no adverse effects and offer greater potential benefits. Hence, the aim of this study was to assess the antioxidant activity of green-synthesised hydroxyapatite nanoparticles using citrus fruit peels.

The primary objective was to generate the green-synthesised hydroxyapatite nanoparticles using citrus fruit peels, and the secondary objective was to evaluate the antioxidant properties of the generated nanoparticles.

MATERIALS AND METHODS

This in-vitro study was carried out at the Nanomedicine Biolab, Department of Pharmacology, Saveetha Dental College, Chennai, Tamil Nadu, India from March to June 2024. The study was conducted with the institute's ethical approval (SRB/SDC/PhD/PROSTHO-2354/23/TH-068). The study involved the preparation of the sample, its characterisation using UV-Vis spectroscopy

and SEM, and the evaluation of antioxidant properties using the Hydrogen Peroxide assay, Ferric Reducing Ability of Plasma (FRAP) assay, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay, and Nitric Oxide Radical Inhibition assay. The usage of citrus fruit peel extracts (*C. reticulata* and *C. limonum*) was the only prerequisite for inclusion, while all other components were excluded.

Study Procedure

Sample preparation: Hydroxyapatite nanoparticles were green-synthesised using extracts of *Citrus reticulata* and *Citrus limonum*. Peels from *C. reticulata* fruits were obtained and ground into a coarse powder after air drying. Two grams of this powder were dissolved in 100 mL of distilled water. This extract was then heated for about 20 minutes at 60-80°C in a heating mantle [26]. After heating, the extract was filtered using a muslin cloth. Fifty milliliters of this filtered extract were mixed with 50 mL of HAP solution, which was prepared by dissolving 200 mg of HAP powder in 50 mL of distilled water. The mixture was then kept still for 48 hours on a magnetic stirrer. Following this, the solution was centrifuged for 10 minutes at 8000 revolutions per minute, and the pellet was collected and preserved for later use. The same procedure was carried out for *Citrus limonum* as well [Table/Fig-1].



[Table/Fig-1]: Green-synthesised *C. reticulata* and *C. limonum* mediated HAP nanoparticles.

Assessment of antioxidant activity of green-synthesised HAP nanoparticles:

The antioxidant activity of green-synthesised HAP nanoparticles was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, Hydrogen Peroxide (H_2O_2) assay, Ferric Reducing Antioxidant Power (FRAP) assay, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, and Nitric Oxide Radical Inhibition assay.

DPPH assay: An unsaturated solution of DPPH at a concentration of 0.1 mM was prepared in methanol. This solution was diluted to a 20 μ M concentration in methanol for carrying out each assay. Two hundred microliters of DPPH working solution were mixed with 10 μ g/mL, 20 μ g/mL, 30 μ g/mL, 40 μ g/mL, and 50 μ g/mL concentrations of *C. reticulata* and *C. limonum* mediated HAP nanoparticles in a 96-well plate.

The DPPH test in 96-well plates is considered advantageous due to its high accuracy and precision, as well as its requirement for smaller reaction volumes compared to conventional test tubes. Ten wells each were designated for the test and control groups for each concentration from 10 μ g/mL to 50 μ g/mL. The solution was then allowed to incubate for half an hour at room temperature in the absence of light. The absorbance was measured at a wavelength of 517 nm using a microplate reader. Methanol (Himedia Carbinol Hi-LTR) was used as the blank since DPPH is soluble in this solvent [27].

The DPPH scavenging activity was determined according to [28]:

$$\% \text{ of Scavenging Activity} = \frac{A \text{ control} - A \text{ sample}}{A \text{ control}} \times 100$$

Where control absorbance (DPPH solution without sample) is A control, and sample absorbance (DPPH solution with green-synthesised HAP nanoparticles) is A sample.

A concentration of 1 mg/mL of ascorbic acid served as the positive control because it is a common antioxidant that scavenges ROS. A positive control is necessary for comparing the sample with a known standard having DPPH scavenging activity [27].

H₂O₂ assay: The antioxidant activity was also assessed using the hydroxyl radical scavenging assay. One milliliter (1 mL) of the reaction mixture was prepared with 100 µL of 28 mM 2-deoxy-2-ribose, followed by the addition of various concentrations (10 to 50 µg/mL) of *C. reticulata* and *C. limonum* mediated HAP nanoparticles. Additionally, 200 µL of EDTA, 200 µL of ferric chloride (200 µM), and 100 µL of ascorbic acid were added. The optical density at 532 nm was measured following a one-hour incubation at 37°C against the blank solution. The positive control used was vitamin C, a powerful antioxidant that neutralises oxidative stress through an electron donation process. This makes it a reliable reference for comparing the antioxidant activity of test samples [28].

$$\text{Hydroxyl radical Scavenging Activity \%} = \frac{A \text{ blank} - A \text{ sample}}{A \text{ blank}} \times 100$$

Where control absorbance (solution without sample) is A blank, and sample absorbance (solution with green-synthesised HAP nanoparticles) is A sample.

A blank is typically used to calibrate measurement instruments (like spectrophotometers), ensuring that measurements reflect only the relevant reactions or signals. Controls are essential for validating experimental conditions; they help determine if any observed effects are due to the experimental manipulation or some other factor.

FRAP assay: The reagents used for the assay were as follows:

- Acetate-based buffer (pH 3.6) at 300 mM: To produce this compound, 16 mL of glacial acetic acid was mixed with 3.1 g of sodium acetate trihydrate. Distilled water was then added to make the volume up to 1 L.
- 10 mM 2,4,6-tripyridyl-s-triazine in HCl (40 mM).
- FeCl₃•6H₂O: 20 mM

The working FRAP reagent was made by combining a, b, and c in a 10:1:1 ratio just before testing. FeSO₄•7H₂O (0.1-1.5 mM) in methanol was used as the standard. All reagents were procured from the German company Merck.

Procedure: To 0.7 mL of distilled water, 2.3 mL of FRAP solution was added, and then incubated for 5 minutes at 37°C. After that, this mixture was combined with a specific concentration of the citrus peel extract-mediated HAP nanoparticles (10 to 50 µg/mL) and incubated at 37°C for 30 minutes. At a wavelength of 593 nm, the absorbance of the reaction mixture was measured using a spectrophotometer. The standard used was ascorbic acid [26,28].

$$\% \text{ of reduction} = \frac{AB \text{ control} - AB \text{ sample}}{AB \text{ control}} \times 100$$

Where control absorbance is AB control, and sample absorbance is AB sample.

ABTS assay: The first step involves the preparation of ABTS radical cation (ABTS+). For this, potassium persulfate (2.45 mM) was mixed with distilled water to react with ABTS (50% ethanol) at 7.0 mM. This prepared solution was then refrigerated for a minimum duration of 24 hours. The above prepared solution was diluted with 50% ethanol until an absorbance of 1.0 (±0.02) was achieved at 734 nm. Then, 20 µL of *C. reticulata* and *C. limonum* mediated HAP nanoparticles (sample) at various concentrations and 250 µL

of ABTS+ were dissolved in ionized water and poured into a 96-well microplate. The standard used for the assay was ascorbic acid, and the blank used was ethanol (20 µL). The reading at 734 nm was recorded using a microplate reader following a 10-minute reaction in the dark [29].

The formula for calculating the radical scavenging activity (I) was:

$$\% \text{ of scavenging activity} = \frac{AB \text{ control} - AB \text{ sample}}{AB \text{ control}} \times 100$$

Where the absorbance of the blank (20 µL ethanol) is AB blank, and the absorbance of the sample (solution with green-synthesised HAP nanoparticles at different concentrations) is AB sample.

Nitric oxide radical inhibition assay: The Griess Illsvooy reaction was used to evaluate nitric oxide radical inhibition. However, instead of 1-naphthylamine (5%) from the Griess Illsvooy reagent, naphthyl ethylene diamine dihydrochloride (0.1% w/v) was used. Furthermore, the standardised protocol of the assay was carried out, and absorbance was measured at 540 nm [30].

$$\% \text{ of scavenging activity} = \frac{AB \text{ control} - AB \text{ sample}}{AB \text{ control}} \times 100$$

Where the control absorbance is AB control, and the sample absorbance is AB sample.

Characterisation using UV-Vis Spectroscopy and Scanning Electron Microscopy (SEM)

The green-synthesised HAP nanoparticles were subjected to characterisation using a UV-Vis spectrometer to assess the absorbance spectrum and SEM to evaluate the morphology and size of the nanoparticles. UV-Vis spectroscopy of the green-synthesised HAP nanoparticle samples was performed using a double beam spectrophotometer (Elico, India) within the spectral range of 250 to 800 nm. SEM analysis of the *C. reticulata* and *C. limonum* mediated HAP nanoparticles was conducted using a ZEISS microscope (Germany) based on standard protocol at different magnification levels [31].

STATISTICAL ANALYSIS

Comparison between the standard and specimen samples was carried out using an independent t-test. ANOVA analysis and post-hoc tests were performed to compare the different assays. A p-value less than 0.05 was considered statistically significant.

RESULTS

The assays used to assess the antioxidant potential of green-synthesised HAP nanoparticles included DPPH, FRAP, H₂O₂, ABTS, and nitric oxide radical inhibition.

Green-synthesised HAP nanoparticles were assessed for the percentage of scavenging activity using the DPPH assay. The percentage of scavenging activity for *C. reticulata* and *C. limonum* mediated HAP nanoparticles was consistent with the control used (standard) (p-value <0.001). The percentage of scavenging or inhibition represents how well an antioxidant prevents free radicals or oxidative damage. The maximum percentage of scavenging shown at a concentration of 50 µg/mL was 89.76% for *C. reticulata* mediated HAP nanoparticles and 91.38% for *C. limonum* mediated HAP nanoparticles [Table/Fig-2,3].

The percentage of scavenging activity of hydroxyl radicals at different concentrations from 10 µg/mL to 50 µg/mL aligned with the standard used. The greatest percentage of scavenging activity was exhibited as 85.6% for *C. reticulata* mediated HAP nanoparticles and 87.2% for *C. limonum* mediated HAP nanoparticles at a concentration of 50 µg/mL [Table/Fig-4,5].

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 µL	Standard	10	66.2500	0.010	<0.001*
	<i>C.reticulata</i>	10	62.2700	0.010	
20 µL	Standard	10	78.5200	0.010	<0.001*
	<i>C.reticulata</i>	10	74.2800	0.010	
30 µL	Standard	10	85.6300	0.010	<0.001*
	<i>C.reticulata</i>	10	82.5900	0.010	
40 µL	Standard	10	88.6800	0.010	<0.001*
	<i>C.reticulata</i>	10	85.8100	0.010	
50 µL	Standard	10	93.1500	0.010	<0.001*
	<i>C.reticulata</i>	10	89.7600	0.010	

[Table/Fig-2]: Comparison between antioxidant activity obtained in different concentrations of DPPH assay (*C.reticulata*).

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 µL	Standard	10	66.2500	0.010	<0.001*
	<i>C.limonum</i>	10	64.0500	0.010	
20 µL	Standard	10	78.5200	0.010	<0.001*
	<i>C.limonum</i>	10	76.3100	0.010	
30 µL	Standard	10	85.6300	0.010	<0.001*
	<i>C.limonum</i>	10	83.4600	0.010	
40 µL	Standard	10	88.6800	0.010	<0.001*
	<i>C.limonum</i>	10	86.2300	0.010	
50 µL	Standard	10	93.1500	0.010	<0.001*
	<i>C.limonum</i>	10	91.3800	0.010	

[Table/Fig-3]: Comparison between antioxidant activity obtained in different concentrations of DPPH assay (*C.limonum*).

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 µL	Standard	10	51.1000	0.126	<0.001*
	<i>C.reticulata</i>	10	47.3000	0.109	
20 µL	Standard	10	56.9000	0.109	<0.001*
	<i>C.reticulata</i>	10	54.1000	0.109	
30 µL	Standard	10	66.1000	0.109	<0.001*
	<i>C.reticulata</i>	10	63.5000	0.109	
40 µL	Standard	10	78.8000	0.109	<0.001*
	<i>C.reticulata</i>	10	75.7000	0.109	
50 µL	Standard	10	89.9000	0.109	<0.001*
	<i>C.reticulata</i>	10	85.6000	0.109	

[Table/Fig-4]: Comparison between antioxidant activity obtained in different concentrations of H₂O₂ assay (*C.reticulata*).

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 µL	Standard	10	51.1000	0.109	<0.001*
	<i>C.limonum</i>	10	48.9000	0.109	
20 µL	Standard	10	56.9000	0.109	<0.001*
	<i>C.limonum</i>	10	54.3000	0.109	
30 µL	Standard	10	66.1000	0.109	<0.001*
	<i>C.limonum</i>	10	65.1000	0.109	
40 µL	Standard	10	78.8000	0.109	<0.001*
	<i>C.limonum</i>	10	75.8000	0.109	
50 µL	Standard	10	89.9000	0.109	<0.001*
	<i>C.limonum</i>	10	87.2000	0.109	

[Table/Fig-5]: Comparison between antioxidant activity obtained in different concentrations of H₂O₂ assay (*C.limonum*).

In the analysis with the FRAP assay, the percentage of reduction exhibited a dose-dependent manner. A concentration of 50 µg/mL exhibited the greatest percentage of reduction for both *C. reticulata* and *C. limonum* mediated HAP nanoparticles (86.91% and 87.19%, respectively) [Table/Fig-6,7].

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 µL	Standard	10	72.9800	0.010	<0.001*
	<i>C.reticulata</i>	10	69.2300	0.010	
20 µL	Standard	10	76.8400	0.010	<0.001*
	<i>C.reticulata</i>	10	75.1500	0.010	
30 µL	Standard	10	81.3100	0.010	<0.001*
	<i>C.reticulata</i>	10	78.6200	0.010	
40 µL	Standard	10	85.8400	0.010	<0.001*
	<i>C.reticulata</i>	10	81.8400	0.010	
50 µL	Standard	10	90.8900	0.010	<0.001*
	<i>C.reticulata</i>	10	86.9100	0.010	

[Table/Fig-6]: Comparison between antioxidant activity obtained in different concentrations of FRAP assay (*C.reticulata*).

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 µL	Standard	10	72.9830	0.011	<0.001*
	<i>C.limonum</i>	10	67.2800	0.109	
20 µL	Standard	10	76.8400	0.109	<0.001*
	<i>C.limonum</i>	10	74.5900	0.109	
30 µL	Standard	10	81.3100	0.109	<0.001*
	<i>C.limonum</i>	10	77.2700	0.109	
40 µL	Standard	10	85.8400	0.109	<0.001*
	<i>C.limonum</i>	10	82.4300	0.109	
50 µL	Standard	10	90.8900	0.109	<0.001*
	<i>C.limonum</i>	10	87.1900	0.109	

[Table/Fig-7]: Comparison between antioxidant activity obtained in different concentrations of FRAP assay (*C.limonum*).

In the ABTS assay, the percentage of radical scavenging activity was consistent with that of the standard ascorbic acid. The greater percentage was exhibited by *C. limonum* mediated HAP nanoparticles (88.46%) at a concentration of 50 µg/mL compared to *C. reticulata* mediated HAP nanoparticles (88.28%) [Table/Fig-8,9]. The results of the nitric oxide radical inhibition assay were similar to those of the other assays performed. The scavenging activity percentage of both *C. reticulata* and *C. limonum* mediated HAP nanoparticles increased with higher concentrations. Furthermore, the results were proportionate to those of the standard. *C. reticulata* mediated HAP nanoparticles demonstrated greater scavenging activity (86.79%) than *C. limonum* mediated HAP nanoparticles (85.22%) [Table/Fig-10,11].

One-way ANOVA was used to compare the groups. The DPPH assay showed a statistically significant difference ($p < 0.001$). This was followed by post-hoc analysis using the Tukey HSD test, which also showed statistically significant differences among all groups ($p < 0.001$) [Table/Fig-12].

In the UV-Vis spectroscopy of *C. reticulata* mediated HAP nanoparticles, the absorbance spectrum exhibited a peak between

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 µL	Standard	10	70.5600	0.010	<0.001*
	<i>C.reticulata</i>	10	68.9400	0.010	
20 µL	Standard	10	75.6800	0.010	<0.001*
	<i>C.reticulata</i>	10	74.6100	0.010	
30 µL	Standard	10	82.4300	0.010	<0.001*
	<i>C.reticulata</i>	10	79.4500	0.010	
40 µL	Standard	10	86.5700	0.010	<0.001*
	<i>C.reticulata</i>	10	81.5900	0.010	
50 µL	Standard	10	91.3870	0.011	<0.001*
	<i>C.reticulata</i>	10	88.2800	0.010	

[Table/Fig-8]: Comparison between antioxidant activity obtained in different concentrations of ABTS assay (*C.reticulata*).

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 μ L	Standard	10	70.5600	0.109	<0.001*
	<i>C.limonum</i>	10	67.6100	0.109	
20 μ L	Standard	10	75.6800	0.109	<0.001*
	<i>C.limonum</i>	10	71.2400	0.109	
30 μ L	Standard	10	82.4300	0.109	<0.001*
	<i>C.limonum</i>	10	78.3500	0.109	
40 μ L	Standard	10	86.5700	0.109	<0.001*
	<i>C.limonum</i>	10	82.8200	0.109	
50 μ L	Standard	10	91.3900	0.109	<0.001*
	<i>C.limonum</i>	10	88.4600	0.109	

[Table/Fig-9]: Comparison between antioxidant activity obtained in different concentrations of ABTS assay (*C.limonum*).

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 μ L	Standard	10	72.4300	0.010	<0.001*
	<i>C.reticulata</i>	10	69.8600	0.010	
20 μ L	Standard	10	77.9400	0.010	<0.001*
	<i>C.reticulata</i>	10	75.1100	0.010	
30 μ L	Standard	10	80.3700	0.010	<0.001*
	<i>C.reticulata</i>	10	79.4700	0.010	
40 μ L	Standard	10	84.2800	0.010	<0.001*
	<i>C.reticulata</i>	10	82.3400	0.010	
50 μ L	Standard	10	88.6700	0.010	<0.001*
	<i>C.reticulata</i>	10	86.7900	0.010	

[Table/Fig-10]: Comparison between antioxidant activity obtained in different concentrations of Nitric Oxide Assay (*C.reticulata*).

Concentration	% of inhibition	N	Mean	Std. Deviation	p-value
10 μ L	Standard	10	72.4300	0.109	<0.001*
	<i>C.limonum</i>	10	70.8900	0.109	
20 μ L	Standard	10	77.9400	0.109	<0.001*
	<i>C.limonum</i>	10	74.9200	0.109	
30 μ L	Standard	10	80.3700	0.109	<0.001*
	<i>C.limonum</i>	10	78.0700	0.109	
40 μ L	Standard	10	84.2800	0.109	<0.001*
	<i>C.limonum</i>	10	81.4300	0.109	
50 μ L	Standard	10	88.6700	0.109	<0.001*
	<i>C.limonum</i>	10	85.2200	0.109	

[Table/Fig-11]: Comparison between antioxidant activity obtained in different concentrations of Nitric Oxide Assay (*C.limonum*).

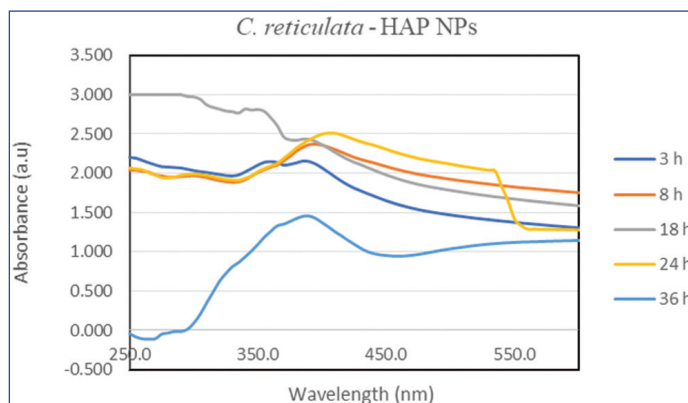
340 to 360 nm, which confirmed nanoparticle synthesis. There were no other elevations in the spectrum. The maximum absorbance

ANOVA (<i>C.reticulata</i>)						
		Sum of Squares	df	Mean Square	F	Sig.
DPPH	Between groups	4771.963	4	1192.991	8947430.250	<0.001*
	Within groups	0.006	45	0.000		
	Total	4771.969	49			
H ₂ O ₂	Between groups	9729.120	4	2432.280	182421.000	<0.001*
	Within groups	0.600	45	0.013		
	Total	9729.720	49			
FRAP	Between groups	1789.410	4	447.352	3355143.750	<0.001*
	Within groups	0.006	45	0.000		
	Total	1789.416	49			
ABTS	Between groups	2125.973	4	531.493	3986199.750	<0.001*
	Within groups	0.006	45	0.000		
	Total	2125.979	49			

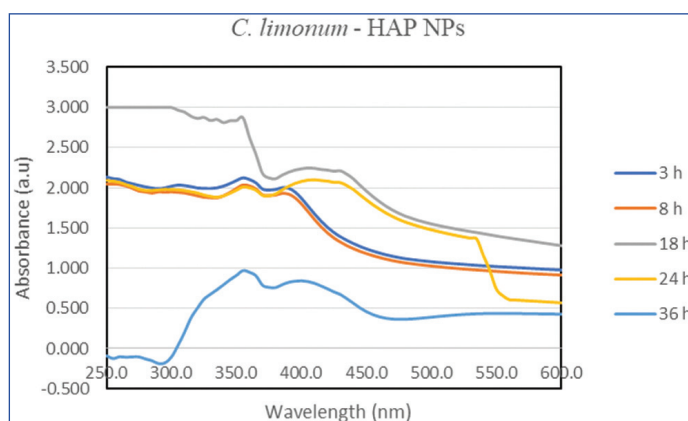
NO	Between groups	1703.233	4	425.808	3193562.250	<0.001*
	Within groups	0.006	45	0.000		
	Total	1703.239	49			
(C.limonum)						
		Sum of squares	df	Mean Square	F	Sig.
DPPH	Between groups	4478.985	4	1119.746	8398097.250	<0.001*
	Within groups	0.006	45	0.000		
	Total	4478.991	49			
H ₂ O ₂	Between groups	9752.520	4	2438.130	182859.750	<0.001*
	Within groups	0.600	45	0.013		
	Total	9753.120	49			
FRAP	Between groups	2308.529	4	577.132	4328491.500	<0.001*
	Within groups	0.006	45	0.000		
	Total	2308.535	49			
ABTS	Between groups	2859.541	4	714.885	5361639.750	<0.001*
	Within groups	0.006	45	0.000		
	Total	2859.547	49			
NO	Between groups	1238.805	4	309.701	2322759.750	<0.001*
	Within groups	0.006	45	0.000		
	Total	1238.811	49			

[Table/Fig-12]: Comparison between different concentrations of DPPH, H₂O₂, FRAP, ABTS, NO of *C. reticulata* and *C. limonum*.

spectrum was observed at 18 hours. After 24 hours, the absorbance rate reduced, possibly due to the agglomeration of the nanoparticles. The absorbance peak was 350 nm for *C. limonum* mediated HAP nanoparticles. Additionally, there were no spectral elevations. The maximum absorbance spectrum was also seen at 18 hours, and after 24 hours, the absorbance rate reduced similarly to that of *C. reticulata* mediated HAP nanoparticles [Table/Fig-13,14].



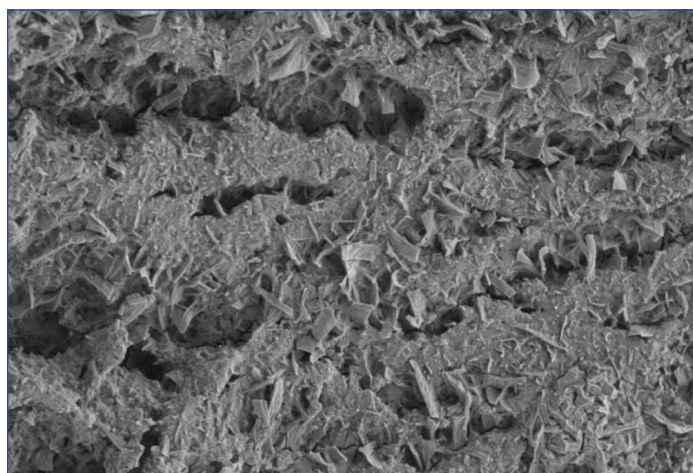
[Table/Fig-13]: UV-Vis spectroscopy of *C.reticulata* mediated HAP nanoparticles.



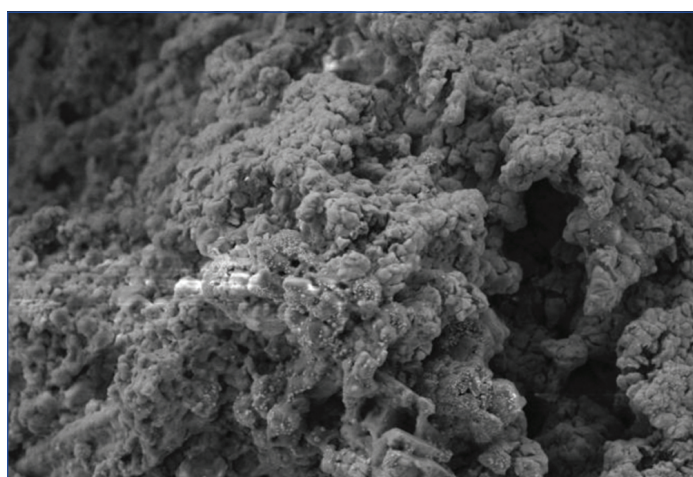
[Table/Fig-14]: UV- Vis spectroscopy of *C.limonum* mediated HAP nanoparticles.

SEM analysis of the *C. reticulata* and *C. limonum* extract mediated HAP nanoparticle samples revealed strong and denser HAP

nanoparticles. For the *C. reticulata* mediated HAP sample, the nanoparticles were rectangular-square and rod-shaped, with sizes ranging from 50 to 150 nm. They exhibited an agglomerated, condensed, and rough morphology. In contrast, for the *C. limonum* extract mediated sample, the nanoparticles were found as spherical agglomerates due to the presence of bioactive compounds. The nanoparticle fibers exhibited a rough distribution with particle sizes ranging from 30 to 90 nm [Table/Fig-15,16].



[Table/Fig-15]: SEM images of *C. reticulata* mediated HAP nanoparticles.



[Table/Fig-16]: SEM images of *C. limonum* mediated HAP nanoparticles.

DISCUSSION

Green synthesis of various compounds has been conducted by numerous researchers due to the potential benefits of environmental compatibility, low cost, scalability, etc. These compounds exhibit a range of beneficial biological properties, such as anti-inflammatory, antioxidant, anti-neoplastic, and antimicrobial effects [32-35]. Synthesising nanoparticles by utilising green technology is more effective because of the synergistic beneficial properties derived from both the nanoparticles and green technology.

This research paper aimed to green synthesise hydroxyapatite nanoparticles using citrus fruit peel extracts (*C. reticulata* and *C. limonum*). Citrus fruits contain polyphenols like flavonoids, which contribute to antioxidant and antimicrobial activities. The antioxidant activity of citrus fruits is often achieved through the reduction or scavenging of ROS and mediators of inflammation [36]. An abundance of literature is available regarding the antioxidant potentials of green-synthesised nanoparticles. For instance, Salari et al., synthesised silver nanoparticles using *Prosopis farcta* and evaluated their antioxidant properties [37].

The green-synthesised *C. reticulata* and *C. limonum* mediated nanoparticles were evaluated for their antioxidant properties using

DPPH, H_2O_2 , FRAP, ABTS, and nitric oxide radical inhibition assays. Except for the FRAP assay, all assays assessed the scavenging activity of the nanoparticles, while the FRAP assay evaluated the reduction potential. The results of these assays suggest the potential antioxidant activity of the green-synthesised nanoparticles (significant p -value < 0.001). This aligns with the test results of Salari et al., [37]. Additionally, dose-dependent antioxidant activity was observed, with the greatest results at a concentration of 50 μ g/mL of the citrus fruit peel-mediated hydroxyapatite nanoparticles. Palierse et al., evaluated the antioxidant properties of hydroxyapatite nanoparticles synthesised using Baicalein and reported that the generated hydroxyapatite nanoparticles exhibited potential antioxidant properties [38].

The normal absorption spectrum of unmodified HAP nanoparticles typically ranges from 200 to 340 nm [39,40]. However, in this study, the green-synthesised *C. reticulata* and *C. limonum* mediated nanoparticles displayed an absorbance range of 340 to 360 nm. This shift is due to reduced band gap energy, allowing the material to absorb light with lower energy. The modification of HAP nanoparticles creates new energy levels within the band gaps, facilitating charge transfer transitions that absorb visible light [41,42]. The increased absorption of the citrus-fruit-modified HAP could be advantageous for bio-imaging and photothermal therapy, owing to its enhanced contrast and therapeutic efficacy. Subramanian et al., reported a similar absorbance peak in the range of 310 to 340 nm, consistent with the results of the current study [43].

The SEM analysis provides information regarding the morphological patterns and surface characteristics. For the *C. reticulata*-mediated HAP nanoparticles, the nanoparticles exhibited a condensed and rough morphology with a pronounced porous nature. This indicates that the extract influenced the formation of tightly packed particles with rough surfaces, potentially enhancing the surface area and porosity, which can be beneficial for applications requiring high reactivity or surface interaction [44]. In contrast, the *C. limonum*-mediated HAP nanoparticles showed nanoparticles forming spherical agglomerates with a porous distribution. The fibers of these nanoparticles exhibited a rough distribution, suggesting that this extract promotes the formation of loosely aggregated, spherical particles. These results are consistent with those of Ali et al., who conducted SEM analysis for green-synthesised hydroxyapatite nanorods using licorice root extract [45]. Devi et al., also observed agglomerated nanoparticles in their SEM analysis of HAP green synthesis using *Carica papaya* leaf and *Acalypha indica* leaf extracts [46]. Agglomerated nanoparticles sized 50 to 80 nm were observed in the SEM analysis during the green synthesis of HAP nanoparticles from *Moringa oleifera* flower extract [47]. The agglomeration of the nanoparticles in the SEM analysis suggests the presence of bioactive compounds.

The presence of these bioactive compounds could be potentially favorable for therapeutic purposes, such as the treatment of infections, cancer, cardiovascular disease, neurodegenerative disorders, and bowel disorders. The antioxidant activity of citrus flavonoids, combined with the osteogenic potential of hydroxyapatite nanoparticles, could be beneficial in bone tissue engineering.

Limitation(s)

The limitations of the current study include the evaluation of only the antioxidant properties, while other properties such as antimicrobial activity could also be assessed. Additionally, the study pertains only to two citrus fruits.

CONCLUSION(S)

The green synthesis of hydroxyapatite nanoparticles mediated by *C. reticulata* and *C. limonum* was successfully completed, and the resulting nanoparticles exhibited potential antioxidant activity. The increased absorption spectrum of citrus fruit peel-mediated

HAP nanoparticles in UV spectroscopy significantly broadens the applicability of these modified HAP nanoparticles in various technological and biomedical fields. The varied morphological characteristics observed in the SEM analysis, brought about by the different citrus extracts, emphasise how organic mediators affect the structural attributes of HAP nanoparticles. The porous and rough characteristics of both samples point to the potential for improved performance in applications where surface area and porosity are critical, such as drug delivery, bone regeneration, and catalysis. This results highlight the importance of selecting suitable natural extracts to modify the shape and performance of HAP nanoparticles for specific applications.

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