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Original Article

Free Radical Scavenging Activity Assessment of Nanocomposite Prepared Using Citrus Fruit Peel (*Citrus reticulata* and *Citrus limonum*) Mediated Hydroxy Apatite

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ABSTRACT

Background: The growing demand for sustainable, biocompatible and cost-effective nanomaterials has led to an increase in exploration in the field of green synthesis. Hydroxy apatite nanocomposites are widely recognized for their biomedical applications; however, improving their functional properties using natural sources is an area of active research owing to its advantages. Citrus fruits and peels, with its abundant reserve of phytochemical, offer an alternative method for the synthesis and functionalization of nanocomposites**. Objectives:** This study aimed to green synthesize hydroxyapatite nanocomposites using citrus peel extracts of *reticulata* and *limonum* and evaluate their antioxidant potential. **Materials and Methods:** Plant-based generation of hydroxy apatite-based nanocomposites was carried out using *C. reticulata* and *C. limonum* peel extracts. For the assessment of antioxidant potential of the generated nanocomposite, the following *in vitro* assays; DPPH, H_2O_2, FRAP, ABTS and NO at varying concentrations were done. **Results:** The results demonstrated effective production of citrus fruit peel mediated hydroxy apatite nanocomposites, with the resultant compound exhibiting scavenging activity comparable to the standards. **Conclusion:** Citrus fruit peel mediated hydroxy apatite nanocomposites exhibited promising antioxidant potential and highlighted the effectiveness of the green synthesis approach.

Keywords: Antioxidant, Biocompatibility, Free radical scavenging, Oxidative stress, Sustainable development.

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INTRODUCTION

According to the discipline of chemistry, free radicals are highly reactive ions, atoms or molecules that contain unpaired electrons in their outermost shell (McNaught *et al*., 2025). Free radical generation is a naturally occurring process during metabolic process like in cellular respiration, ATP production using oxygen, immunological processes etc. Two types of free radicals are produced during these activities Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). Reactive oxygen species are promising green oxidants present in the form of Hydroxyl Radicals (OH) Superoxide Anions ($O2^-$) and Hydrogen Peroxide (H_2O_2). Reactive nitrogen species include Nitric Oxide (NO) and Peroxynitrile (ONOO-) (Hayyan *et al*., 2016). Both these free radicals have potential benefits and harmful effects. They potentially serve as anti-tumorigenic species owing to their ability to trigger cellular senescence and apoptosis. Small amounts of free radicals can help in growth and maturation of the cell structures, detoxification and wound healing (Pham-Huy *et al*., 2008).

Harmful effects include oxidative stress generation leading to cellular destruction, DNA damage thereby resulting in carcinomas, lipid peroxidation, etc. (Valko *et al*., 2006). Apart from the generation of free radicals from the human body, it also produced from environmental pollutants, cigarettes, radiation, ozone, some drugs, etc. (Ebadi, 2001). The oxidative stress from the free radicals is responsible for various pathologies in body such as cancers, inflammatory disorders [arthritis, glomerulonephritis, etc.]; Atherosclerosis leading to cardiovascular accidents, ischemic heart diseases, hypertension, stroke, preeclampsia; neurological disorders like Parkinson's, Alzheimer's disease, multiple sclerosis, depression; respiratory disorders like asthma, chronic obstructive pulmonary disorder and affects much more organs. Oxidative damage to proteins results in changes to the signal transduction circuits, vulnerability to proteolysis resulting

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in rapid aging process. Oxidative stress is also reported to cause damage to DNA (Lobo *et al*., 2010; Pizzino *et al*., 2017).

Antioxidants are compounds which limit or inhibit the free radical's formation by supressing the oxidation reactions. Biologically there are enzyme based and non-enzyme-based antioxidants. Enzyme based antioxidants include glutathione reductase and peroxidase, catalase and superoxide dismutase. Non-enzyme-based antioxidants are further classified into metabolic (endogenous antioxidants generated from the body itself which include melatonin, uric acid and bilirubin) and nutrient antioxidants (exogenous ones which has to be supplemented like Vitamin C, E, Omega-[3,6] fatty acids, etc.). (Pham-Huy *et al*., 2008; Willcox *et al*., 2004). Currently there are synthetic antioxidants available like propyl gallate that are used in pharma and food processing industries.

Antioxidants are further classified by mechanism as primary and secondary. The primary ones include Vitamin C, E, phenolic compounds that act by scavenging ROS or RNS species by Hydrogen atom (H•) or donating electron which in turn inhibits the free radical chain reaction. The secondary group hinder the production of free radicals similar to metal chelators and act as preventive antioxidants. Few other mechanisms of antioxidant activity are enzymatic antioxidant defence system where body provides enzymes that convert ROS into harmless molecules, suppression of lipid peroxidation and thereby cell membrane protection (Losada-Barreiro *et al*., 2022).

Under healthy circumstances ROS and RNS are tightly monitored and regulated by the antioxidant system of the human body. However, when ROS and RNS levels exceeds the body's antioxidant capacity it results in oxidative stress leading to significant cellular component damage leading to wide range of diseases. Due to concern of these detrimental effects, there has been a surge in research focusing on the discovery and development of potential antioxidants. Various methods are experimented for antioxidant generation like its extraction from natural sources, chemical synthesis and green synthesis. The chemical synthesis involves the direct production of synthetic antioxidants like Butylated Hydroxyanisole (BHA) Butylated Hydroxytoluene (BHT), or synergistic chemical- natural antioxidant formulations, nano-formulations, etc. (Gulcin, 2025).

Compounds of plant origin have recognized and proven to have antioxidant properties which highlights the emphasis of green synthesis (Flieger *et al*., 2021). The green synthesis of antioxidants involves the process of designing and generation of antioxidants using sustainable and eco-friendly synthetic pathways through plant extracts, microbes etc. Zielińska *et al.* reported the antioxidant nature of apple peel and flesh owing to presence of phenolic compounds within them. The antioxidant assay revealed the presence of catechin, epicatechin, phloretin and various other antioxidant compounds (Zielinska *et al*., 2020). Medlej MK *et al.* demonstrated antioxidant potential of a wild species of *Ornithogalum billardieri* plant (Medlej *et al*., 2021). A hydroalcoholic extract prepared from *Aegopodium podagraria* L. plant exhibited significant antioxidant activity (Flieger J, Flieger M., 2020). Uma *et al* confirmed the antioxidant potential of endophytic fungus *Achaetomium* sp. extracted from *Euphorbia hirta* owing to the high levels of phenolics like flavonoids and tannins (Uma *et al*., 2017).

Among these the green nano-formulations have gained popularity in recent years owing to the biological potentials and unique properties of nanoparticles. There is an abundance of literature available in this context. Potential antioxidant activity was exhibited by gold nanoparticles green synthesised using avocado seed aqueous extract, silver nanoparticles using *Ficus benghalensis, Camellia sinensis, Ilex paraguariensis, Salvia officinalis, Viscum album, Porphyra yezoensis, Tilia cordata, Urtica dioica, Aegopodium podagraria, Capsicum baccatum, Levisticum officinale*, iron oxide nanoparticles using *Phoenix dactylifera* L. (Ngungeni *et al*., 2023; Singh *et al*., 2021; Flieger *et al*., 2021; Abdullah *et al*., 2022). Nanocomposites, which combine nanoparticles with organic or inorganic materials, also exhibit excellent properties such as increased surface area, targeted delivery etc. and have been researched and found to have an antioxidant effect (Noohpisheh *et al*., 2020).

One such promising material is hydroxy apatite possessing inherent antioxidant qualities in addition to biocompatibility, osteoconductivity and similarity to bone. Studies have focused on synthesizing hydroxy apatite nanocomposites using natural sources, with the aim of exploring their antioxidant activity (Subramanian *et al*., 2025). One such material is citrus fruits, that are abundant with biologically active compounds like flavonoids, phenolic acids. Different components of these citrus fruits are employed for the sustainable and ecofriendly development of compounds with scavenging potential (Zhang *et al*., 2015).

In addition to the antioxidant benefits, citrus fruits also act as natural reducing agents and stabilization agents which could help in the enhancement of the structural and functional properties of the hydroxy apatite nanocomposites. Hence, this article aimed to evaluate the free radical scavenging activity of a nanocomposite prepared using hydroxy apatite green synthesized through citrus fruit peel extract. The outcome of this study was intended to contribute to the expanding body of knowledge on the development of novel antioxidant materials and offer insight into sustainable use of green resources in the creation of functional nanocomposites.

MATERIALS AND METHODS

Sample preparation

C. reticulata and *limonum* peel extract-based production of hydroxy apatite nanoparticles was carried out according to

standard protocol and it was used as the test solution (Ajithan *et al.*, 2025). The resultant hydroxy apatite nanoparticles were used for nanocomposite preparation using chitosan solution (Ajithan *et al.*, 2025). The Nanocomposite (HAP NCs) was subjected to DPPH radical scavenging assay, Hydrogen peroxide radical scavenging assay, ABTS, FRAP and Nitric oxide radical inhibition assay.

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

The procedure of the assay was carried out following standard protocol. The absorbance value was recorded at 517 nm using a spectrophotometer, with methanol serving as the blank (Ajithan *et al.*, 2025). Percentage of DPPH radical scavenging was calculated.

$$\%\ of\ DPPH\ Radical\ Scavenging\ Activity = \frac{Acontrol\ -\ Asample}{A\ control} \times 100$$

where absorbance values of the test solution (DPPH solution with the green synthesised hydroxy apatite nanocomposite) are designated as Asample, absorbance values of the control (DPPH solution without green synthesised hydroxy apatite nanocomposite) is designated as Acontrol. Ascorbic acid was chosen as the reference standard for the study.

H_2O_2 assay

In this assay, Vitamin C was employed as the reference standard. For the comparative antioxidant efficacy, Vitamin C and *Citrus reticulata* peel mediated hydroxy apatite nanocomposite were prepared at a dose range of 10 to 50 µg/mL and added to 0.6 mL of 40 mM H_2O_2 phosphate buffer solution. In a dark environment, the reaction was allowed to proceed for about 10 min. Post-incubation, the spectrophotometric absorbance at 230 nm was used to quantify H_2O_2 depletion. Scavenging activity was calculated comparing the absorbance values of the treated samples against the untreated control using the given formula.

$$\%\ of\ Hydroxyl\ radical\ Scavenging\ Activity\ = \frac{Acontrol\ -\ Asample}{Acontrol} \times 100$$

Where Asample is absorbance values of test solution and Acontrol is absorbance values of untreated solution.

ABTS assay

To assess the radical scavenging activity, the ABTS reagent was prepared according to the standard protocol (Ajithan *et al.*, 2025). Before the commencement of analysis, the prepared solution was diluted with 50% ethanol to an absorbance value of (1.0±0.02) at 734 nm. In 96-well microplates, 20 µL of the test sample (*Citrus reticulata* peel mediated hydroxy apatite nanocomposite dissolved in distilled water at 10-50 µg/mL) was treated with 250 µL of prepared ABTS solution. The reaction was allowed to proceed for 10 min in the dark environment. Followed by the measurement of absorption at 734 nm using a microplate reader. The reference standard used was ascorbic acid and ethanol served as blank. The scavenging activity percentage was calculated by comparing the sample absorbance values to the blank.

FRAP (Ferric Reducing Antioxidant Power) assay

FRAP assay was carried out by using reagents from Merck (Germany). The working solution was freshly prepared by the standard procedure. For carrying out the assay, 0.7 mL of *C. reticulata* mediated hydroxy apatite nanocomposite solution at doses ranging from 10-50 µg/mL was reacted with 2.3 mL of the FRAP reagent. After 30-min incubation at 37°C in the dark environment, the absorbance values at 593 nm were recorded using a spectrophotometer. The conversion of the ferric-tripyridyltriazine complex to ferrous form was monitored by measuring the increase in absorbance values. Increased absorbance values directly correlated to higher antioxidant reduction potential. Ascorbic acid served as the reference standard and all analyses conducted in triplicate to ensure reproducibility.

Nitric oxide radical inhibition assay

The nitric oxide scavenging potential was evaluated using a modified Griess-Illosvoy reaction. In this study, the Griess-Illosvoy reaction's 1-naphthylamine (5%) was replaced with 0.1% (w/v) naphthylethylene diamine dihydrochloride to enhance the reagents efficacy. Standard procedure was carried out and absorbance was measured spectrophotometrically at 540 nm (Ajithan *et al.*, 2025). The percentage of inhibition was calculated as follows:

$$\%\ of\ Scavenging\ Activity\ = \frac{Abcontrol\ -\ Ab\ testsample}{Abcontrol} \times 100$$

where Ab test sample is absorbance values of test group, Abcontrol is absorbance values of control.

The same procedure of all assays was repeated using *C. limonum* mediated hydroxy apatite nanocomposites as well.

RESULTS

The radical scavenging potential of *C. reticulata* and *limonum* peel mediated hydroxy apatite nanocomposite was quantified using five distinct assays including, DPPH, H_2O_2, ABTS, FRAP and NO. Measurements of all parameters were conducted in triplicate to ensure reproducibility. Across all parameters, the hydroxy apatite nanocomposites of *C. reticulata* and *limonum* peel exhibited a robust dose-dependent inhibitory profile.

When free radical scavenging activity assessed using DPPH assay, marked increase in scavenging activity was observed between 10 and 30 µg/mL, after which the rate of increase became gradual in both *C. reticulata* and *limonum* peel mediated hydroxy apatite nanocomposite. However, the highest free radical scavenging potential was observed at 50 µg/mL; which was 90.12 µg/mL for *C. reticulata* and 89.44 µg/mL for *C. limonum* peel mediated hydroxy apatite nanocomposite. At all tested concentrations,

the scavenging activity of hydroxy apatite nanocomposites was slightly diminished than that of the reference standard; however, the values remained closely comparable showing the antioxidant potential of the test group (Figures 1 and 2).

Results demonstrate that the standard, *C. reticulata* and *limonum* mediated hydroxy apatite nanocomposites exhibit significant scavenging activity of hydroxyl radical with increased concentration. From 10 - 50 µg/mL concentration, standard showed slightly higher scavenging activity compared to the test groups. However, the *C. reticulata* and *limonum* mediated hydroxy apatite nanocomposites demonstrated comparable antioxidant potential, achieving a maximum scavenging activity of 86.5% for *C. reticulata* and 86.8% for *C. limonum* mediated hydroxy apatite nanocomposites at 50 µg/mL concentration closely, approaching the standard (89.9%) (Figures 3 and 4).

FRAP assay analysis indicated a clear concentration dependant increase in reducing power for standard, *C. reticulata* and *limonum* mediated hydroxy apatite nanocomposites. At the highest dose of 50 µg/mL, *C. reticulata* mediated hydroxy apatite nanocomposite displayed a strong ferric reducing capability reaching 87.38% and *C. limonum* mediated hydroxy apatite nanocomposite 87.96% (Figures 5 and 6).

Effective ABTS scavenging potential was exhibited by *C. reticulata* and *limonum* mediated hydroxy apatite nanocomposites. The increase in antioxidant activity was dose dependent. At 10 µg/mL concentration the standard exhibited 70.56% inhibition whereas, 67.43% and 68.34% inhibition was exhibited by *C. reticulata* and *limonum* mediated HAP nanocomposites. When the dose reached 50 µg/mL, the inhibition percentage increased to 89.03% and 89.87% which approached that of the reference standard (Figures 7 and 8).

A progressive dose-dependent increase in nitric oxide scavenging was observed similar to other assays. Maximum scavenging activity was observed, 87.84% for *C. reticulata* mediated hydroxy apatite nanocomposite and 84.78% for *C. limonum* mediated HAP nanocomposite at the dose of 50 µg/mL. *C. reticulata* mediated hydroxy apatite nanocomposite showed only a minute difference in antioxidant potential with the reference standard (88.67%) (Figures 9 and 10).

DISCUSSION

This study comprehensively evaluated the antioxidant potential of *C. reticulata* and *limonum* based hydroxy apatite nanocomposites using multiple *in vitro* analysis including DPPH, H_2O_2, FRAP, ABTS and NO. The use of multiple analysis enabled a broader understanding of the underlying mechanism of free radical scavenging and reducing ability of the green synthesized nanocomposites.

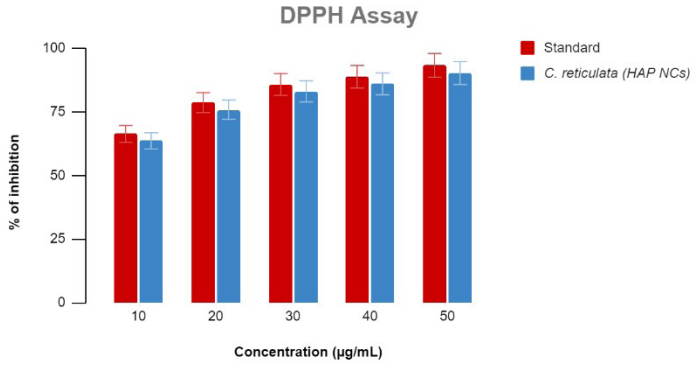


Figure 1: DPPH assay of *C. reticulata* mediated hydroxy apatite nanocomposites

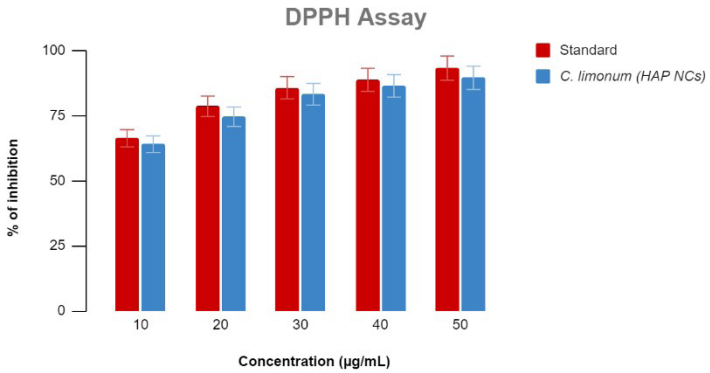


Figure 2: DPPH assay of *C. limonum* mediated hydroxy apatite nanocomposites.

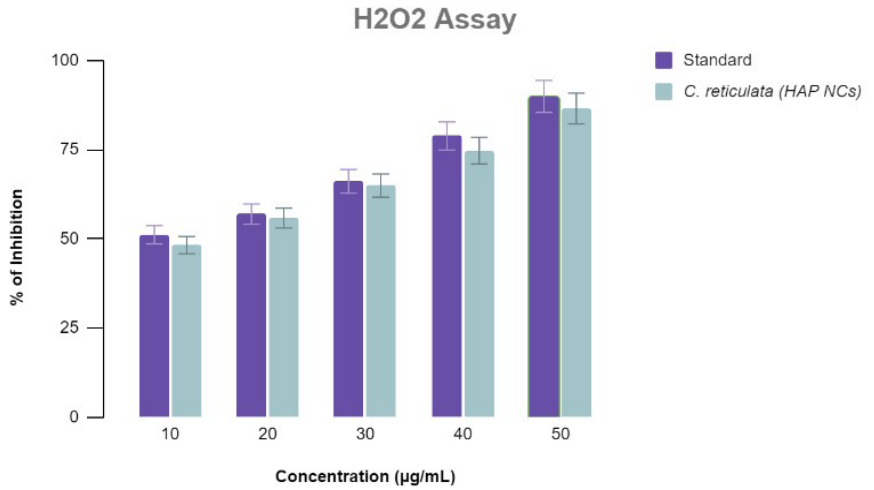


Figure 3: H_2O_2 assay of *C. reticulata* mediated hydroxy apatite nanocomposites.

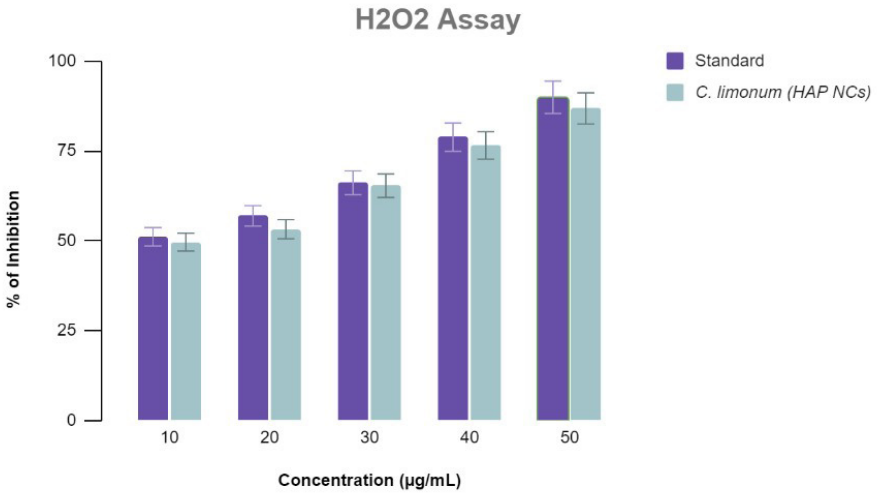


Figure 4: H_2O_2 assay of *C. limonum* mediated hydroxy apatite nanocomposites.

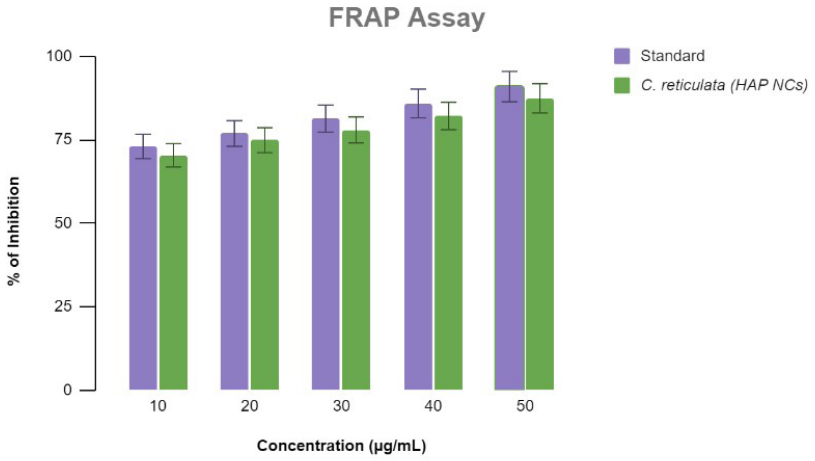


Figure 5: FRAP assay of *C. reticulata* mediated hydroxy apatite nanocomposites.

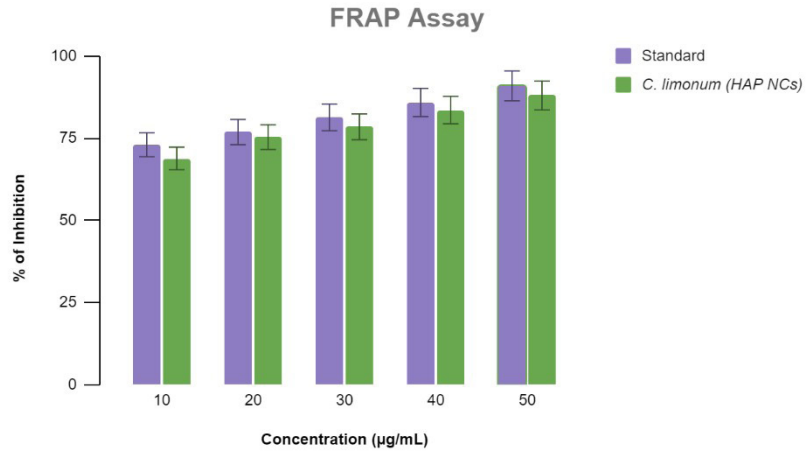


Figure 6: FRAP assay of *C. limonum* mediated hydroxy apatite nanocomposites.

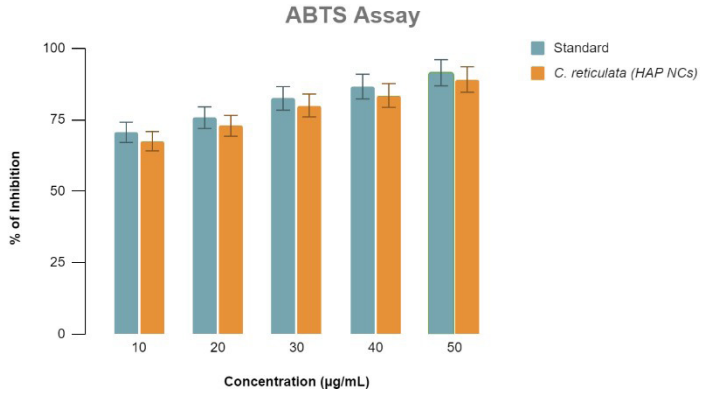


Figure 7: ABTS assay of *C. reticulata* mediated hydroxy apatite nanocomposites.

The green synthesised nanocomposite's ability to contribute hydrogen atoms or electrons to neutralize stable free radicals was demonstrated by DPPH assay, indicating effective radical quenching capability. The H_2O_2 analysis indicated the nanocomposite's capability to decompose H_2O_2 and thereby limiting the formation of highly reactive hydroxyl radicals and in turn the reduction of oxidative damage. The strong reducing power of the generated nanocomposites was highlighted by the FRAP assay owing to its electron donating capacity and its potential to convert oxidized intermediates into stable forms. Whereas, the ABTS assay validated the nanocomposite's broad- spectrum antioxidant activity through electron transfer mechanisms. The NO assay revealed the nanocomposite's potential to inhibit reactive nitrogen species. The overall antioxidant performance of the generated nanocomposites can be attributed to a combination of mechanisms, including hydrogen atom transfer, electron transfer and metal ion reduction.

The results of the assays demonstrated the ability of *C. reticulata* and *C. limonum* based hydroxy apatite nanocomposite's ability for radical scavenging and there by the antioxidant potential in a dose-dependent manner. In DPPH assay, at lower doses (ranging from 10 to 30 µg/mL), the scavenging activity of the generated green nanocomposites showed a marked increase followed by a gradual increase at higher concentrations. This pattern suggests that at lower concentrations, the greater number of active sites on the nanocomposite surface are readily available for interaction with DPPH radicals, resulting in rapid neutralization. As the concentration increases, the system may approach saturation, which leads to a slower rate of increase in scavenging efficiency. Al Baloushi *et al* in their study observed similar results (Al Baloushi *et al.*, 2024). Govindappa *et al.* also observed similar patterns of antioxidant potential of green generated nanoparticles (Govindappa *et al.*, 2018). However, there was a dose-dependent increase in scavenging activity when concentration increased from lower to higher. All the remaining assays also exhibited similar trend.

The antioxidant activity of the green synthesized nanocomposites is fundamentally rooted in the synergistic integration of the inorganic mineral phase with the organic bioactive component. Citrus peels are reservoirs of phenolic acids and flavonoids such as hesperidin, naringin, and other flavones which in turn provide these materials the redox-active properties (Singh *et al.*, 2020;

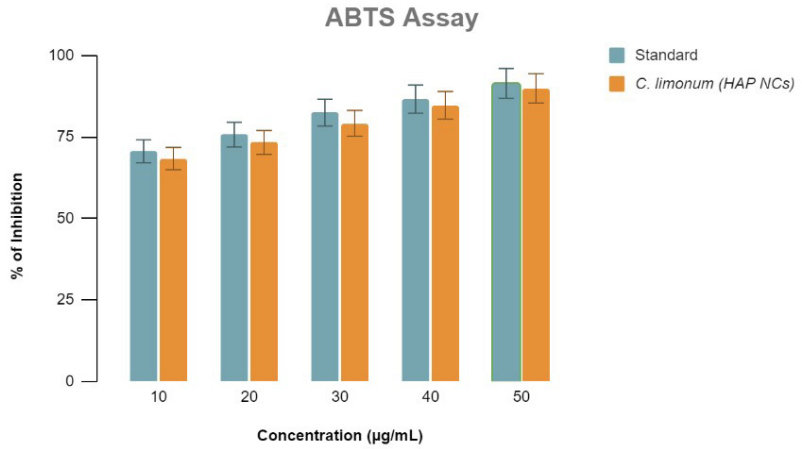


Figure 8: ABTS assay of *C. limonum* mediated hydroxy apatite nanocomposites.

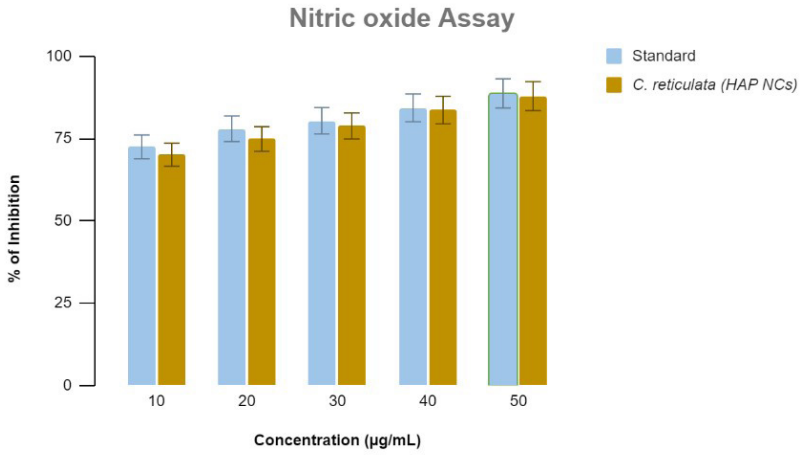


Figure 9: Nitric oxide assay of *C. reticulata* mediated hydroxy apatite nanocomposites.

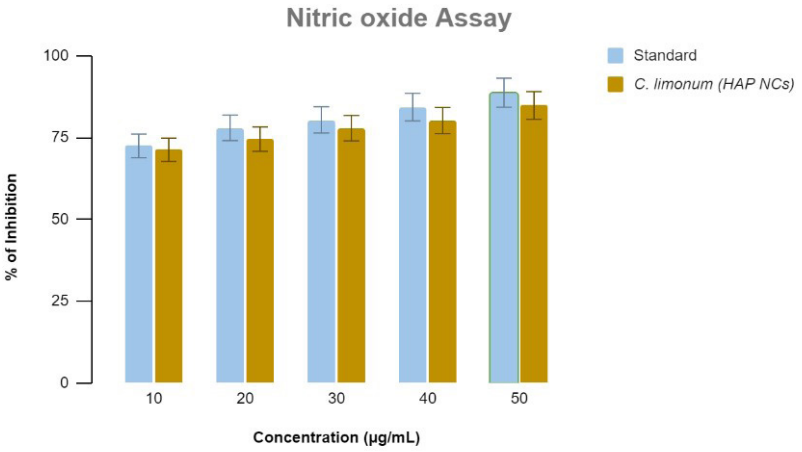


Figure 10: Nitric oxide assay of *C. limonum* mediated hydroxy apatite nanocomposites.

Zou *et al.*, 2016). They not only acts as reducing and stabilizing agents during nanoparticle synthesis but also remain attached to nanoparticle surface and thereby providing active sites for the radical scavenging.

Furthermore, the nanoscale particle size and hydroxy apatite's high surface area to volume ratio promotes the increased interaction between the reaction species and active sites. The adsorption and neutralization of free radicals are enhanced by the porous nature of hydroxy apatite (Fox *et al.*, 2012). Hence the synergistic interaction between the inorganic hydroxy apatite and organic phytochemicals result in the generation of a multifunctional nanocomposite with better antioxidant qualities than its individual counterparts.

The outcomes of this study are in line with previous researches reporting the potential antioxidant activity exhibited by the plant-mediated nanomaterials (Naveed *et al.*, 2022; Qubtia *et al.*, 2024; Dathan *et al.*, 2025; Joseph *et al.*, 2025). The green synthesized hydroxy apatite nanocomposite is a promising option for biomedical and pharmaceutical applications due to its triple advantage of biocompatibility, environment friendly manufacture and cost-effectiveness compared to the chemically synthesized ones. Additionally, the generated nanocomposite's antioxidant capacity points to its possible uses in the prevention of oxidative stress related harm in biological systems. This opens avenues for its usage in drug delivery, tissue engineering, and as a protective ingredient in formulations meant to mitigate oxidative deterioration.

This study also has certain limitations, like only the scavenging potential under *in vitro* conditions was evaluated. The scenarios could change under the *in vivo* situations. Also, further evaluations are required pertaining to the nanocomposites long term stability, bioavailability, metabolism, safety, mechanical properties at molecular level for its practical usage. We also suggest the future optimization of synthesis parameters for the process's scalability and antioxidant efficacy.

CONCLUSION

An effective synthesis of citrus fruit peel (*reticulata* and *limonum*) mediated hydroxyapatite nanocomposites were completed. The developed nanocomposite exhibited strong and multifaceted antioxidant activity as demonstrated by DPPH, H_2O_2, FRAP, ABTS and NO assays. This enhanced scavenging potential is attributed to the synergistic action of the phytochemical functionalization of peels of those citrus fruits and nanoscale characterisation of the hydroxy apatite. This study highlights the potential of green synthesized nanocomposites as an effective, sustainable, biocompatible and cost-effective antioxidant system for future perspective of bio-medical application.

ABBREVIATIONS

ROS: Reactive Oxygen Species; **RNS:** Reactive Nitrogen Species; **NO:** Nitric Oxide; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **ABTS:** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); **FRAP:** Ferric Reducing Antioxidant Power; **H_2O_2:** Hydrogen Peroxide; **HAP NCs:** Hydroxyapatite Nanocomposites.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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