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Original Article

In vitro Cytotoxic Effect of Green Synthesized Hydroxyapatite Nanoparticles and Nanocomposites using Fibroblast Cell Line

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ABSTRACT

Introduction: Hydroxy apatite nanoparticles are a material of choice for biomedical application owing to its properties like biocompatibility and bioactivity. But there are concerns regarding the safety of these nanoparticles. Green synthesis offers an alternate method for the development of these nanoparticles with improved functionalization, eco-friendliness and lesser harmful effects. **Materials and Methods:** *Citrus reticulata* and *Citrus limonum* peel extract mediated hydroxy apatite nanoparticles and nanocomposites were synthesized. The cytotoxicity evaluation of the synthesized samples was carried out using MTT assay on fibroblast cell line. **Results:** At lower concentrations, *Citrus reticulata* and *limonum* mediated hydroxy apatite nanoparticles and nanocomposites exhibited negligible or mild toxicity. When concentrations increased, moderate levels of cytotoxicity were observed but nowhere near IC_{50} lethal dose. **Conclusion:** Dose dependant cytotoxicity was observed for both *Citrus reticulata* and *limonum* mediated hydroxy apatite nanoparticles and nanocomposites. Since IC_{50} is not reached, the material is considered relatively biocompatible and safe.

Keywords: Biocompatible materials, Cell viability, Cytotoxicity, Green synthesis, Nanocomposite.

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INTRODUCTION

Hydroxy apatite is inorganic calcium phosphate ceramic mineral with close resemblance to mineral composition of human bone and teeth. This property makes them an ideal biomaterial of choice for both dental and medical applications. Its extensive use in bone grafts, implant coating and tissue engineering is attributed to its properties like excellent biocompatibility, bioactivity and osteoconductivity. The biological properties are highly specific and influenced greatly by the physiochemical parameters like size and morphology and mode of synthesis (Habibah *et al*., 2022).

In addition to the trending use of hydroxy apatite mineral, its nano counterparts are gaining popularity due to improved surface area, mechanical properties and enhanced interaction with biological systems. However, the conventional method of synthesis often involves harsh toxic chemicals and complex procedures (Jagwani and Hari, 2021). It raises concerns on its cytotoxicity effects and environmental safety. This highlights the necessity of alternative technique for the synthesis.

Among the available methodologies, green synthesis represents a premier eco-friendly alternative for nanoparticle synthesis. Green synthesis is the technique of using non-toxic and biosafe materials for synthesis of products. Usually natural materials like plants, fungi, algae, bacteria, agricultural waste, etc are used in this process. Almost all parts of plants are used for green synthesis like peel, seed, fruits, etc. (Gawande *et al*., 2020). The phytochemicals present in these natural sources reduce the metal ions and stabilize the formed nanoparticles through the bottom-up approach. The size, shape and crystallinity of the nanoparticles also can be controlled by regulating reaction rates and surface energy of certain phytochemicals (Hanna *et al*., 2025).

Abundant literatures and researches are available regarding the use of green synthesis for the generation of nanoparticles and those generated particles exhibiting various properties like anti-inflammatory, anti-oxidant, anti-microbial potential. Iron nanoparticles were green synthesized from *Lawsonia inermis* and *Gardenia jasminoides* leaves extract and the synthesized nanoparticle exhibited antibacterial activity (Naseem and

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Farrukh, 2015). Similarly, Wei *et al* also synthesized iron nanoparticle; from *Eichhornia crassipes* and observed the reduction of hexavalent chromium (Wei *et al*., 2017). Copper nanoparticles were synthesized using *Citrus grandis* peel extracts and observed its photocatalytic efficiency to degrade methyl red dye (Sinha and Ahmaruzzaman, 2015). Successful synthesis of gold nanoparticles using leaf extracts of *Kalanchoe pinnata* (Mallepaka *et al*., 2026). Antimicrobial activity was exhibited by silver nanoparticles synthesized using *Tectona grandis* seeds extract (Rautela and Rani, 2019).

Literature reviews give an insight of utilizing green synthesis for hydroxy apatite nanoparticles production and are expected to exhibit enhanced biological and mechanical properties, biocompatibility and controlled degradation; which make them suitable for biomedical application. However, from experimental formulations to clinical application, the safety margin of generated nanomaterial has to be thoroughly evaluated.

Even though hydroxyapatite is considered biocompatible, when it is converted to its nano scale there might be changes in their physio-chemical properties like surface-area to volume ratio, cellular uptake mechanism, etc. (Szczyglewska *et al*., 2023). There has been reports of unexpected nanomaterial toxicity owing to its potential to interact with biological structures like proteins and triggering inflammatory and immunological responses (Mitra *et al*., 2023). Especially the smaller sized nanoparticles demonstrated higher cellular uptake and higher toxicity. Numerous factors could contribute to the cytotoxicity of nanoparticles like size, shape, surface charge and hydrophobicity (Dong *et al*., 2020).

Study by Clark *et al* reported that carbon nanotubes disrupted the cellular reproduction, metabolic activity, membrane integrity and potential of Caco-2 cell model which simulates human intestinal enterocytes (Clark *et al*., 2012). Potential cytotoxicity and genotoxicity could result from silver and gold nanoparticles as they induce oxidative stress (Foldbjerg *et al*., 2011). Sharma *et al* demonstrated that the sub-acute oral expose led to accumulation of zinc oxide nanoparticles in liver causing oxidative stress which resulted in DNA damage and apoptosis (Sharma *et al*., 2012).

Hence understanding the interaction of green synthesized nanoparticles and nanocomposites with cellular structures is necessary to eliminate the safety concerns. The incorporation of citrus based secondary metabolites offer protective synergistic effect as it is coated with phytochemicals or biomolecules. It could potentially reduce cytotoxicity and oxidative stress as compared to the chemical-based nanoparticles. However, the influence of green synthesized nanoparticles or nanocomposites on cell viability or proliferation is unknown (Gawande *et al*., 2020).

In vitro cytotoxicity assessment is the primary step to determine the biocompatibility of generated nanoparticles with the living cells. The *in vitro* cytotoxicity assessment will give information regarding the changes in cell viability, proliferation and morphology when exposed to the green synthesised nanoparticles and nanocomposites under experimental conditions (Awashra and Mlynarz, 2023). Therefore, the objective of this study was to evaluate the *in vitro* cytotoxic potential of green synthesized hydroxyapatite nanoparticles and nanocomposites using fibroblast cell line to bring insights on their safety and potential usage in biomedical applications and thereby bridging the gap in between sustainable material science and safe biomedical engineering.

MATERIALS AND METHODS

In this study citrus fruits, *Citrus reticulata* and *Citrus limonum* are used for the green synthesis of hydroxy apatite nanoparticles and nanocomposites; followed by *in vitro* cytotoxicity assessment.

Sample Preparation

Preparation of Citrus reticulata and limonum mediated hydroxy apatite nanoparticles

The sample preparation began with the procurement of *Citrus reticulata* and *limonum* fruits. The fruit peels were separated, dried and ground into coarse powder. 2 g of this coarse peel powder was diluted with 100 mL distilled water and heated to 50-60°C in a heating mantle for 20 min. The obtained solution was filtered using a muslin cloth and stored.

Afterwards, 200 mg of hydroxy apatite particles were diluted with 50 mL distilled water. Then 50 mL of this hydroxy apatite suspension was added to 50 mL of *Citrus reticulata* and *limonum* peel extract. For next 28 hr this solution was kept undisturbed in a magnetic stirrer. Following a colour change, the mixture was centrifuged for 10 min at 8000 rpm. The centrifugation resulted in the formation of *Citrus reticulata* and *limonum* peel extract mediated hydroxy apatite nanoparticle pellets.

Preparation of *Citrus reticulata* and *limonum* mediated hydroxy apatite nanocomposites

For the preparation of nanocomposites, the above prepared nanoparticle pellets were diluted. Followed by chitosan solution preparation. To 500 mg of medium molecular weight chitosan, 1 mL of glacial acetic acid was added. Into this mixture 49 mL distilled water was incorporated and was kept in a magnetic stirrer at 650 rpm for 24 hr. Later 1 mL of hydroxy apatite nanoparticle solution is mixed with 4 mL of chitosan solution. This solution was also kept in a magnetic stirrer for 3 hr at 450 rpm. The resultant solution was used as stock solution for cytotoxicity evaluation.

Cytotoxicity assessment

The MTT assay was opted for the *in vitro* cytotoxicity assessment of *Citrus reticulata* and *limonum* mediated hydroxy apatite nanoparticles and nanocomposites. The MTT assay was carried out using 3T3-L1 mouse fibroblast cells. The 3T3-L1 mouse fibroblast cells were procured from the National Centre for

Cell Science (NCCS), Pune. The obtained cells were cultured in 25 cm^2 vented flasks under controlled incubation conditions, maintained at 37°C with 5% CO_2 in humidified atmosphere. This was done for ensuring optimal cell growth and pH stability. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) [Invitrogen, USA] and enriched with 10% Fetal Bovine Serum (FBS) [Thermo Fisher Scientific, USA] and 1% Penicillin-Streptomycin [Life Technologies, USA]. Upon reaching the confluence of 70-80%, the cells were harvested and seeded into a 96 well plated at a density of 1x104 cells per well and incubated for 1 to 2 days for attaining a uniform monolayer.

For experimental treatments, stock solutions of *Citrus reticulata* and *limonum* mediated nanoparticles were prepared at a concentration of 100 mg/mL (weight/volume). It is then serially diluted to obtain a range of working concentrations: 3.175, 6.25, 12.5, 25, 50,100 and 200 µg/mL. The control group consisted of cells that received no treatment. Once the fibroblasts reached confluence, they were exposed to various concentrations for 24-hr period.

To assess the viability of cells, 50 µL of MTT reagent (5 mg/mL in PBS; Sigma-Aldrich) was added to each well and the plates were incubated at 37°C for 2 hr, facilitating the reduction of the reagent into formazan crystals by the metabolically active cells. The formazan crystals were then dissolved in 150 µL of Dimethyl Sulfoxide (DMSO) and at 490 nm the resultant absorbance was measured using a TECAN microplate reader. Additionally, using a phase- contrast microscopy, any variation in the morphology of the cells was evaluated and documented using digital imaging (Mansoor *et al*., 2022). Same procedure was repeated with *Citrus reticulata* and *limonum* mediated hydroxy apatite nanocomposites.

RESULTS

MTT assay was used for the assessment of cytotoxicity of *Citrus reticulata* and *limonum* green synthesized hydroxy apatite nanoparticles and nanocomposites. Optical density and percentage of cell viability was measured at different concentrations (3.175, 6.25, 12.5, 25, 50,100 and 200 µg/mL)

The control group exhibited high absorbance values (0.945, 0.924 and 0.949) with a mean value of 0.939. The viability rate was also high 100.04%. In the test group, for *Citrus reticulata* mediated hydroxy apatite nanoparticles, gradual decrease in the mean absorbance value was observed with increased nanoparticle concentration. At lower concentrations 3.175 µg/mL and 6.25 µg/mL the optical density values (0.934, 0.918) remained close to the control group. However, when the concentration increased to 12.5, 25 and 50 µg/mL, the optical density decreased moderately to 0.906, 0.887 and 0.862. At higher concentrations of 100 and 200 µg/mL further reduction of optical density was observed; 0.824, 0.730 respectively. The percentage of cell viability also followed a similar pattern. Lower concentrations from 3.175 µg/mL to 12.5 µg/mL percentage of cell viability was above 96% suggesting very minimal cytotoxicity. Viability percentage decreased to 94.34% and 91.76% when concentration increased to 25 and 50 µg/mL. When concentrations increased to further higher rates of 100 and 200 µg/mL more prominent cytotoxic effect with decline in viability percentage to 87.79% and 77.74% was observed. The standard deviation and standard error of mean were low for all groups, indicating reliability of the obtained data (Table 1).

The phase contrast microscopy of the control and test groups were done. The digital image of the control group demonstrated adherent cells of normal morphology with dense well spread monolayer pattern and elongated or spindle shape. The cell membranes were intact with distinct boundaries. The test group at 3.175 µg/mL concentration showed elongated or spindle shaped cell with intact membrane and dense monolayer pattern similar to the control group. However, the digital imaging of 25 µg/mL concentration showed noticeable change in cell morphology compared to the control group and low concentration test groups. The cells remained elongated or in spindle shaped morphology. But there were visible changes like few partially rounded cells, diminished cell spreading and increased inter cellular spaces. Some cells were less defined and few debris were observed. Even with these findings significant number of cells remain viable. Pronounced morphological changes were observed at higher concentration of 200 µg/mL. A significant number of cells lost the conventional spindle like cell structure and attachment potential. Cells became round and shrunken. There was increase in cellular debris and floating cells with loss of monolayer pattern. However, limited number of cells still retained normal morphology (Figure 1).

Citrus limonum mediated hydroxy apatite nanoparticles showed similar pattern as that of *Citrus reticulata.* As the concentration increased, the cytotoxicity also increased. At lower concentrations like 3.175 µg/mL to 12.5 µg/mL, even though the optical density slightly decreased, the cell viability remained quite high (95-99%). When concentration further increased to 25 and 50 µg/mL the optical density further decreased with gradual decline in percentage of cell viability (93.82% at 25 µg/mL and 90.45% at 50 µg/mL). At higher concentrations of 100 and 200 µg/mL along with optical density, the cell viability decreased profoundly to 86.19% at 100 µg/mL and 80.44% at 200 µg/mL (Table 2).

Comparing *Citrus reticulata* and *limonum* mediated hydroxy apatite nanoparticles, *Citrus reticulata* exhibited higher cytotoxicity than *limonum* with marked reduction in percentage of cell viability at 200 µg/mL (77.74% vs 80.44%).

Digital images of the phase contrast microscopy of the *Citrus limonum* mediated hydroxy apatite nanoparticles are in consistent with *Citrus reticulata* however it was less pronounced. At lower concentration of 3.175 µg/mL normal morphology of cells were observed. Spindle in shape with intact cell membrane

and monolayer dense pattern similar to control. At 25 µg/mL concentration, minor changes in morphology were observed like few rounding of cells, debris formation, reduced cell spreading. When concentration reached 200 µg/mL the changes become more prominent like more rounding of cells, debris formation and adherence loss. But a significant number of cells remain intact compared to *Citrus reticulata* mediated hydroxy apatite nanoparticles (Figure 2).

The MTT assay of *Citrus reticulata and limonum* mediated hydroxy apatite nanocomposites were also carried out. The cytotoxic activity was evaluated across a concentration range of 3.175 to 200 µg/mL similar to the nanoparticles.

The control group exhibited a mean optical density of 0.939 and percentage cell viability of 100.04%. The concentration range between 3.175 µg/mL to 12.5 µg/mL of *Citrus reticulata* mediated hydroxy apatite nanocomposites demonstrated a mean optical density of 0.937, 0.927 and 0.914; and cell viability range from 99.82% to 97.37% indicating very minimal cytotoxicity. At intermediate concentration there was reduction in optical density and cell viability. At 25 µg/mL concentration, the optical density decreased to 0.899 and cell viability to 95.78%; which then further reduced to 0.879 and 93.61% at 50 µg/mL. When concentrations of the group increased to 100 and 200 µg/mL, there were evident decrease in both parameters (Optical density 0.854 and 0.757; Cell viability 90.98% and 80.62%. The standard deviation and standard error of mean remained low at all concentrations suggesting the reliability of the experimental data (Table 3).

Both *Citrus reticulata* mediated hydroxy apatite nanoparticles and nanocomposites exhibited decrease in optical density and percentage of cell viability as concentrations increased from 3.175 µg/mL to 200 µg/mL. However, the more pronounced reduction was shown by the nanoparticles. The nanocomposites exhibited a higher 80.62% viability at 200 µg/mL.

Phase contrast microscopic images of control group revealed cells of normal morphology representing viable cells. At 3.175 µg/mL concentration of *Citrus reticulata* mediated hydroxy apatite nanocomposites, the cells had no or minimal change compared to the control group. When the concentration of 25 µg/mL was microscopically evaluated, noticeable changes were seen like cell shrinkage and rounding, diminished cellular density. At higher concentration of 200 µg/mL evident cytotoxic effects were seen. Cell shrinkage, fragmented and rounded cells, attachment loss and reduced confluency were observed. This is in agreement with the decreased values of cell viability. Compared to the green synthesized nanocomposites, nanoparticles exhibited reduced cell adherence and density. Nanocomposites demonstrated better preservation of cell structure and more viable cells (Figure 3).

A pattern similar to *Citrus reticulata* mediated hydroxy apatite nanocomposites were observed for *Citrus limonum* mediated hydroxy apatite nanocomposites as well. Lowest concentration 3.175 µg/mL exhibited 99.89% cell viability and highest concentration 200 µg/mL exhibited cell viability of 84.95%. The cell viability of all other concentrations remained between these (Table 4).

When comparing *Citrus limonum* mediated hydroxy apatite nanoparticles and nanocomposites, the nanocomposite group showed higher cell viability among all tested concentrations. At lower doses both groups exhibited minimal toxicity. The comparison between *Citrus reticulata and limonum* mediated hydroxy apatite nanocomposites shows that at lower doses both groups exhibited greater cell viability and at higher concentrations *Citrus limonum* based nanocomposites exhibited greater cell viability, 84.95% compared to 80.62% at 200 µg/mL of *Citrus reticulata* based nanocomposites.

The findings of digital images of phase contrast microscopy of *Citrus limonum* mediated hydroxy apatite nanocomposites are also consistent with quantitative data. Mild or negligible morphological variation seen in lower concentration and predominant variation seen at higher doses. Image comparison

Table 1: Optical density and percentage of cell viability of *Citrus reticulata* mediated hydroxy apatite nanoparticles.

Citrus reticulata										
Concentration (µg/mL)	OD 1	OD 2	OD 3	Mean	% cell viability			Mean	SD	SEM
Control	0.945	0.924	0.949	0.939	100.64	98.40	101.06	100.04	1.43	0.83
3.175	0.935	0.938	0.93	0.934	99.57	99.89	99.04	99.50	0.43	0.25
6.25	0.918	0.922	0.915	0.918	97.76	98.19	97.44	97.80	0.37	0.22
12.5	0.905	0.91	0.902	0.906	96.38	96.91	96.06	96.45	0.43	0.25
25	0.89	0.888	0.882	0.887	94.78	94.57	93.93	94.43	0.44	0.26
50	0.865	0.862	0.858	0.862	92.12	91.80	91.37	91.76	0.37	0.22
100	0.828	0.825	0.82	0.824	88.18	87.86	87.33	87.79	0.43	0.25
200	0.723	0.736	0.731	0.730	77.00	78.38	77.85	77.74	0.70	0.40

Table 2: Optical density and percentage of cell viability of *Citrus limonum* mediated hydroxy apatite nanoparticles.

Citrus limonum										
Concentration (µg/mL)	OD 1	OD 2	OD 3	Mean	% cell viability			Mean	SD	SEM
Control	0.945	0.924	0.949	0.939	100.64	98.40	101.06	100.04	1.43	0.83
3.175	0.93	0.928	0.935	0.931	99.04	98.83	99.57	99.15	0.38	0.22
6.25	0.912	0.917	0.92	0.916	97.12	97.66	97.98	97.59	0.43	0.25
12.5	0.9	0.897	0.905	0.9	95.85	95.53	96.38	95.92	0.43	0.25
25	0.878	0.885	0.88	0.881	93.50	94.25	93.72	93.82	0.38	0.22
50	0.85	0.846	0.852	0.849	90.52	90.10	90.73	90.45	0.33	0.19
100	0.81	0.803	0.815	0.809	86.26	85.52	86.79	86.19	0.64	0.37
200	0.764	0.747	0.755	0.755	81.36	79.55	80.40	80.44	0.91	0.52

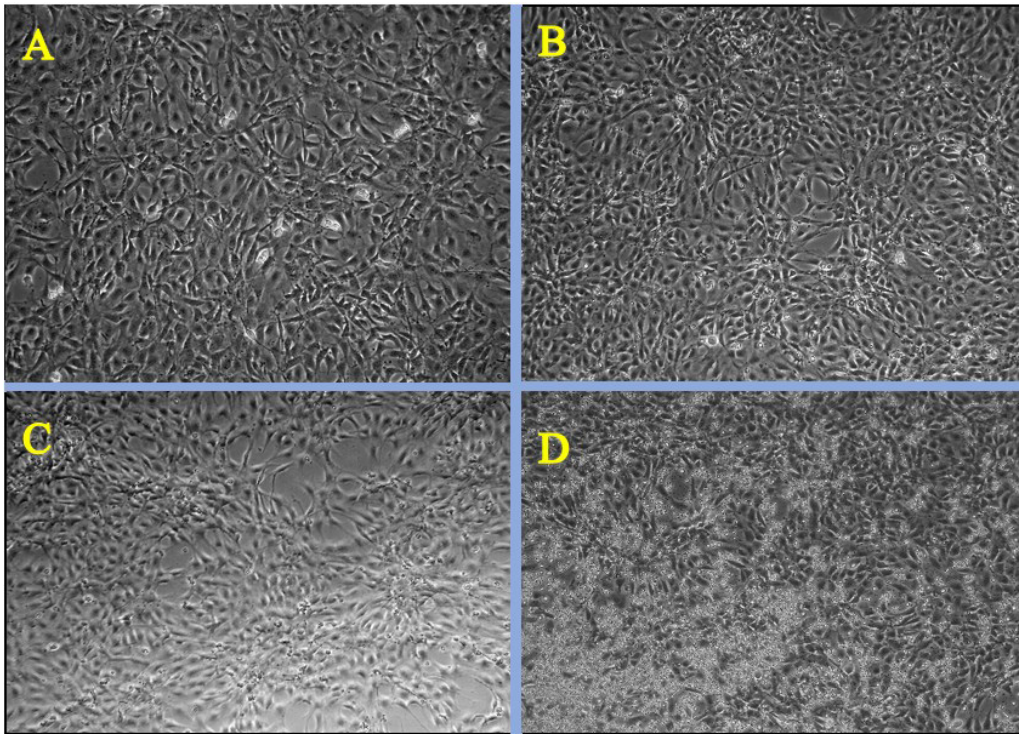

Figure 1: Phase contrast microscopy images of *Citrus reticulata* mediated hydroxy apatite nanoparticles at different concentrations A) control B) 3.175 µg/mL C) 25 µg/mL D) 200 µg/mL.

also reveals *Citrus limonum* based nanocomposites showed better viability to nanoparticles and to *Citrus reticulata* (Figure 4).

The results also reveal that all the tested groups, even at highest tested concentration of 200 µg/mL, Half Maximal Inhibitory Concentration (IC_{50}) remained above 77%, indicating that 50% inhibition was not reached.

DISCUSSION

This study evaluated the cytotoxic effects of *Citrus reticulata* and *limonum* mediated hydroxy apatite nanoparticles and nanocomposites using fibroblasts cell line to identify the biocompatibility and its potential safe use for biomedical application. In *Citrus reticulata* mediated hydroxy apatite nanoparticles, the high optical density values and viability rate of control group suggested its normal cell growth in the optical density value with increasing concentrations of test group may be attributed to the reduced cellular metabolic activity (Van *et al.*, 2011). Cell viability rate was above 96% at lower concentrations which suggests that the green generated nanoparticle does not significantly interfere with cellular metabolic activity and exhibiting good biocompatibility at these concentrations. Even when concentrations increased to up to 50 µg/mL, only a slight reduction in the cell viability was observed, ensuring its safety levels. More pronounced decline was observed at 100 and 200 µg/

mL but still remained above 75%. This confirms a dose dependent cytotoxic effect similar to findings of (Hoogstraten *et al.*, 2022).

A phase- contrast microscopy imaging was done to evaluate any variation in the morphology of the cells. The control and test group of lower concentrations showed cells of good proliferation and attachment with no membrane alteration which is suggestive of non-significant cytotoxicity effects. When concentration reached 25 µg/mL even though there were morphological changes, the monolayer integrity was still maintained. This demonstrated that concentrations like 25 µg/mL induced only moderate cellular stresses without significant cell deaths. This is in agreement with the higher cell viability (94.43) of the MTT assay, suggestive of the relatively good cellular tolerance. In contrast, 200 µg/mL concentration showed marked cytotoxic effects. Significant number of cells showed morphological changes and increased cellular debris and floating cells. This indicates loss of cell membrane integrity and cell death (Khalid and Azimpouran, 2026). The reduced percentage of cell viability of 77.74% supports the significant cytotoxicity at higher concentrations. This clearly shows a concentration dependant cytotoxicity pattern

Table 3: Optical density and percentage of cell viability of *Citrus reticulata* mediated hydroxy apatite nanocomposites.

Citrus reticulata										
Concentration (µg/mL)	OD 1	OD 2	OD 3	Mean	% cell viability			Mean	SD	SEM
Control	0.945	0.924	0.949	0.939	100.64	98.40	101.06	100.04	1.43	0.83
3.175	0.938	0.934	0.94	0.937	99.89	99.47	100.11	99.82	0.33	0.19
6.25	0.928	0.924	0.93	0.927	98.83	98.40	99.04	98.76	0.33	0.19
12.5	0.915	0.91	0.918	0.914	97.44	96.91	97.76	97.37	0.43	0.25
25	0.9	0.895	0.903	0.899	95.85	95.31	96.17	95.78	0.43	0.25
50	0.88	0.875	0.882	0.879	93.72	93.18	93.93	93.61	0.38	0.22
100	0.855	0.85	0.858	0.854	91.05	90.52	91.37	90.98	0.43	0.25
200	0.756	0.746	0.769	0.757	80.51	79.45	81.90	80.62	1.23	0.71

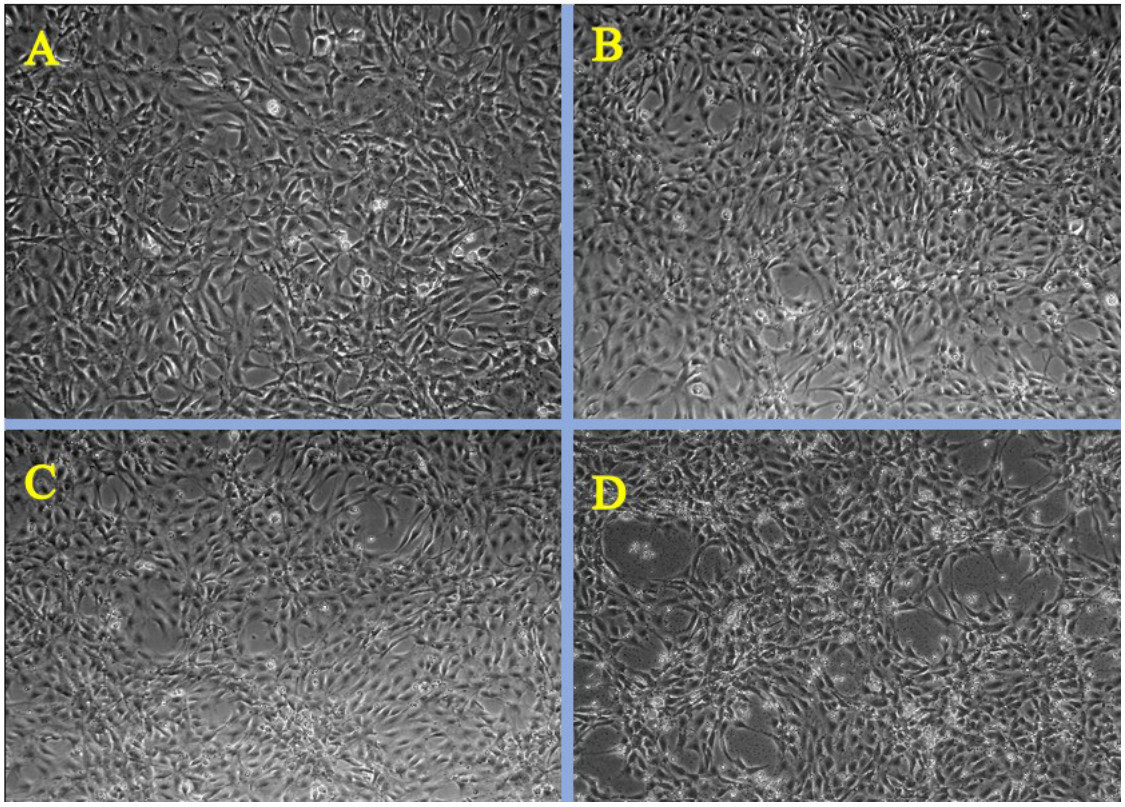

Figure 2: Phase contrast microscopy images of *Citrus limonum* mediated hydroxy apatite nanoparticles at different concentrations A) control B) 3.175 µg/mL C) 25 µg/mL D) 200 µg/mL.

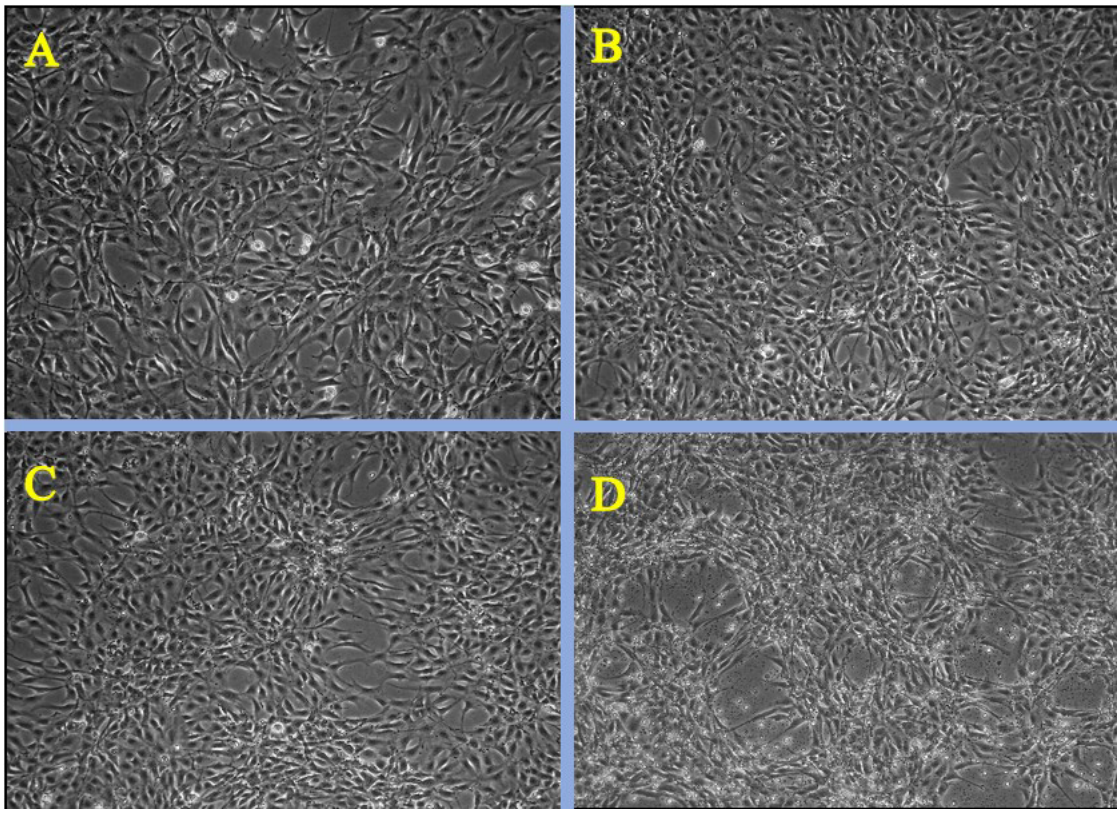

Figure 3: Phase contrast microscopy images of *Citrus reticulata* mediated hydroxy apatite nanocomposites at different concentrations A) control B) 3.175 µg/mL C) 25 µg/mL D) 200 µg/mL.

Table 4: Optical density and percentage of cell viability of *Citrus limonum* mediated hydroxy apatite nanocomposites.

Citrus limonum										
Concentration (µg/mL)	OD 1	OD 2	OD 3	Mean	% cell viability			Mean	SD	SEM
Control	0.945	0.924	0.949	0.939	100.64	98.40	101.06	100.04	1.43	0.83
3.175	0.94	0.938	0.936	0.938	100.11	99.89	99.68	99.89	0.21	0.12
6.25	0.927	0.925	0.92	0.924	98.72	98.51	97.98	98.40	0.38	0.22
12.5	0.912	0.91	0.905	0.909	97.12	96.91	96.38	96.81	0.38	0.22
25	0.898	0.895	0.892	0.895	95.63	95.31	94.99	95.31	0.32	0.18
50	0.878	0.875	0.87	0.874	93.50	93.18	92.65	93.11	0.43	0.25
100	0.852	0.85	0.845	0.849	90.73	90.52	89.99	90.42	0.38	0.22
200	0.794	0.802	0.797	0.798	84.56	85.41	84.88	84.95	0.43	0.25

as lower concentration maintained cellular integrity and at higher concentration it was disrupted similar to the findings of Arland and Kumar, who reported that concentration dependent reduction in cell viability was observed when exposed to green synthesized silver nanoparticles (Arland and Kumar, 2024).

Citrus limonum mediated hydroxy apatite nanoparticles also demonstrated a dose dependant cell viability. Concentrations up to 12.5 µg/mL showed a cell viability above 95% which indicated a minimal effect of the nanoparticle on the cell metabolism. When concentration increased to 25 and 50 µg/mL, decrease in cell viability suggests generated cellular stress. There was further decrease in percentage of cell viability at higher concentrations of 100 and 200 µg/mL but still remained above 80%. These findings indicate non-lethal dose within tested concentration range.

The digital imaging revealed almost normal morphology at lower concentrations; but with increasing concentration features like partial rounding of cells, increased debris formation, reduced cell spreading were observed suggesting early cytotoxic response (Montes-de-Oca-Saucedo *et al.*, 2025; Biswas *et al.*, 2025; Nandhini *et al.*, 2021). Relatively higher cell viability at the highest

tested concentration indicated the weak cytotoxic potential of *Citrus limonum* mediated hydroxy apatite nanoparticles. When compared, *Citrus reticulata* mediated hydroxy apatite nanoparticles showed more pronounced morphological changes at equivalent concentration. This might be attributed to the varied composition of phytochemicals, its concentration or the lack of cellular interaction and uptake amplification by the nanoparticles (Makni *et al*., 2018; Wang *et al*., 2019).

The optical density and cell viability values of control group of *Citrus reticulata* mediated hydroxy apatite nanocomposites confirm the normal cell growth and optimal experimental environment. This is consistent with green synthesized hydroxy apatite nanoparticles. The minimal cytotoxicity at lower concentrations indicates good cellular compatibility of the generated nanocomposite. The decline in optical density values and percentage of cell viability at moderated concentration of 25 and 50 µg/mL reflects cellular stress induced by the material. At higher concentration even with reduction in the values more than 80% of cells remained viable indicating relatively lower cytotoxicity profile.

The nanoparticle formulation exhibited a slightly higher cytotoxicity potential compared to the nanocomposites. This difference is attributed to the difference in physiochemical properties. The increased surface area and reactivity, direct interaction with cell membrane leading to the increased stress and decreased viability is the reason for higher toxicity effects of nanoparticles. On the contrary, nanocomposites provide a stabilizing matrix that helps in the release of active compounds which decreases direct cellular interaction leading to lesser cytotoxicity.

In the phase contrast microscopy of *Citrus reticulata* mediated hydroxy apatite nanocomposites, minimal or absence of morphological changes at the lowest 3.175 µg/mL concentration represent a lower or no cytotoxicity and suggests that lower doses does not affect cellular integrity or metabolism. Morphological changes at 25 µg/mL indicate the beginning of cellular stresses caused by the interaction between nanocomposite and the cell membrane. The evident changes at 200 µg/mL are an indicator that the higher concentration of nanocomposites might result in cell death through oxidative stress, leading of reactive oxygen species and mitochondrial dysfunction mechanism (Fallah *et al*., 2024). This is potentially due to the presence of bioactive compounds of *Citrus reticulata*; which enhances therapeutic and cytotoxic potential at higher doses. When comparing *Citrus reticulata* mediated hydroxy apatite nanoparticles and nanocomposites, both expressed significant cytotoxicity at higher concentration. However, nanocomposites showed lesser effects to nanoparticles. This is due to controlled release and interaction, reduced particle aggregation and bioavailability of nanocomposites.

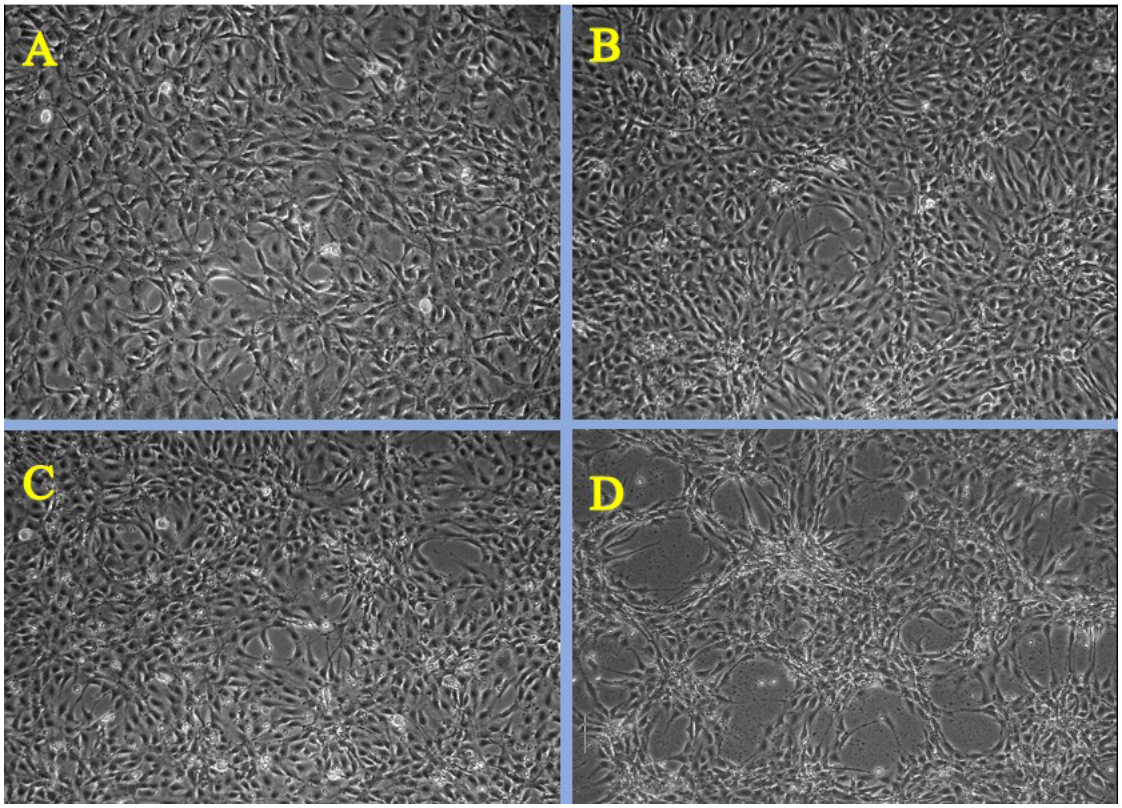

Figure 4: Phase contrast microscopy images of *Citrus limonum* mediated hydroxy apatite nanocomposites at different concentrations A) control B) 3.175 µg/mL C) 25 µg/mL D) 200 µg/mL.

Citrus limonum mediated hydroxy apatite nanocomposites also demonstrated a dose dependant decrease in optical density and cell viability. However, the reduction was relatively moderate, values remaining above 80%, which suggestive of good biocompatibility. The comparative findings of both nanoparticle and nanocomposite from *Citrus limonum* indicate that the nanocomposite expressed relatively higher cell viability owing to the increased biological activity and cellular interaction. In the comparison between nanocomposites of both *Citrus reticulata* and *limonum*, they also showed concentration dependant cell viability. *Citrus limonum* exhibited slightly lower cytotoxicity to *Citrus reticulata* and its suitability for biomedical application owing to lower toxic effects.

Another important matter of fact is none of the tested concentrations exceeded the IC_{50} lethal dose. Even at 200 µg/mL, 50% of cell death was not observed. This suggests that under experimental conditions the IC_{50} of *Citrus reticulata* and *limonum* mediated hydroxy apatite nanoparticles and nanocomposites is greater than 200 µg/mL; demonstrating its low cytotoxicity levels. Materials of such high IC_{50} values are often considered as relatively safe for biological usage (Berrouet *et al*., 2020). The overall minimal cytotoxicity effects of the nanomaterials are attributed to the synergistic effect of green chemistry and nanoparticles. This is in agreement with findings of Khorrami *et al* (Khorrami *et al*., 2018).

The presence of bioactive phytochemicals like polyphenols, Flavonoids, essential oils in the *Citrus reticulata* and *limonum* at higher concentrations induce oxidative stress or affect integrity of cellular structures. This might be reason for the observed moderate cytotoxicity effects at higher concentrations of test group. However, at lower concentrations these phytochemicals offer antioxidant or protective effects which explains the maintenance of cell viability (Di Giacomo *et al*., 2026).

There are few limitations to this study like only MTT assay was considered for *in vitro* cytotoxicity assessment. Also, there could be changes under *in vivo* conditions. So future researches have to be conducted considering more assays for *In vitro* and *in vivo* assessment.

CONCLUSION

Findings of the study suggests that both *Citrus reticulata and limonum* mediated hydroxy apatite nanoparticles and nanocomposites showed a concentration dependant cytotoxicity. Lower concentration of *Citrus reticulata* group exhibited minimal or no toxicity. Moderate cytotoxic effects were shown at higher concentrations but not below the Lethal Dose (IC_{50}). Comparable results were demonstrated by *Citrus limonum* groups. Also, nanocomposites exhibited better cytocompatibility and more gradual cytotoxic effects; which emphasize its merit to be used as nanocomposite formulations for biomedical purposes.

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ABBREVIATIONS

CBD: Cannabidiol; **DMEM:** Dulbecco's Modified Eagle Medium; **DMSO:** Dimethyl Sulfoxide; **FBS:** Fetal Bovine Serum; **IC_{50}:** Half-Maximal Inhibitory Concentration; **NCCS:** National Centre for Cell Science; **OD:** Optical Density; **SD:** Standard Deviation; **SEM:** Standard Error of the Mean.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AI USE STATEMENT

The author used ChatGPT for grammar checking and improving sentence clarity. The author reviewed and edited the output and takes full responsibility for the final content.

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