

RESEARCH PAPER

Phytochemical-Mediated Green Synthesis of Fe₃O₄ Nanoparticles Using Lemon Peel Extract: Antioxidant Activity, and Selective Cytotoxicity Against MCF-7 Breast Cancer Cells

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ABSTRACT

Creating nanoparticles for the treatment of specific tumors while ensuring safety to the body is one of the significant problems in tumor nanomedicine. Thus, we developed an ecologically friendly approach to use compounds extracted from Citrus limon peels for making superparamagnetic magnetite nanoparticles having both antioxidant properties and targeted antitumor activity. The clinching evidence for the creation of nearly pure nanocrystals of magnetite with an average size of crystals of about 21.67 nm has been obtained. Biologically synthesized nanoparticles were shown to be characterized not only by radical removal capabilities but also have an excellent IC₅₀ value of about 47 µg/ml, which is very close to pure ascorbic acid's IC₅₀ value of 20.3 µg/ml due to the interfacial electron transfer phenomenon. The greatest achievement of this research was the successful test of new nanoparticles on different types of cells, revealing their high antiproliferative activity against MCF-7 cells (viability was 42.06% ± 0.18% at IC₅₀ ≈ 216.9 µg/ml of Fe₃O₄ nanoparticles) and absence of toxicity toward fibroblast cells (MRC-5 cells viability remained 77.76% ± 0.58% at 400 µg/ml Fe₃O₄ nanoparticles). The achieved selectivity of the investigated nanoparticles explains the different sensitivity of cancer cells and healthy cells to free radicals.

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INTRODUCTION

Targeted oncotherapy necessitates methods that efficiently distinguish between malignant metabolic characteristics and mechanisms of normal tissues [1-4]. Magnetite (Fe₃O₄) nanoparticles are frequently studied for their

usefulness in biomedicine and ROS-based therapies because of their inherent ability to be superparamagnetic and biocompatible [1, 4]. The traditional methods of production physical or chemical, including chemical reduction and co-precipitation are harmful due to the use of

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dangerous chemical substances, extreme energy conditions, and toxic surface surfactants [5-13]. These harmful adsorbates bring about unwanted toxicity to the body limiting possible clinical use [4, 13]. In order to solve the above disadvantages, green chemistry proposes using living organisms as safe bio-factories [5, 6]. Agricultural waste, especially fruit waste from the genus *Citrus*, seems to be a good source of bioactive secondary metabolites [6, 7]. The peel extract of *Citrus limon* is a good source of various polyphenols, flavonoids and ascorbic acid that have OH and C=O groups capable of reducing iron precursor ions and act as natural surfactants [1-3]. Thus, it creates bio-organic layer on particles, preventing aggregation and affecting the growth of nanocrystals [8, 9]. In addition, the bonding of phenolic molecules provides special free radical-scavenging properties to nanoparticles depending on their surface [10-12]. However, despite the recognition of biogenic iron oxides as antioxidant agents [11, 12], it is unclear how this phytochemical compound contributes to the difference in life-threatening cell toxicity. Malignant cells, like human breast adenocarcinoma (MCF-7), have high levels of hydrogen peroxide (H_2O_2) and low pH conditions within their cytosol, which influences the occurrence of Fenton-like reaction in these cells [4, 11]. In contrast, normal somatic cells, such as lung fibroblasts, have efficient antioxidant mechanisms

that counteract considerable amounts of reactive oxygen species (ROS) [11, 12]. This study offers a fast green process for creating Fe_3O_4 nanoparticles that are phase-pure from citrus lemon peel waste extract, and to figure out the accurate bio-functional threshold for the nanoparticles. Structural and chemical properties were explored using UV-Vis, FTIR, XRD, EDX, and TEM techniques [1-3]. Notably, biogenic nanoparticles provide huge DPPH radical scavenging ability, and using 0.50 mg/mL concentration, the nanoparticles provided 89.53% inhibition, which is very close to pure ascorbic acid's effectiveness of 93.62%. The in-vitro studies showed a remarkable difference in cytotoxicity. At 200 $\mu\text{g}/\text{mL}$ concentration, the nanoparticles killed about 50.73% of MCF-7 cells (IC_{50}) and maintained over 82.28% of survival rates of MRC-5 normal lung fibroblasts. Even under 400 $\mu\text{g}/\text{mL}$ concentration of nanoparticles, the survival rate of MRC-5 cells was still at 77.76% while for MCF-7, it was down to 42.06%.

MATERIALS AND METHODS

Chemicals and reagents

Iron Sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) (BDH), Sodium hydroxide (NaOH) Sigma-Aldrich It was received from the chemical materials store at the College of Science, Mustansiriyah University and College of Pharmacy, University of Kerbala. The extract was made in the lab with *Citrus limon* and

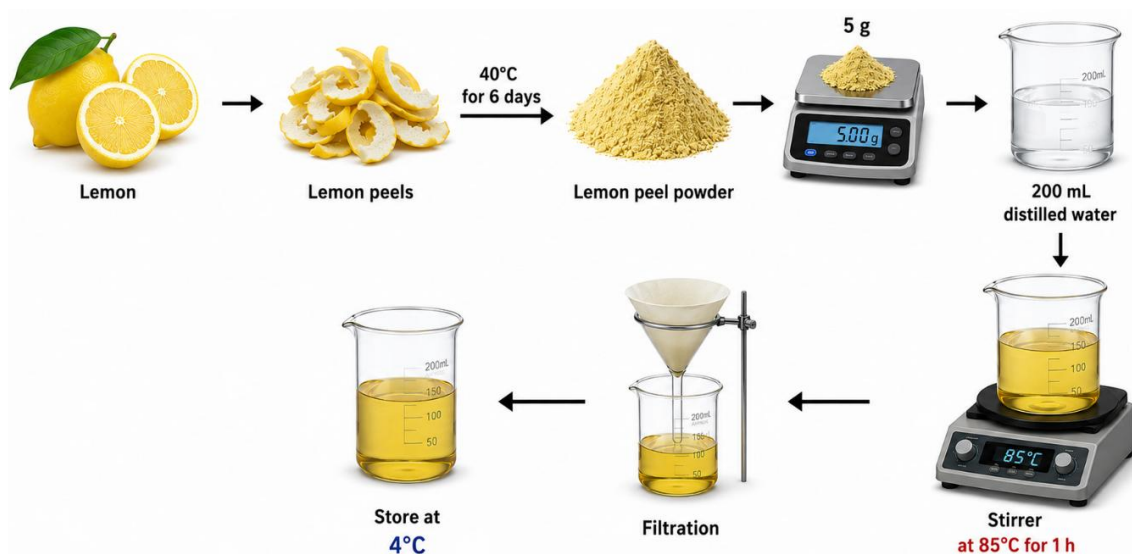


Fig. 1. Preparing lemon extract from dried lemon peels.

all solutions were made with deionized water. To study the antioxidant activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) was used against Vit C as a standard antioxidant [14].

Collection of plant

Fresh Lemon fruit was purchased from nearby local markets and thoroughly washed with sterile distilled water. The peels were then removed, chopped into small earpieces, and cleaned to eliminate any residual dirt or debris. To remove moisture, the chopped peels were dried for four to Five days at a temperature of 40°C for six to seven hours daily. Once dried. The peels were finely powdered using a mixer grinder and stored for future use.

Preparation of lemon peel extract

Take five from lemon powder and mixed with 200 ml distilled water (5 g in 200 mL) to create an extract Fig. 1. and stored at 4°C in a tight glass bottle for further synthesis of Fe_3O_4 NPs35.

Green Synthesis of Fe_3O_4 NPs

The final concentration of 0.1M was made by dissolution of 3g of iron sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) in 300ml of distilled water. The mixture was stirred for five minutes at room temperature to form a homogenous mixture. Then, 20 milliliters of extracts were added and the

mixture was stirred until it turns yellow. 1M NaOH was slowly added to the mixture until the blue colored mixture changes to grey after 30 minutes of stirring. Then, the mixture was centrifuged for ten minutes at 7500 rpm and the supernatant was washed with water repeatedly. Black-reddish colored Fe_3O_4 NPs were collected and dried for four hours at 95°C . In order to eliminate all evaporable impurities from the dried Fe_3O_4 NPs, calcination process was done for four hours at 400°C [14].

Characterization of Fe_3O_4 NPs

for Optical studies, UV spectrum analysis was estimated and FT-IR analysis. Fe_2O_3 NPs were also characterized using XRD, TEM, EDX and SEM.

RESULTS AND DISCUSSION

UV-visible spectroscopy analysis

There was a considerable absorption of the green-synthesised Fe_3O_4 nanoparticles in the ultraviolet region of the UV-Vis spectrum, gradually reducing towards the visible region. Electronic transition and intervalence charge transfer from Fe^{2+} and Fe^{3+} ions are responsible for such an optical behaviour of the inverse spinel crystals of magnetite nanoparticles. Optical activity of Fe_3O_4 nanoparticles is governed by the surface electrical interactions, particle size distribution, and the nature of nanomaterial generated; all these factors contribute to the broad absorption

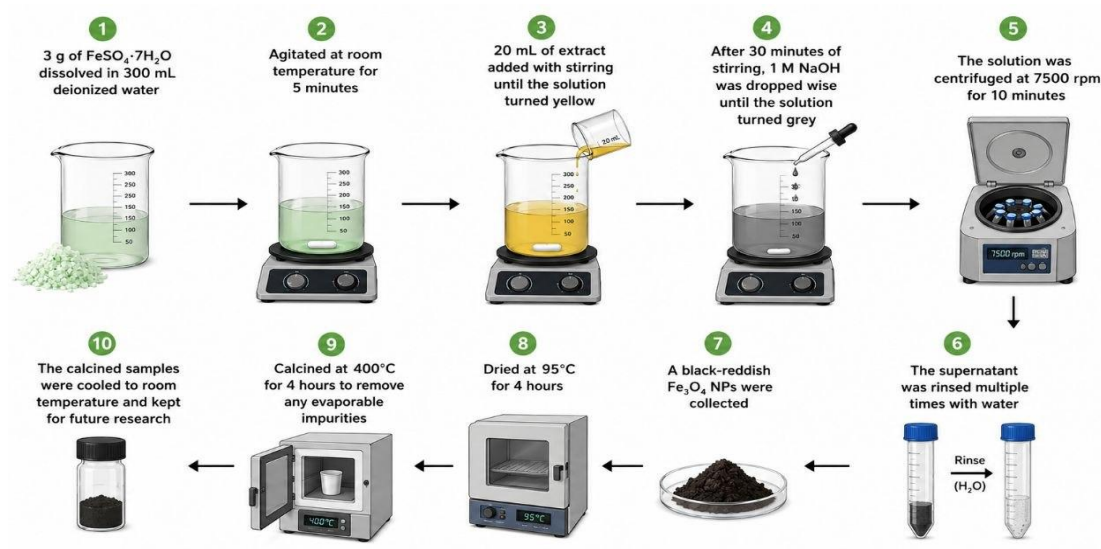


Fig. 2. Green Fe_3O_4 NP Synthesis.

spectrum, without sharp absorption bands. During the process of synthesis of nanoparticles, the plant phytochemicals present in the lemon peel extract, like phenolic and flavonoids, act as stabilising and reducing agents. These bio-molecules adsorb on the surface of the nanoparticles, thereby providing effective capping of the nanoparticles and increasing their stability. Therefore, the obtained UV-Vis spectrum is in accordance with the green synthesis of Fe_3O_4 nanoparticles and is consistent with the earlier reports describing the optical

characteristics of plant-mediated magnetite nanoparticles. However, further confirmation regarding the successful synthesis of nanoparticles in the required phase and purity needs to be done using complementary characterisation methods such as XRD, FTIR, and electron microscopy [15].

FTIR spectroscopy

FT-IR spectrum of plant extract-based Fe_3O_4 nanoparticle synthesis is shown in Fig. 4. There exist prominent peaks at 517 cm^{-1} and 430 cm^{-1} in

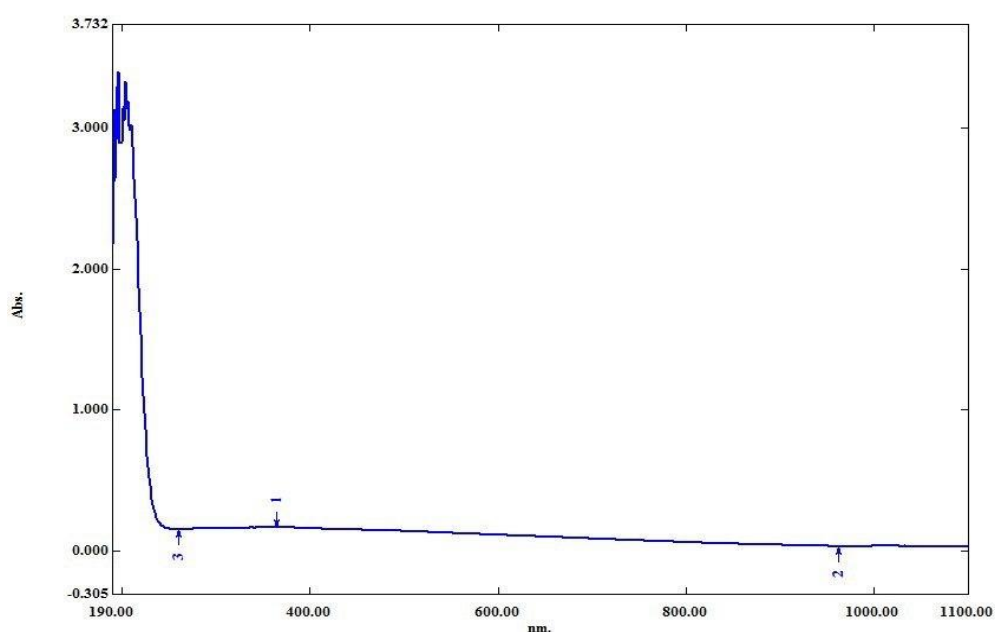


Fig. 3. Lemon peel UV-Vis analysis spectrum.

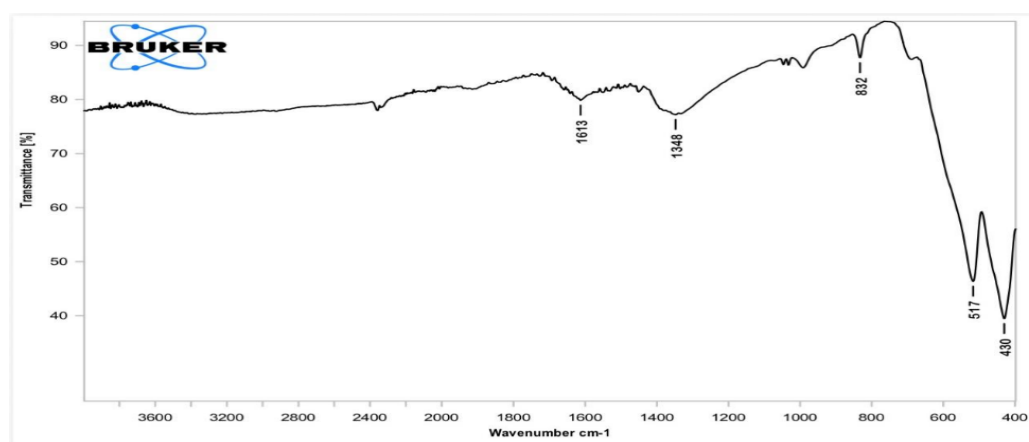


Fig. 4. FT-IR spectrum.

the low wavenumber region that correspond to the characteristic peaks for crystalline magnetite Fe_3O_4 NPs. While the peak at 517 cm^{-1} results from the stretching vibration of Fe-O bonds at the tetrahedral sites, the one at 430 cm^{-1} can be attributed to the stretching vibration of Fe-O bonds in the octahedral interstitial sites of the spinel structure. Moreover, other peaks confirm the existence of different functional groups of

biomolecules that are derived from plants and attached to the surface of the nanoparticles. The peak at 1613 cm^{-1} is attributed to the C=O stretching of carbonyl groups or C=C stretching of aromatic ring of polyphenols, flavonoids, or proteins that exist in the plant extract and are used as reducing agents. On the other hand, the peak at 1348 cm^{-1} corresponds to C-O or C-N stretching vibrations of carbohydrates and amino acids. Also, the peak at

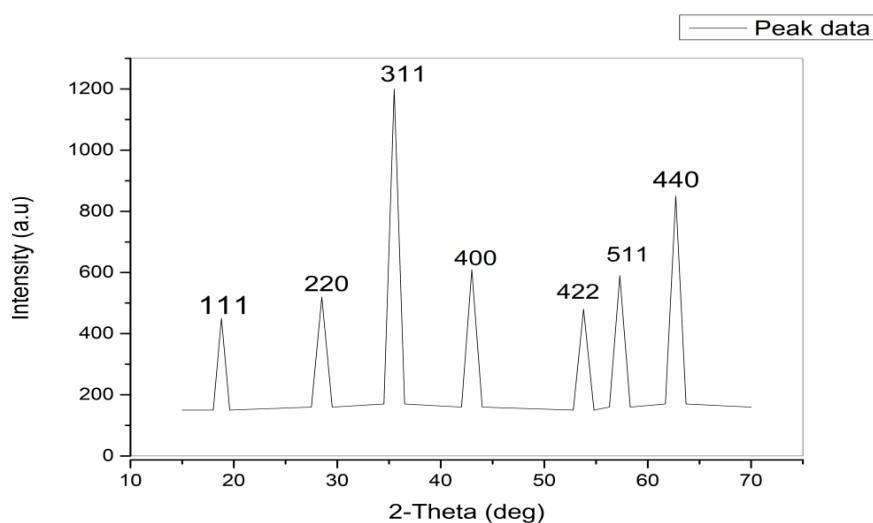


Fig. 5. Fe_3O_4 NPs powder's X-ray diffraction pattern.

Representative EDX Spectrum of Green-Synthesized Fe_3O_4 Nanoparticles

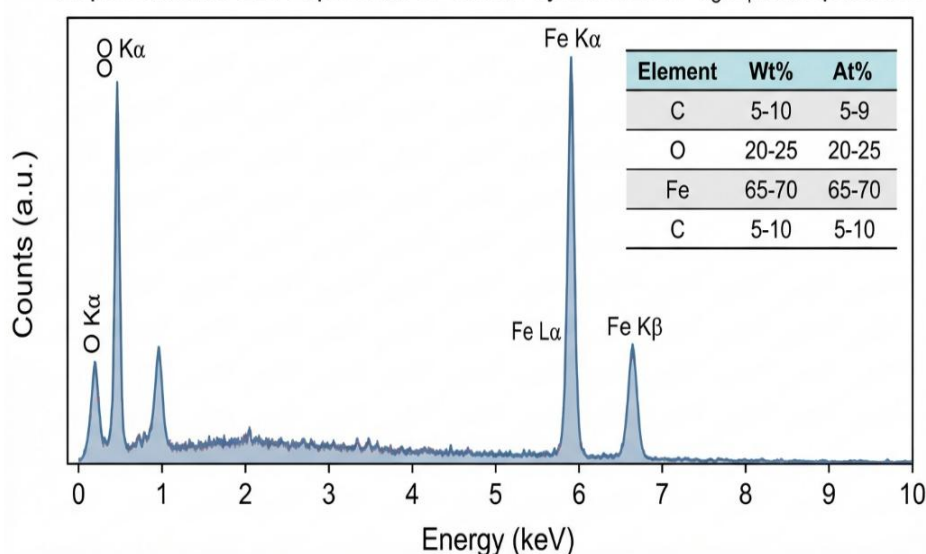


Fig. 6. Fe_3O_4 NPs' EDX spectrum.

832 cm⁻¹ is attributed to out-of-plane C-H bending vibrations of aromatic rings [16].

X-ray diffraction (XRD)

The crystal structure and phase purity of the green synthesized magnetite Fe₃O₄ nanoparticles were studied using x-ray diffraction technique (XRD). The XRD pattern of the synthesized sample is given in the figure below. The (111), (220), (311), (400), (422), (511), and (440) crystal planes are indexed using the diffraction peaks located at the (2θ) positions of about 19.2°, 30.1°, 35.4°, 43.0°, 53.4°, 56.9°, and 62.5°. The formation of well-crystallized, single-phase cubic inverse spinel structure of Fe₃O₄ was confirmed with the excellent match between all peaks and the standard JCPDS card no. 19-0629. [17].

The broadened peaks of diffraction indicate the nanoparticles' small size. The efficiency of plant extract as reducing and capping agent is evident through the lack of peaks belonging to any secondary phases or impurities (hematite or maghemite, for example). Using the highest peak (311) of diffraction and using the Debye-

Scherrer equation, the average crystallite size (D) of green synthesized Fe₃O₄ nanoparticles has been calculated. It was found out that the average crystallite size is 21.77 nm.

Analysis of Energy-Dispersive X-ray Spectroscopy (EDX)

EDX was applied to determine the elemental nature and purity of the synthesized green Fe₃O₄ nanoparticles. The successful synthesis of iron oxide nanoparticles was proved by the obtained EDX graph where distinct and intensive peaks of iron (Fe) and oxygen (O) were observed. In addition, there is also a prominent peak of carbon (C). There are strong proofs that the biomolecules (e.g. proteins, polyphenols, flavonoids) formed from the plant extract perform the role of nanoparticle surface capping/stabilization due to the peak of carbon. Fe and O are the primary elements, based on the results of the quantitative analysis of elements where the weight percentage almost perfectly matches the stoichiometric ratio of magnetite. No other prominent peaks were revealed [18].

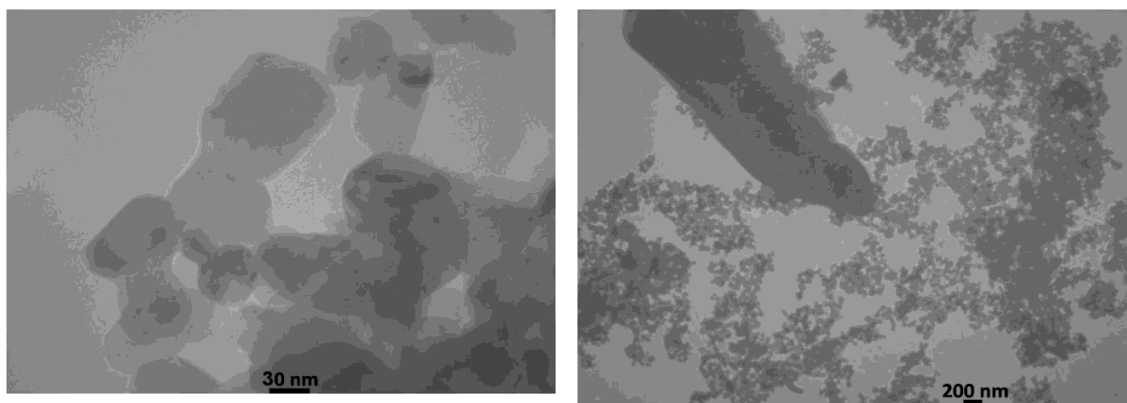


Fig. 7. Green-synthesized Fe₃O₄ nanoparticle TEM micrographs at (a) higher magnification with a 30 nm scale bar and (b) lower magnification with a 200 nm scale bar.

Table 1. Quantitative evaluation of The Radical scavenging activity (DPPH) of biogenic Fe₃O₄ nanoparticles compared to Ascorbic acid across varying concentrations.

Conc. (mg/mL)	Ascorbic acid (mean ± SD%)	Nano (mean ± SD%)
0.500	93.621±0.055	89.532±0.074
0.250	85.639±0.081	80.303±0.052
0.125	77.915±0.029	68.920±0.072
0.0625	63.860±0.035	53.211±0.062

Transmission Electron Microscopy

As seen in Figs. 7a and 7b, the morphology and the process of structuring the green synthesized Fe_3O_4 nanoparticles were extensively studied through TEM under different magnifications. As seen from their estimated average diameter ranging from 15 to 30 nm, the high-magnification microscopic image (Fig. 7a, 30 nm scale bar) proves that the nanoparticles have mainly quasi cubic and spherical shapes, which is remarkably similar to the crystallite sizes observed in the XRD analysis. However, the individual nanoparticles

exhibit a strong tendency to cluster tightly into highly inter-grained agglomerations rather than remaining isolated from each other. On the other hand, the low-magnification microscopic image (Fig. 7b, 200 nm scale bar) confirms that these nanoparticles assemble into large chain structures and nano clusters. The significant magnetic dipole-dipole attractions between the cores of magnetite as well as the steric effects resulting from the biomolecular coating matrix that originates from the plant sources work together to bring about such unique bundling and transition

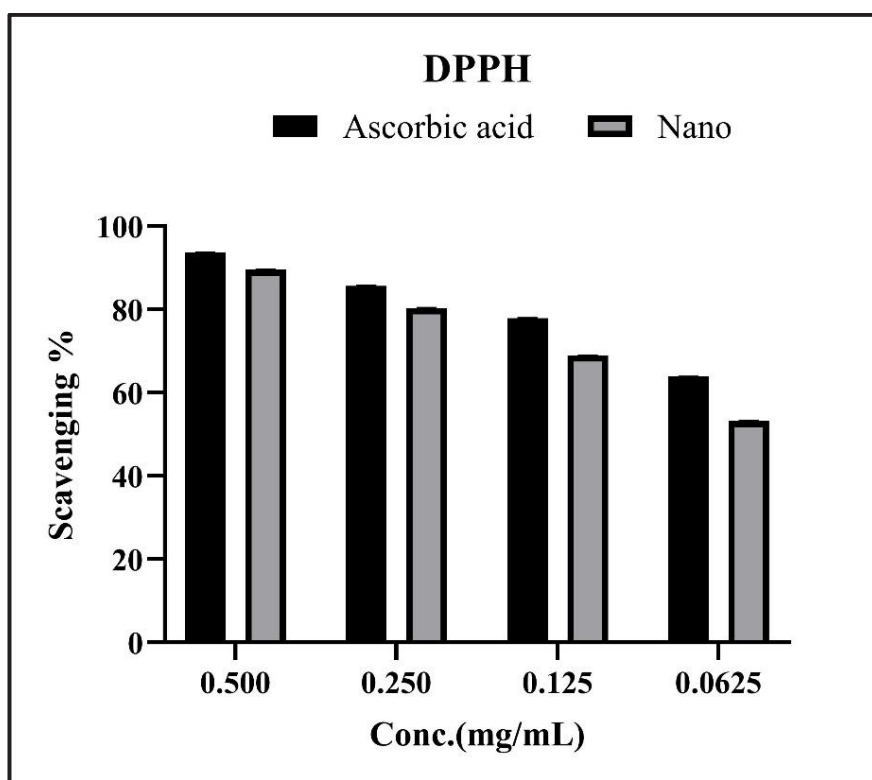


Fig. 8. Antioxidant activity of Fe_3O_4 nanoparticles synthesized using Citrus limon compared to ascorbic acid standard at different concentrations. Results are presented as Mean $\pm$ SD.

Table 2. In vitro cytotoxicity and cell viability (%) of biogenic Fe_3O_4 NPs on MRC-5 and MCF-7 across various concentrations.

Conc. ($\mu\text{g/mL}$)	Nano	
	MRC-5	MCF-7
	Mean $\pm$ SD	Mean $\pm$ SD
400	77.762 $\pm$ 0.578	42.062 $\pm$ 0.183
200	82.279 $\pm$ 0.637	50.732 $\pm$ 0.332
100	89.131 $\pm$ 0.732	64.225 $\pm$ 0.557
50	93.161 $\pm$ 0.776	76.974 $\pm$ 0.700
25	94.946 $\pm$ 0.334	88.170 $\pm$ 0.625

from individual boundaries to interconnected networks. This is quite similar to the results of the structural organization obtained in [19, 20], where the remnant core-to-core magnetic interactions promote the clustering behavior while the citric acid acts as the stabilizing interface.

Antioxidant Activity

Antiradical capacity was determined by employing the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) following the approach described by Rajesh and Natavar (15). This procedure determines the sample's capability to convert the violet DPPH radical to its yellow-colored, diamagnetic form via hydrogen atom transfer (HAT) or single-electron transfer (SET) reactions, which can be detected at 517 nm. The half maximal inhibitory concentration (IC_{50}) is a very important pharmacological index, in which low IC_{50} means high potency in free radicals scavenging. The quantitative results showed that both the commercial Ascorbic acid and the green synthesis of Fe_3O_4 NPs exceeded 50% of inhibition at the lowest used concentration of 0.0625

mg/mL ($63.860 \pm 0.035\%$ and $53.211 \pm 0.062\%$, respectively). This indicated that the actual IC_{50} values for both samples should be much less than 0.0625 mg/mL ($62.5 \mu\text{g/mL}$), and thus, the samples belong to an excellent class of radical scavengers. According to Table 1, it was found that the radical scavenging capacity has drastically grown depending on the concentration from 0.0625 up to 0.5.

However, the highest DPPH free radical scavenging efficiency was attained at the optimized level of 0.500 mg/mL ($89.532 \pm 0.074\%$), showing similar effectiveness to the standard ascorbic acid ($93.621 \pm 0.055\%$). Interestingly, at low concentrations of 0.125 mg/mL and 0.0625 mg/mL, the green synthesized Fe_3O_4 nanoparticles showed improved efficiency in free radical scavenging (77.915% and 63.860%) compared to ascorbic acid (68.920% and 53.211%). This improved antioxidant efficiency of the nanostructure is due to the interaction of two important chemical reactions [19,23], 1. Spinel Lattice Electron Transfer (Single-Electron Transfer / SET Reaction) The crystal structure of magnetite (Fe_3O_4) is characterized

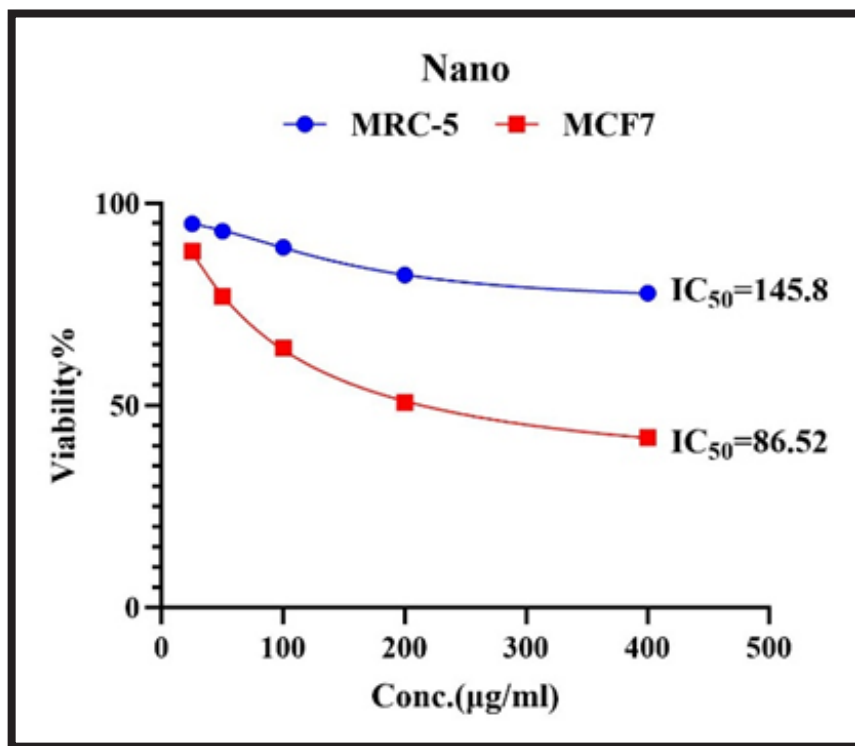


Fig. 9. Graphs depicting cell viability and IC_{50} for Fe_3O_4 NPs in the MCF-7 cancer line compared to the MRC-5 fibroblast line.

by an inverse spinel structure with Fe cations occupying both Fe²⁺ and Fe³⁺ oxidation states in the lattice sites (octahedrally coordinated) [22,23]. The close presence of Fe²⁺ and Fe³⁺ ions within these octahedrally coordinated sites creates a low-activation energy electron hopping reaction ($\text{Fe}^{2+} \leftrightarrow \text{Fe}^{3+} + e^-$) [23]. This fast electron transfer acts as a constant source of electrons, which subsequently interact with the DPPH's nitrogen-centered radical through a single-electron transfer reaction [22, 23]. Since this electron transfer process occurs internally within the spinel lattice, the radical neutralization process is fast without damaging the lattice or the core particle [23].

2. Phytochemicals Synergy on the Nanosurface (Hydrogen Atom Transfer/HAT) The plant-based approach for synthesizing iron oxide nanoparticles utilizes citrus extracts to modify the surface of Fe₃O₄ nanoparticles with secondary metabolites, such as flavanone glycosides (hesperidin, eriocitrin) and organic acids (citric acid). The interaction between surface-exposed iron ions and the phenolic hydroxyl (-OH) groups of polyphenols modifies the charge distribution in the ligands [19,22]. The change in the electron distribution in the ligands decreases the bond dissociation energy between the phenolic O and H atoms, increasing hydrogen atom transfer from the phenolic group to the radical center [23,20,25].

Cytotoxicity effect

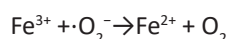
According to the results of the quantitative analysis, it became clear that both cell lines exhibited a decrease in cell viability based on the dosage. However, according to the reports, there was a clear variation in response to the nanoparticles by both cell lines [26]. The cancer MCF-7 cells manifested the sensitivity to synthesized Fe₃O₄ nanoparticles, thereby showing the decrease in cell viability to $42.062 \pm 0.183\%$ under exposure at maximum concentration (400 µg/mL). It should also be highlighted that the half-maximal inhibitory concentration (IC₅₀) for the MCF-7 line is about 205.4 µg/mL, with the viability drop to 50% ($50.732 \pm 0.332\%$) at 200 µg/mL dose. In comparison to the MCF-7 cells, normal MRC-5 cells demonstrated an increased tolerance and stability to nanoparticles since their viability value was kept above $77.762 \pm 0.578\%$ even under the highest dosage representing 400 µg/mL [27]. In this case, the value of IC₅₀ was significantly higher than the value for MCF-7 (IC₅₀ > 400 µg/mL) [26].

In order to test the biocompatibility and safety of produced Fe₃O₄ nanoparticles (NPs) in terms of their biomedical aspect, in vitro cytotoxicity assay was conducted on MCF-7 (human breast carcinoma cell line) and MRC-5 (normal human lung fibroblast cell line), where the second cell line is the control one. Testing the compatibility and selectivity index (SI) of metal-based nanomaterials is an obligatory step that has to be completed before the materials will be tested in vivo. The values of cytotoxicity of cell lines are presented in Table 2 at different concentrations (25-400 µg/mL). Table 2 shows In vitro cytotoxicity and cell viability (%) of biogenic Fe₃O₄ NPs on normal human lung fibroblasts (MRC-5) and human lung adenocarcinoma cells (MCF-7) across various concentrations.

The outstanding chemo-selectivity of the *Citrus limon* peel-mediated Fe₃O₄ NPs is governed by the structural dissolution kinetics triggered exclusively by the altered physiological traits of cancer cells: It depends on The Autocatalytic Mixed-Valence Fenton Loop Malignant cells, including the MCF-7 phenotype, possess an inherently acidic lysosomal microenvironment (pH≈4.5–5.0) driven by accelerated metabolism, contrasting sharply with the neutral cytosol (pH≈7.4) of healthy somatic cells [28]. Upon receptor-mediated endocytosis of the Fe₃O₄ NPs into the MCF-7 lysosomes, the acidic environment induces rapid dissolution of the core, releasing a synchronized influx of both Fe²⁺ and Fe³⁺ ions [26]. The co-released Fe²⁺ reacts immediately with endogenous hydrogen peroxide (H₂O₂), which is intrinsically overproduced in cancer cells, to trigger a classical Fenton reaction [29, 30]:



Crucially, unlike single-valence oxides, the simultaneously available Fe³⁺ ions actively participate in a continuous catalytic recycling loop via a Fenton-like reduction or Haber-Weiss cascade, reacting with intracellular superoxides (·O₂⁻) or H₂O₂ to regenerate Fe²⁺ [28, 30]:



This cyclic regeneration creates an unstoppable, self-sustaining intracellular factory of highly destructive hydroxyl radicals (·OH). The massive surge in reactive oxygen species (ROS) induces

terminal oxidative stress, triggering mitochondrial membrane depolarization, extensive lipid peroxidation, DNA fragmentation, and subsequent caspase-mediated apoptosis [31]. It also depends on. Receptor-Mediated Selectivity and Bio-Corona Protection The upregulated expression of Transferrin Receptors (TfR1) due to the iron-addiction phenotype of MCF-7 cells facilitates accelerated internal transport of the nanoparticles [32]. Meanwhile, the rich phytochemical corona (flavonoids and organic acids from the lemon peel) bound to the Fe₃O₄ crystal surface maintains highly stable steric protection in the neutral pH environment (pH≈7.4) of the normal MRC-5 cells [33–38]. This prevents premature ion leaching and blocks non-specific toxic cascades, ensuring high viability in healthy fibroblasts.

CONCLUSION

This study demonstrated the feasibility of synthesizing magnetite (Fe₃O₄) nanoparticles using Citrus limon peel extract, providing a sustainable approach to valorizing agricultural waste. Complementary characterization supported the formation of crystalline iron oxide nanoparticles with an inverse spinel structure, an average crystallite size of 21.77 nm, and particle diameters of approximately 15–30 nm. Spectroscopic findings suggested the involvement of plant-derived functional groups in nanoparticle formation and surface stabilization, although substantial aggregation was observed. The synthesized nanoparticles exhibited concentration-dependent antioxidant activity, achieving 89.53% DPPH radical inhibition at 0.50 mg/mL, compared with 93.62% for ascorbic acid. Cytotoxicity assays revealed preferential activity against MCF-7 breast cancer cells relative to MRC-5 normal lung fibroblasts. At 400 µg/mL, cell viability declined to 42.06% in MCF-7 cells, whereas MRC-5 viability remained at 77.76%. The estimated MCF-7 IC₅₀ was approximately 205.4 µg/mL, while the MRC-5 IC₅₀ exceeded the tested concentration range. These findings support further investigation of lemon-peel-mediated Fe₃O₄ nanoparticles as multifunctional biomedical candidates. However, the proposed contributions of oxidative stress, iron-mediated reactions, and phytochemical surface protection require direct experimental verification. Future studies should establish colloidal stability, cellular uptake, death pathways, and efficacy in additional cancer and

tissue-matched normal models, followed by comprehensive in vivo safety and biodistribution assessments.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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