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ORIGINAL STUDY

Formulation and Evaluation of *In-Vitro* Anti-Cancer Activity of Iron Nanoparticles on MCF-7 & A-475 Cells

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ABSTRACT

Nanomedicine is an interdisciplinary field within the developing nano medication delivery system. Ayurveda, a traditional form of healing, employs nanoparticles known as Bhasmas. Nanoparticles can be selectively designed to simultaneously deliver many medications. This innovative functionality enables meticulous regulation of drug release, therefore obviating the necessity for intricate dosing schedules. This enhances patient compliance with treatment. The aim of this work is to synthesise iron nanoparticles using an environmentally friendly process, evaluate the quality of the produced nanoparticles, and examine their possible anti-cancer properties by *in-vitro* experimental testing utilizing MCF-7 and A-475 cell lines. To produce neem leaf extract, fresh leaves of *Azadirachta indica* plants are used. The extraction procedure involves suspending the leaves in an Erlenmeyer flask filled with 100 mL of deionized water. The mixture is thereafter agitated and heated for a period of 20 min. The sample was incubated for the length of 30 min at a temperature of 25°C. The iron nanoparticle-containing solution underwent centrifugation at 5000 revolutions per minute (rpm) for a period of 15 to 20 min. Our findings align with prior research that have shown a notable decrease in cell viability of A-475 cells and breast cancer cells at inhibitory concentration (IC₅₀) levels of 30 µg/mL and 37 µg/mL, respectively, when exposed to FeNPs produced using environmentally friendly methods. Green FeNPs exhibited anticancer properties in MCF-7 and A-475 cells, suggesting their potential for therapeutic use in the treatment of lung and breast cancer in humans.

Keywords: Nanotechnology, Patient compliance, Neem leaf extract, Anti-cancer activity, Iron nanoparticles

1. Introduction

Cancer, a complex group of disorders, arises from aberrant cell division, resulting in the formation of

abnormal cells that can subsequently metastasize to other anatomical sites [1, 2]. Cancer is a prominent contributor to global mortality, ranking among the foremost causes of death across various diseases [3].

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Prior to the onset of this disease, there are observed abnormalities in cancer cell migration, proliferation, differentiation, and apoptosis [4]. Nanostructured materials have become increasingly significant in the field of biomaterials for the detection and management of intricate disorders, encompassing various infectious diseases and cancers.

The field of cancer treatment has made significant progress due to an enhanced comprehension of cancer biology, the intricate interactions within the microenvironment, the intricate signaling pathways, and the mechanisms underlying metastatic progression [5–7]. Currently, there is ongoing development of various medications such as angiogenesis inhibitors, transferase inhibitors, migration inhibitors, matrix metalloproteinase inhibitors, mitotic inhibitors and inhibitors of the epidermal growth factor receptor [8].

However, the efficacy of current cancer treatment methods has been constrained in terms of achieving significant improvements across various types of cancer due to the intricate nature of tumor progression, mechanisms of drug resistance, tumor compositions, and blood vessel structures. Paclitaxel is a chemotherapy medication used to treat various cancers by inhibiting the growth of cancer cells. It stabilizes microtubules, disrupting cell division and leading to cell death [9, 10]. It is typically administered as an intravenous infusion over hours, depending on the treatment regimen [11]. Some typical adverse effects include alopecia, emesis, lethargy, and heightened susceptibility to infections caused by reduced levels of white blood cells. Additional potential adverse effects encompass neuropathy, hypersensitivity responses, and cardiac toxicity. The neem tree contains various natural substances that are known to have beneficial properties [12–14]. This group of compounds comprises Limonin, Azadirachtin, Kaempferol, Beta carotene, and Ascorbic acid. Furthermore, apart from reducing oxidative damage in the human body, these phytochemicals have the capacity to enhance the immune system, reduce inflammation, and hinder the growth of pathogenic cells [15]. The application of neem leaf extracts has been found to induce apoptosis, thereby inhibiting the proliferation of leukemia and melanoma cell lines. The user has provided a numerical reference. The utilization of silver nanoparticles that have been modified with neem phytochemicals with anticancer properties could potentially enhance the precision of cancer treatment.

MCF-7 and A-475 are human cell lines used in scientific research, particularly in studying cancer. MCF-7, derived from breast cancer tissue, expresses estrogen receptors, aiding in studying hormone-responsive breast cancers and developing treatments

[16]. A-475, derived from human skin, studies melanoma, a type of skin cancer, aiding in understanding its genetic and molecular basis and testing the efficacy of anti-melanoma drugs and therapies.

Cytotoxicity in cancer cell lines like MCF-7 and A-475 involves multiple signaling pathways, which can vary depending on the treatment or stressor applied. Common pathways associated with cytotoxicity include phosphatidylinositol 3-kinase (PI3K)/Protein Kinase B (PKB), Mitogen activated protein kinases (MAPK)/Extracellular signal-regulated kinase (ERK), Estrogen Receptor Signaling, p53, B-Raf proto-oncogene, serine/threonine kinase (BRAF)/Neuroblastoma Rat Sarcoma, viral (v-ras) oncogene homolog (NRAS) [17].

In MCF-7, PI3K/PKB is activated in response to growth factors, leading to apoptosis and increased sensitivity to chemotherapeutic agents. MAPK/ERK is activated by various mitogens and growth factors, promoting cell proliferation and survival. Estrogen Receptor Signaling is driven by Estrogen in Estrogen receptor-positive breast cancer cells, and can be targeted by anti-estrogen therapies to induce cell death in hormone-responsive breast cancers [18]. p53 is activated in response to DNA damage and stress, leading to cell cycle arrest and apoptosis.

In A-475, MAPK/ERK is activated due to mutations in BRAF or NRAS, and inhibitors can induce apoptosis in melanoma cells with specific mutations. PI3K/PKB is frequently activated in melanoma, and inhibition can induce cell death and sensitize cells to other treatments. JNK is activated by stress and cytokines, and p53 is activated in response to genotoxic stress, promoting apoptosis and cell cycle arrest [19]. These nanoparticles possess distinctive physical and chemical characteristics, as well as the ability to easily undergo synthesis and surface modification. Furthermore, they exhibit bactericidal effects and demonstrate favourable biodistribution and biosafety profiles [20, 21].

The compound known as Nimbolide, which is derived from the neem tree (*Azadirachta indica*), has garnered significant interest in scientific research due to its notable abilities to inhibit cell proliferation and induce apoptosis. The utilization of neem compounds for functionalization as both synthesis and capping agents has demonstrated significant anti-cancer properties against Gastric cancer cells in vitro. The concentrations of biochemical factors such as albumin, glucose, and DNA were adjusted in conjunction with the activation of protease inhibitor and catalase. Additionally, specific cancer-related proteins, including p53, GRD 70–78 kDa, and other proteins ranging in size from 35–40 kDa corresponding to the H⁺K⁺ATPase protein [22] were modulated.

Nanotechnology is a multidisciplinary field that has significantly impacted the medical field, including cancer treatment, cardiovascular conditions, and vaccine development. The nascent discipline of “nanomedicine” uses nanoscience principles and techniques to address disease diagnosis, treatment, and prevention. The FDA-approved first cohort of nanoparticle-based therapeutics includes lipid systems like liposomes and micelles [23], as well as inorganic nanoparticles like silver, iron nanoparticles, and gold.

Iron oxide nanoparticles are biocompatible nanoscale materials that induce cellular bioactivity when introduced into bodily fluids.

The mechanisms of action of iron nanoparticles (FeNPs) vary depending on their given use. Magnetic characteristics of these materials facilitate precise administration of drugs and application of high temperatures for cancer therapy.

They participate in redox reactions, making them effective in environmental remediation. Their specific surface area enhances their reactivity, making to adsorb and relate with other molecules or ions [24].

FeNPs can affect cell mechanisms through reactive oxygen species (ROS) production, gene expression, enzyme activity, signal transduction, and environmental mechanisms. They can generate ROS, which can induce cell death in cancer therapy. They can also influence enzyme activity by acting as cofactors or altering the local environment of enzymes [25]. They can interfere with cellular signaling pathways, impacting processes like cell proliferation, apoptosis, and differentiation.

Iron nanoparticles (FeNPs) find extensive use in several medical fields such as targeted medication delivery, cancer treatment, imaging, environmental cleanup, and industrial technologies. These materials have the potential to serve as carriers for precise drug delivery, break down pollutants via redox processes, and efficiently eliminate toxins from water.

FeNPs can also be used as catalysts in chemical reactions, enhancing reaction rates and selectivity [26]. Additionally, their high surface area and conductivity can improve the performance of batteries and supercapacitors, making them a valuable resource in various industries.

2. Materials and methods

Ferric chloride, ethanol, deionized water, concentrated hydrochloric acid, and ammonia are all obtained from SD Fine Chem Limited. The laboratory equipment utilized in this study includes a conical flask, beakers, a burette, a burette stand, a stirrer, a

measuring cylinder, magnetic beads, a magnetic stirrer, a volumetric flask, a thermometer, and a heating mantle.

2.1. Formulation of leaf extracts from *azadirachta indica* neem leaves

Fresh *Azadirachta indica* leaves were obtained from the premises of the Munganoor village.

The leaves underwent a thorough washing process in order to eliminate any dust particles present. The substance was subjected to solar drying in order to eliminate moisture, after which it was pulverized into a fine powder. A quantity of 15 g of powdered Neem leaves was introduced into a 250 mL Erlenmeyer flask, which already contained 100 mLs of deionized water. The mixture was then subjected to stirring and heated for a duration of 20 min. The sample was allowed for incubation for a duration of 30 min at a temperature of 25°C. The solution was subjected to centrifugation at a speed of 5000 rpm for a duration of 30 min at a temperature of 25°C. A transparent solution was obtained and subsequently underwent filtration, after which the resulting filtrate was subjected to preliminary phytochemical investigations.

2.2. Synthesis of iron nanoparticles

A solution of ferric chloride with a concentration of 1 mM was prepared, and subsequently, a recently prepared plant extract was transferred into an Erlenmeyer flask. A solution of FeCl₃ was prepared and transferred into a burette. The FeCl₃ solution was carefully dispensed from the burette into the flask containing the plant extract, while maintaining constant agitation using a magnetic stirrer. Throughout the entire process, a noticeable alteration in the colour of the solution was observed, transitioning from a reddish-brown hue to black. This change in colour signifies the reduction of iron and the subsequent formation of iron nanoparticles. Iron nanoparticle solutions were prepared using three different concentrations, namely 1:2, 1:4, and 1:5, which represent the ratio of FeCl₃ solution to plant extract. The solution containing iron nanoparticles was subjected to centrifugation at a speed of 5000 rpm for a duration of 15 to 20 min. Subsequently, the resulting pellet was diluted.

2.3. Characterization of iron nanoparticles

2.3.1. UV visible spectral analysis

The aqueous component of this biological sample was reduced and subsequently utilized to quantify the absorbance of the solution. The UV-visible spectra

of the concentrations 1:2, 1:4, and 1:5 was obtained, with the 1:5 ratio demonstrating the most favourable outcome. The UV-Vis spectral analysis was conducted using a UV-Vis spectrophotometer within the wavelength range of 200–800 nm.

2.3.2. FTIR spectral analysis

The ferric chloride solution that has undergone bio reduction was centrifuged at 12000 rpm for 20 min. The resultant pellet was collected and characterised using Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) in the 4000–400 cm^{-1} spectral region.

3. Results and discussion

3.1. NANOTRAC

3.1.1. Procedure

The nanotrac cuvette should be cleansed using deionized water, followed by the implementation of the standard operating procedure (SOP) specific to the colloidal solution. Once the program has been configured, it is necessary to execute the background setup. The background value must fall within the specified limit values; otherwise, it will be necessary to repeat the process and reconfigure the program. In order to obtain accurate measurements of the size of the colloidal solution, it is necessary to first clear the background before running the software.

3.1.2. Spectroscopic studies

3.1.2.1. UV visible spectroscopy and colour change analysis of iron nanoparticles synthesised using green processes. The process of reducing Fe^{+3} in aqueous solutions was observed by periodically extracting samples from the mixture and subsequently analyzing the UV-Vis spectra. The UV-Vis spectral analysis was conducted using the Systronics 118 UV-Vis spectrophotometer within the wavelength range of 300–500 nm. The analysis revealed the presence of an absorption peak at 270 nm, which can be attributed to the excitation of surface plasmon vibrations in the iron nanoparticles. This absorption peak closely resembles the characteristic UV-visible spectrum of metallic iron, and it was duly recorded. Verification of the creation of iron nanoparticles was shown by the observation of suitable surface plasmon resonance (SPR) with high band intensities and peaks in the visible spectrum, utilising a leaf extract (Fig. 1).

The size of the colloidal iron nanoparticles was determined using the Nanotrac instrument equipped with Microtrac-ink software (version 10.6.2). The analysis revealed mean average diameter to be 37.5 nm (Fig. 2).

Table 1. Mammalian cell culture.

Cell lines	Cell type (Human)	Tissues	Medium
293	Fibroblast	Embryonic kidney	MEM & FBS
A549	Epithelial	Lung carcinoma	F-12k & FBS
BHL-100	Epithelial	breast	McCoy5A & FBS
Chang	Epithelial	liver	BME & calf serum
Jurkat	Lymphoblast	lymphoma	RPMI1640 & FBS

3.1.2.2. TEM of iron nano particles.

3.1.3. Cytotoxicity assays

3.1.3.1. MTT test. The assessment of cytotoxicity was conducted using the MTT colorimetric assay, 24 hrs after incubation with FeNPs. A stock solution of dimethylthiazolyl-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, 1 mg ml^{-1}) in PBS was prepared immediately prior to its utilization. Each culture dish without a coverslip received a 500 μL volume of the MTT solution, which contained 50 μg ml^{-1} MTT in culture medium. The cells were incubated for a duration of 3 hrs. Following this, the reduced formazan was extracted using 500 μl of dimethylsulfoxide. The absorbance of the extracted formazan was then measured at a wavelength of 570 nm using a Spectra Fluor spectrophotometer manufactured by Tecan Group Ltd, located in Männedorf, Switzerland. Cell viability was quantified as the percentage of absorbed treated cells relative to the absorption of control cells.

3.1.3.2. Trypan-blue exclusion test. The quantification of cell viability was performed using the Trypan blue dye exclusion method. In a concise manner, trypsin was introduced to both control and treated cells following a 24 hr incubation period. Following the detachment of cells from the plate, they were subsequently resuspended in culture media (Table 1). The experiment involved combining equal volumes of cell suspension and trypan blue solution (0.2% in phosphate-buffered saline) for the purpose of cell counting using a hemocytometer. The cells that exhibited a blue stain were classified as nonviable cells, while the cells that did not exhibit any staining were classified as viable cells. The optimum temperature for culturing of cells depends on the body temperature of the host from which they have been isolated. Most of the human and mammalian cell lines are maintained at 36°C–37°C for their optimum growth. Simple medium such as MEM supplemented with serum is suitable for the growth of continuous mammalian cell lines.

3.1.3.3. Dose-dependent cytotoxicity/cell viability of FeNPs in MCF-7 and A-475 cells. The present study

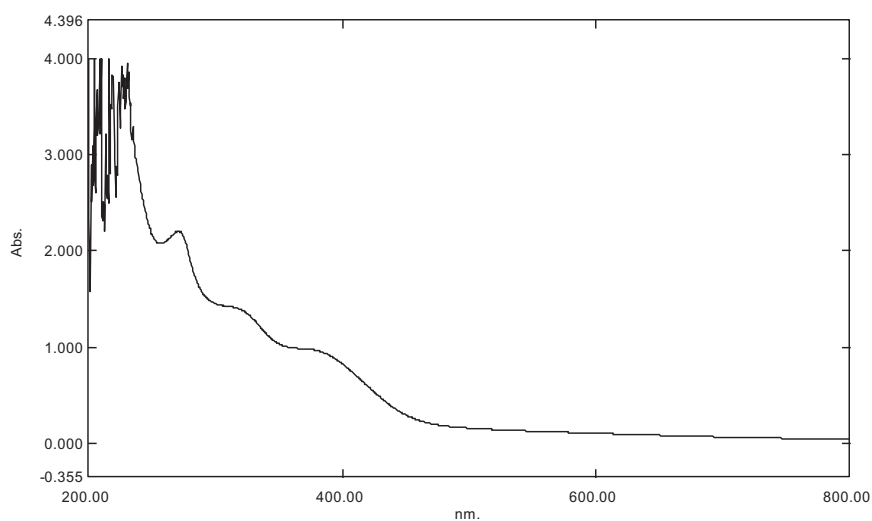


Fig. 1. UV-Visible spectrum green synthesized iron nanoparticles.

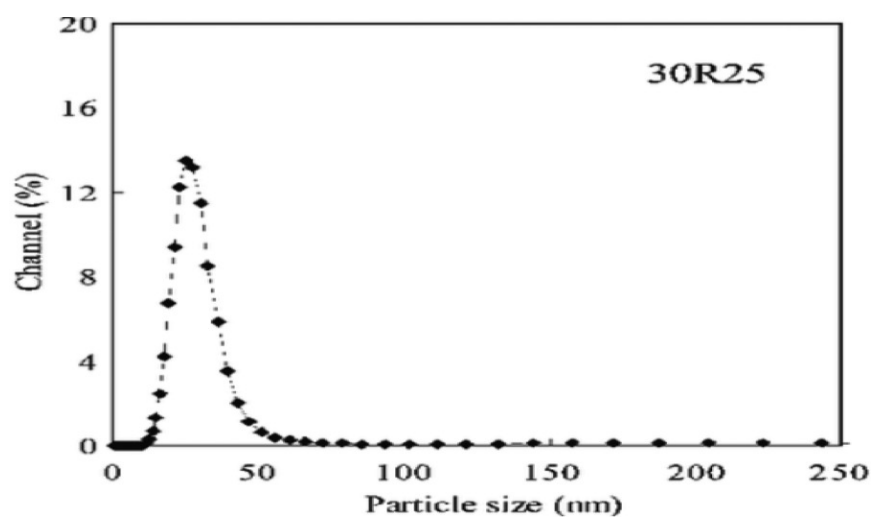


Fig. 2. Nanotracer of ferric chloride nano-particles.

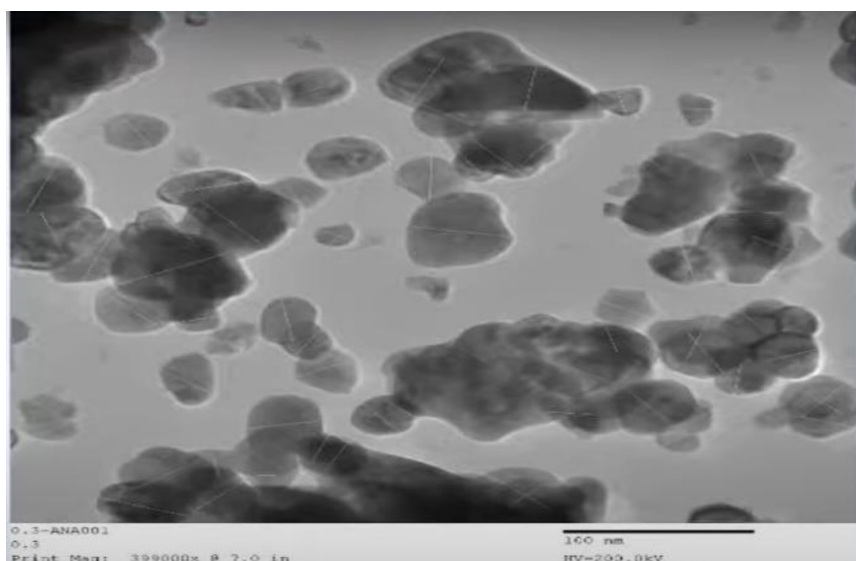


Fig. 3. Transmission electron microscopic image of iron nanoparticles.

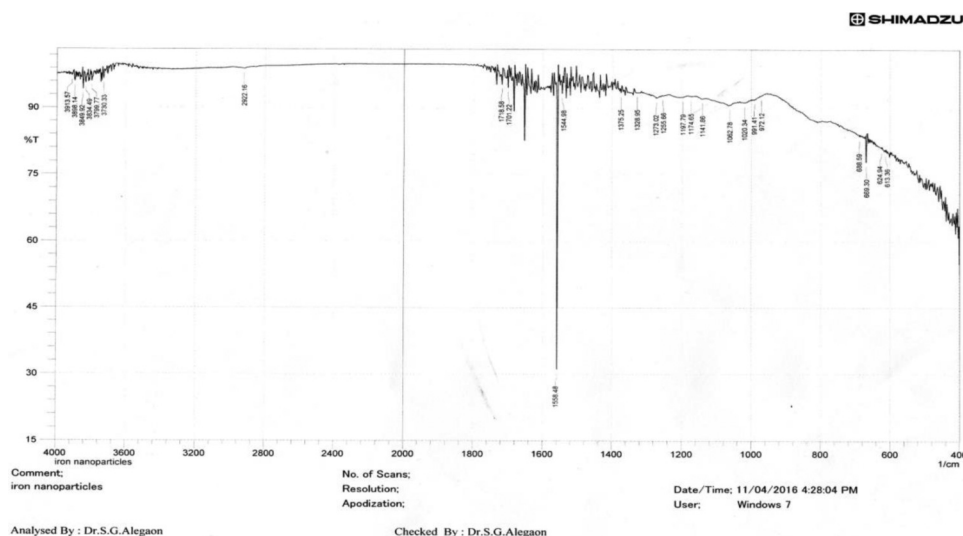


Fig. 4. FTIR of iron nanoparticles.

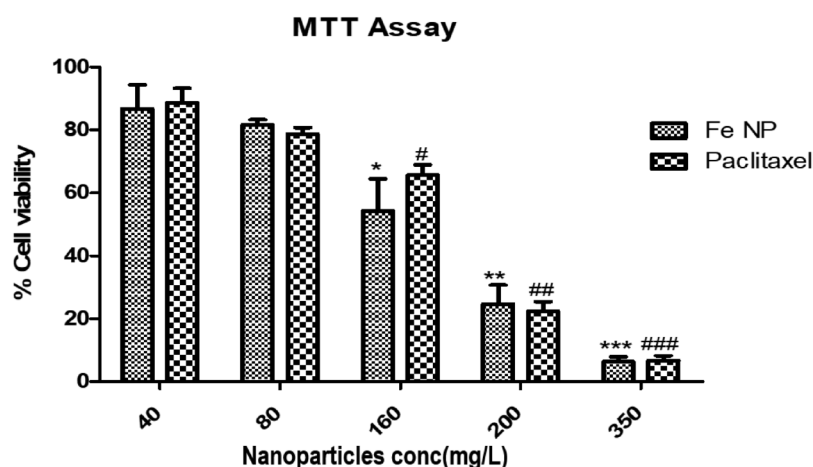


Fig. 5. MTT assay of *A. indica* mediated FeNPs on MCF-7 cells.

investigated the capacity of green FeNPs to reduce MTT in MCF-7 cells. Our findings indicate a significant enhancement in cytotoxicity within cells following treatment with 350 ppm of *A. indica*-mediated FeNPs. The application of varying concentrations (80, 160, 200, and 350 ppm) of *Azadirachta indica*-mediated FeNPs demonstrated a notable suppression of cell proliferation in MCF-7 cells. The application of 350 ppm of green iron nanoparticles (FeNPs) resulted in a mere 2% cell viability in MCF-7 cells. The impact of FeNPs synthesized through a green method on the percentage of cell viability in MCF-7 cells was assessed (Fig. 5). The cells were subjected to treatment with green and chemically synthesized FeNPs at varying concentrations ranging from 0 to 350 ppm for a duration of 24 hrs. The assessment of cytotoxicity was conducted using the MTT method. The data presented in this study are represented as the mean $\pm$ standard

deviation (S.D) obtained from three separate and independent experiments. *,# $p < 0.05$, **,## $p < 0.01$, ***,### $p < 0.001$ significantly different from control.

The use of *Azadirachta indica* to mediate the synthesis of FeNPs results in the modification of cell shape or morphology. The capacity of green FeNPs to reduce MTT in A-475 cells. The findings of our study indicate a significant enhancement in cellular cytotoxicity upon exposure to 240 mg/L of *Azadirachta indica*-mediated FeNPs. The application of various concentrations (40, 80, 160, 200, and 240 mg/L) of *Azadirachta indica*-mediated FeNPs resulted in a notable suppression of cell proliferation in A-475 cells. When subjecting A-475 cells to the treatment of 240 mg/L of green FeNPs, it was observed that only 4% of the cells exhibited viability.

The provided diagram, labeled as Fig. 6, illustrates the visual representation of the data. The impact of

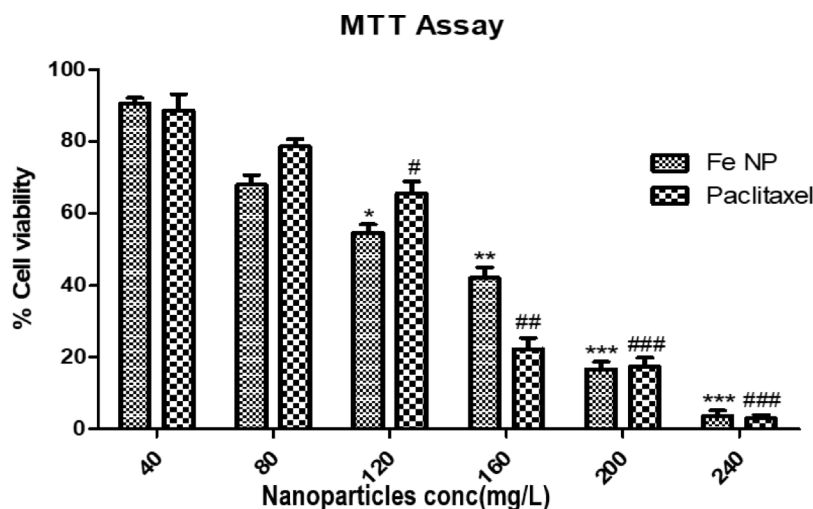


Fig. 6. MTT assay of *A. indica* mediated FeNPs on A-475 cells.

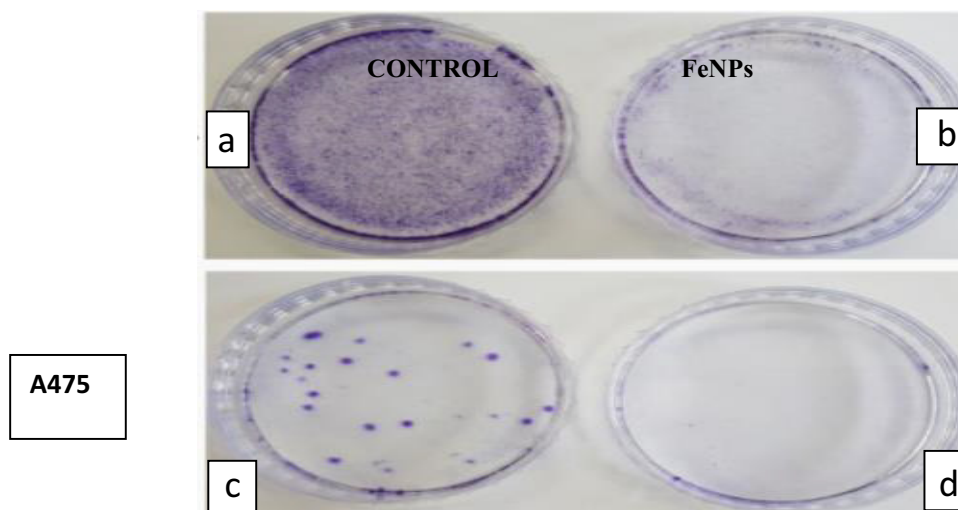


Fig. 7. Effects of FeNPs on colony formation or clonogenic ability of MCF-7 and A-475 cells. Culture dishes represent the number of colonies formed in control and FeNPs treated cells.

FeNPs synthesized through a green method on the percentage of cell viability in A-475 cells. The cells were subjected to treatment with green and chemically synthesized FeNPs at various concentrations ranging from 0 to 240 mg/L for a duration of 24 hrs. The assessment of cytotoxicity was performed using the MTT method. The data presented in this study are represented as the mean $\pm$ standard deviation (S.D) obtained from three separate and independent experiments. *,# $p < 0.05$, **,## $p < 0.01$, ***,### $p < 0.001$ significantly different from control.

Photomicrographs from phase-contrast microscopy (10 $\times$). Cell death induced by 24 h exposure to different concentrations of FeNPs in MCF-7 and A-475 cancer cells, replaced with fresh cell culture medium

or treatment with medium containing FeNPs. The cell morphology images were taken after 24 h.

4. Conclusion

The utilization of *Azadirachta indica* plant extract in the process of green synthesis has demonstrated the ability to generate FeNPs, which exhibit favorable stability in colloidal form. The nanoparticles exhibit favorable surface plasmon resonance behavior within the UV-visible wavelength range. Analysis using Nanotracer and TEM techniques revealed the presence of well-defined nanoparticles. Additionally, the formation of iron nanoparticles was confirmed

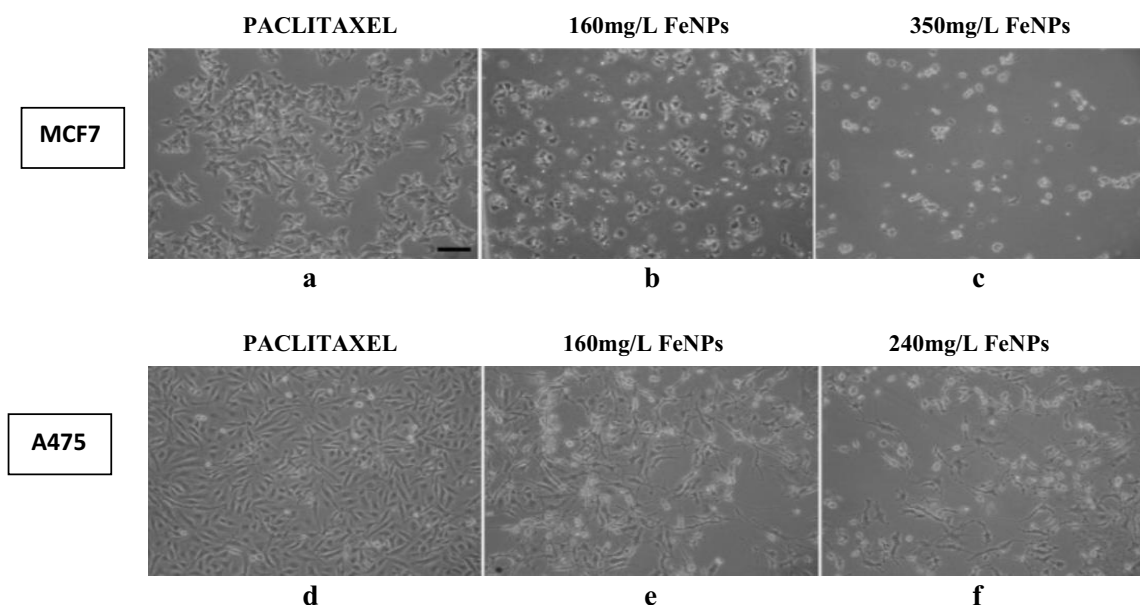


Fig. 8. FeNPs induced cytotoxicity in MCF-7 and A-475 cancer cells.

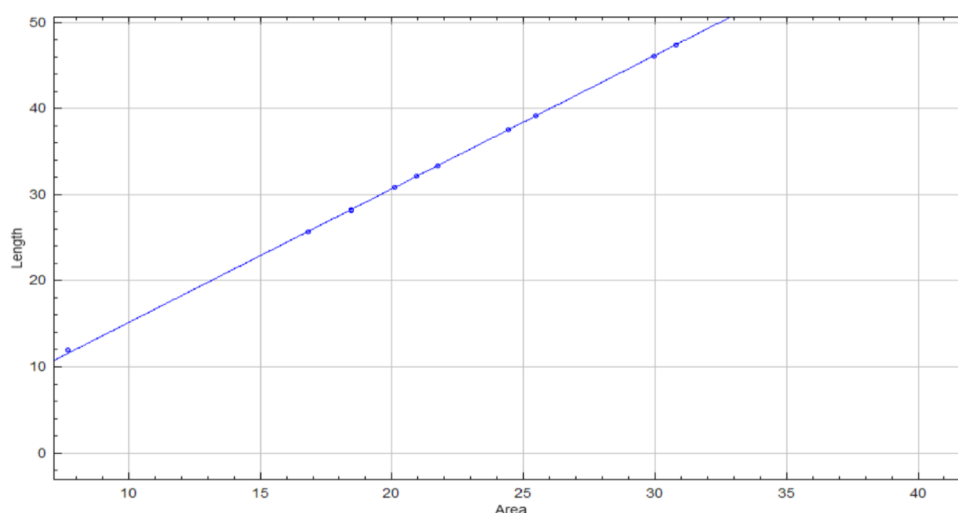


Fig. 9. Mean length and mean area of nanoparticles was found to be 37.52 nm & 24.43 nm respectively. Linear plot indicates uniformity in length and area of nanoparticles.

through the results obtained from FTIR analysis. The anticancer activity of green-synthesized FeNPs was observed in MCF-7 and A-475 cells, indicating that these nanoparticles hold promise for potential therapeutic applications in human lung cancer and breast cancer. Furthermore, it has been observed that green FeNPs exhibit a decrease in cell viability that is dependent on the dosage administered.

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Abbreviations

FDA: Food and Drug Administration; mM: millimolar; °C: Degree Celsius; h: hour; m: minute; rpm: revolutions per minute; ATR-FTIR: Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy; SOP: standard operating procedure; MTT: Mean transit time; FeNPs: iron nanoparticles; SD: standard deviation; TEM: Transmission electron microscopy.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interests with respect to research, authorship and/ or publication of this research paper.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Clinical trial

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

Conception: S,P,N,B; A, B
 Design: S,P,N,B; T, S, S; Y, N; S, V
 Supervision: S,P,N,B; T, S, S
 Literature: S,P; N,P; A, B
 Review: T, S, S; Y, N; S, V
 Writing: S,P,N,B; T, S, S; Y, N; S, V
 Critical Review: A, B; T, S, S

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