

Plant Extract Intervened Metal-based Ternary Nanoparticles Synthesis For Safer Pharmaceutical Application with Special Reference to Anti - Cancer Activity

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ABSTRACT

Biologically applicable Ternary nanostructured materials, fabricated by integrating three diverse metals, have been considered as promising useful material because of their synergetic as well as ligand effects. Owing to continued improvements in the production of ternary nanostructured materials, it is imperative to focus on recent advancements in medical field. Our paper work related with the synthesis and application of a novel Ternary (Cu-Ag-Fe) nano composite and by the way of Green chemistry method, it was successfully prepared using *Moringa oleifera* leaf extract with one-to-one metal salts precursors. The synthesised Cu-Ag-Fe nanoparticles were characterized using TEM, FT-IR, GC-MS, DLS (dynamic light scattering), and Zeta potential. We also reported the anticancer activity of Cu-Ag-Fe nanoparticles against Hela cancer cell line using MTT assay. The cytotoxicity data revealed that the IC₅₀ of Cu-Ag-Fe nanoparticles was 125.4 µg/ml. Our work provides promising advantages in biomedical applications. The Cu-Ag-Fe Nanoparticles may act as a potential beneficial molecule in cancer chemoprevention.

Key words: *Moringa oleifera*, Ternary nanoparticles, Hela cell, MTT assay, Cytotoxicity

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INTRODUCTION

Nanotechnology, a multidisciplinary field in constant expansion, has triggered remarkable advances in science and technology, with applications ranging from electronics to medicine. Within this fascinating scenario, an area of growing interest emerges is the "Ternary Elements in Nanotechnology". Understanding and manipulating systems composed of three distinct elements at the nanometric scale presents unique challenges and opportunities, promising to unveil new frontiers for innovative applications. The synthesis of uniform sized Ternary nanocomposite is very important because their properties include optical, magnetic, electrical and biological properties depending on their size and dimensions [1]. The synthetic methods are frequently classified in to three classes i.e. solution based synthesis, vapor phase synthesis, gas phase synthesis and green route synthesis[2]. Trimetallic particles are composed of three diverse metals with distinctive morphological features, are of keen interest in this regard. Trimetallic alloyed nanomaterials exhibit improved ORR catalytic performance, exceeding that of other reported nanocatalysts due to a wide range of fascinating properties in the ternary systems[3]. So, the construction of ternary nanostructured particles with metallic elements has been a key topic in current nanotechnology research[3].

Because of their adjustable features and markedly enhanced ORR electro-catalytic abilities makes it compared to single and binary nanocatalysts, such ternary structures have attracted a great deal of [4]. Furthermore, the ability to modify the compositions of trimetallic nanomaterials easier to prevent a general alteration in physio-chemical characteristics thanks to the presence of the third metallic component. The development of ternary nanomaterials are

one of the main strategies to overcome bimetallic catalyst limitations. The synergy between distinct metal elements, high selectivity, electronic and geometric effects, resistance to degradation, and structural flexibility of ternary nanomaterials attracts the research community to explore more about these materials[5].

Generally, biologically-derived chemicals or natural product extracts obtained from bio-renewable sources with their promising reducing, stabilizing and capping potentials can be deployed for the greener production of mono- and multi-metallic NPs[6]. Plant-mediated systems for the synthesis of mono/bi/trimetallic NPs, tubes, wires, rods, and alloys exploit the chemical constituents and ingredients of various plant systems, such as flavonoids, phenolic acid, glycosides, terpenoids, enzymes, proteins, vitamins, carbohydrates, alkaloids, and antioxidants as capping, stabilizing, and reducing agents[7].

Trimetallic nanoparticles (TMNPs) have emerged as a pivotal area of research due to their unique properties and diverse applications across medicine, agriculture, and environmental sciences. TMNPs, specifically addressing their unique properties and applications, which have been less examined compared to other multimetallic nanoparticles[8]. Secondly, they emphasize innovative biosynthesis methods utilizing microorganisms and plant extracts, positioning these green synthesis approaches as environmentally friendly alternatives to traditional chemical methods[9]. Furthermore, they integrate discussions on both medical and agricultural applications, highlighting their multifunctional potential across diverse fields[10]. Ternary metal nanoparticles advancements have led to the development of its allied fields, such as nanoparticle synthesis and their applications in the field of biomedicine. Nanotechnology driven innovations have given a hope to the patients as well as physicians in solving the complex

medical problems. Moreover, the physico-chemical and biological properties of Ternary nanoparticles can be modified depending on the applications[11]. Different Ternary nanoparticles have been documented with a wide range of applications in various fields of medicine and biology including cancer therapy, drug delivery, tissue engineering, regenerative medicine, biomolecules detection, and also as antimicrobial agents. Our current research paper summarizes the important approaches for the green synthesis of Ternary nanoparticles(**Cu-Ag-Fe Nanoparticles**), and their characterization using TEM, FTIR, GC-MS, and Zeta potential techniques. Finally the biomedical application related with anti- cancer activity also evaluated.

2. MATERIALS AND METHODS

2.1 Preparation of extract:

The leaf extract was prepared by collecting the selected *Moringa oleifera* and washed in running tap water and then rinsed with distilled water for the removal of impurities. 100g of leaves were chopped into small pieces with a sterile knife and were grinded using mortar and pestle and was then boiled for 15 min at 60 °C and filtered using Whatman's no. 1 filter paper. The filtrate was collected in clean, dry 250ml conical flask and stored in refrigerator for further use.

2.2. Preparation Precursors

For tri-metallic nanoparticle preparation, 50 mL of mixed precursor solution ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}:\text{AgNO}_3:\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (2:1:1)) at a concentration of 0.01 M was magnetically stirred [800 rpm] with temperature control at 60°C.

2.3. Biosynthesis of Nanoparticles

For the synthesis of Cu-Ag-Fe Nanoparticles, the crude *Moringa oleifera* leaf extract in aqueous solution having volume of about 10-20 mL was added drop wise into the conical flask containing 50 mL of mixed precursor solution ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}:\text{AgNO}_3:\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (2:1:1)) at a concentration of 0.01 M. 20 mL was added drop wise until pH at the end is about pH (9–10) (adjusted with water and NaOH [0.1M]). The reaction was carried out for 2 hours and the development of the reaction was checked by observing colour change from blue-green to dark brown, suggesting reduction process. After an hour of the brown colour formation, centrifugation at 10,000 rpm for 30 min was made, and the resultant sediments were washed with 90 percent ethanol, and dried in an oven at 60°C. A fine Cu-Ag-Fe nanoparticles in powder form was obtained.

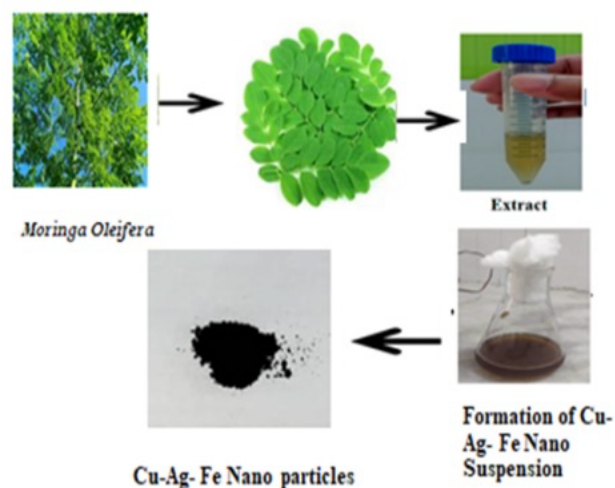


Figure 1. Explicit of CU-Ag-Fe nanoparticle synthesis

2.4.Characterization of CU-Ag-Fe nanoparticle

(a).Characterization by TEM

Transmission electron microscope(TEM), offers information about the crystal structure with high spatial precision. It generates a magnified image by focusing an electron beam on a sample. Objects can be magnified up to a million times with a TEM. Therefore, TEM is a highly effective method for characterizing materials. We can see objects to the order of a few angstrom (10-10 m). TEM instrument used for our characterization is Transmission Electron Microscope (Make: Philips, Model CM 200, Operating voltages : 20-200kv Resolution : 2.4 Ao). The resulted colloidal particles were characterized to determine their shape and size by high-resolution transmission electron microscopy (TEM). For the preparation of the TEM grid, a carbon-coated copper grid was used. A drop of the CU-Ag-Fe nanoparticle solution was placed over the grid and dried at room temperature. The solution was first dried for 1 hour in oven and then put 15 minute in front of IR lamp and after that TEM characterization is taken. The TEM image of different CU-Ag-Fe nanoparticle morphology that was captured with 200Kv was found that the size of CU-Ag-Fe nanoparticle spheres in the range from was obtained.

(b).FTIR assessment

FTIR Analysis of leaf extracts was used to identify the functional groups of compounds in crude powder of the leaves. The wavelength of light absorbed corresponds to features in the chemical bond, which can be observed in the annotated FTIR spectrum. The infrared absorption spectrum in FTIR is used to interpret the chemical bonds in the compound. For FTIR analysis, 10 mg dried powder of leaf sample was used to identify the functional groups of compounds in crude powder of the plants. The wavelength of light absorbed corresponds to features in the chemical bond, which can be observed in the annotated FTIR spectrum. The infrared absorption spectrum in FTIR is used to interpret the chemical bonds in the compound. For FTIR analysis, 10 mg dried powder of each sample was encapsulated in 100 mg of potassium bromide (KBr) pellet and translucent sample discs were prepared. The samples were run at infrared region between 400-4000 cm^{-1} and standard DLATGS detector was used at 2.8 mm/sec mirror speed. DLATGS stands for Deuterated L-Alanine-doped

Triglycine Sulfate. It is an advanced organic crystal material widely used as a high-performance pyroelectric sensor in infrared (IR) detectors, especially for Fourier-transform infrared (FTIR) spectrometers.

(c).GC-MS

analysis

The *Moringa oleifera* leaf extract was analyzed based on the optimized method using an Agilent 6890/5973 GC-MS system (Santa Clara, CA, USA). The separation was performed with an HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm). The injector was set at 325°C (split mode with the ratio adjusted to 5:1, injection volume = 1 µL). The oven temperature started at 70°C for 2 min, increased to 280 °C, then was held for 20 min at 20°C ramp/min. The flow rate of the carrier gas (helium) was maintained at 1.2 mL/min. The mass spectrometer was operated with ionization in the electron impact mode using 70 eV (ionization energy) to obtain the mass spectra that ranged from m/z 40 to 700. Identifications were made by comparison with NIST library data.

(d).DLS and Zeta potential analysis

For CU-Ag-Fe nanoparticle size distribution measurement in liquid and colloidal suspension, dynamic light scattering measurements technique was followed. In DLS Instrument 12 mm cell (DTS 0012) was used. One milliliter of distilled water (or ethylene glycol) was added to the cell and then 50 µl from CU-Ag-Fe nanoparticle stock dispersions were added. Samples were again sonicated for 5 minutes. Size distributions of the nanoparticles were determined with a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., GB) by the DLS technique. The suitable parameters (viscosity, absorption and refractive index) were chosen for each CU-Ag-Fe nanoparticle dispersants. For CU-Ag-Fe nanoparticle sample: absorption 0.1, refractive index 2.0, for water: viscosity 1.0031cP, refractive index 1.33, for ethylene glycol viscosity 1.98 cP, refractive index 1.44. The pH value of each suspension was adjusted by adding either NaOH or HCl. Histograms with the distributions of sizes were recorded.

The zeta potential was measured by analyzing 0.1 g of CU-Ag-Fe nanoparticle in 10 ml of water (or additives solutions) using the Zetasizer Nano ZS (Malvern Instruments Ltd., GB). Before zeta potential measurements all samples were sonicated for 5 minutes. Zetasizer Nano ZS uses Laser Doppler Velocimetry to determine electrophoretic mobility. The zeta potential was obtained from the electrophoretic mobility by the Smoluchowski equation. The dynamic method consisted of using ZS Malvern Zetasizer device coupled with an automatic titrator (Malvern MPT-2). pH of the suspensions was automatically adjusted by this automatic titrator using hydrochloric acid (0.25 and 0.025 mol/l) and sodium hydroxide (0.25 mol/l). Concentrations of additives (SDS, CTAB, NaCMC) were 5mmol/l.

(e) Anticancer activity

(i) MTT assay

The MTT test (EZ4U, Biomedica, Austria), on different cell lines. For the experiment, the cells were plated in 96-well plates of 100 µl at the concentrations of 5,000 cells/well (substrate-dependent cells) or 10,000–15,000 cells/well (suspension cells) overnight. Then, aliquots of 100 µl of experimental Cu-Ag-Fe nanoparticles (0, 1, 10, and 100 µg/ml) as well as the reference compound doxorubicin (0, 1, and 10 µg/ml) in culture medium were added to the cells

and incubated for the next 72 hours. Furthermore, the 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT reagent) was appended to the cells following the manufacture recommendations (Biomedica, Austria). The results of the reaction were determined by an Absorbance Reader Bio Tek ELx800 (BioTek Instruments, Inc., Winooski, VT) at 490 and 630 nm. The relative number of living cells (parts per unit) was determined by the ratio of optical densities measured for the wells with tested compounds to the wells with control (non-treated) cells. The half-maximal inhibitory concentration (IC50) of tested extracts was calculated as the extract concentration that reduced the cell viability to 50%. Doxorubicin (Actavis, Romania) was chosen as a reference drug as a positive control. The extracts were added to the cultured cells at the indicated concentrations (0–100 µg/ml) and then were treated for 72 hours. The results were analyzed and illustrated using GraphPad Prism 6 software (GraphPad Software., San Jose, CA). The obtained data are presented as the average ± standard deviation (SD) of three replications in two parallels (n = 6).

(ii). Analysis of apoptosis

Cell line Human Cervical Cancer cells (HeLa) were kindly provided by Cancer Institute, adayar, Chennai, The stored cells were first suspended in DMEM, to wash away DMSO, and collected by centrifugation at 1200rpm for 5min and 25°C. The washed cells were re-suspended and cultured in DMEM medium (Dulbecco's Modified Eagle Medium) supplemented by 10% heat-inactive Fetal Bovine Serum (FBS) and 1% Pen-Strep Antibiotic (100U/mL Penicillin and 100µg/mL streptomycin); at 37°C in 5% CO₂ incubator. Cells were sub-cultured after they formed a monolayer on the culturing flask and detached by treating with trypsin for 15min. cell suspension was mixed by pipetting to remove any clumps of cells just before a sample was taken. Then Cu-Ag-Fe nanoparticles determined earlier concentration were added and after 24 hours, complete medium was added to inhibit the further chemical reaction.

(iii).Cancer cell death image analysis

Morphological changes and death of HeLa cells treated with the different concentrations of Cu-Ag-Fe nanoparticles were observed by Operetta high-content imaging system (Perkin Elmer, Buckinghamshire, UK) for fluorescent microscopy. HeLa cells (2.5 × 10⁴ cell/mL, 0.1 mL/well) were incubated in a 96-well plate with 0.2 mL/well of three different doses (62.5, 125.0, and 250.0 µg/mL) of the selected Cu-Ag-Fe nanoparticles concentrations for 48 h at 37 °C with 5% CO₂. The cells were directly imaged for phase contrast microscopy, whereas Hoechst 33,342 dye (Invitrogen, Paisley, UK; Cat. No.: H1399) was applied for fluorescent microscopy following the manufacturer's instructions. The cells were stained with 0.5 µL dye (0.4 mg/mL) and incubated for 15 min prior to viewing.

(iv) Evaluation cancer cell death by flow cytometric method

Flow cytometer with Cell cycle analysis software, ModFit LT™ (Verity Software House)

Cell Culture

1. Suspend 0.5×10^6 MCF-7 breast cancer or PE01 ovarian cancer cells in 25 mL RPMI/PS/10% FCS and transfer to a 75-cm² tissue culture flask. Place in a 5% CO₂ humidified incubator at 37 °C.
2. Allow cells to grow to confluence, feeding twice per week by aspirating the spent media, washing with 20 mL PBS, and then adding 25 mL fresh tissue culture media.
3. When cells become confluent, remove spent tissue culture media and wash cells in 10 mL PBS. Remove excess PBS.
4. Add 5 mL Trypsin/EDTA to each flask and place back in the incubator until the cells have become detached (Note 5).
5. Transfer cell suspension to a sterile Universal container using a sterile pipet; pour 20 mL RPMI/PS/10% FCS (to deactivate the Trypsin) into the flask in order to wash it out and then pool with the cell suspension in the Universal container (total volume of 25 mL).
6. Centrifuge at 600 g for 5 minutes.
7. Pour off the media and resuspend the pellet in 5 mL RPMI/PS/10% FCS. Syringe with a 21G needle three times to break up the pellet, and then make up to 25 mL with RPMI/PS/10% FCS
8. Count total number of cells using a hemocytometer.
9. Transfer aliquots of 1×10^6 cells to FACS tubes (regardless of volume) and then centrifuge again at 600 g for 5 minutes.
10. Resuspend pellet in 100 μ L citrate buffer, cover tubes, and store at -40 °C prior to analysis.

Preparation of Samples for Analysis

1. Thaw Solutions A and B, along with the frozen whole cell suspensions (in 100 μ L) (but not heated) in a water bath at 37° before preparing and staining nuclei for DNA analysis. Allow Solution C to thaw at room temperature before placing on ice.
2. Digest cell suspensions down to nuclei by adding 450 μ L of 0.003% Trypsin solution (Solution A), mix and leave at room temperature for 10 minutes (Notes 6 and 7).
3. Prevent further degradation by adding 0.05% (w/v) Trypsin inhibitor solution and 0.01% (w/v) RNase A (Solution B) in a final volume of 375 μ L, mix, and leave for 10 minutes.

Finally, stain cells by adding 416 μ L/mL ice-cold propidium iodide/1.16 mg/mL spermine tetrahydrochloride solution (Solution C) in a final volume of 250 μ L and leave the samples on ice in the dark for an additional 10 minutes prior to analysis.

2.5. Statistical analysis

All experiments were conducted in triplicate. The data were assessed through one-way and two-way ANOVA, followed by Tukey's post-hoc test for multiple comparisons. Statistical evaluations were performed using Origin software (Version 2025, OriginLab Corporation, Northampton, MA, USA), with significance at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

TEM analysis was performed to know the morphological

shape and size of the synthesized CU-Ag-Fe nanoparticle, the particles are spherical in shape and size was averagely 70.6nm. This result indicates that the synthesized CU-Ag-Fe were crystalline and monodispersed. Selected area electron diffraction (SAED) pattern of CU-Ag-Fe Cu NPs were shown in Figure 2 a. and b.

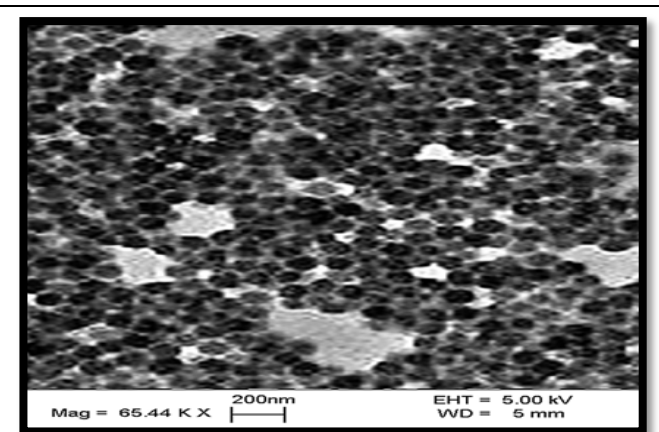


Figure 2.(a). Transmission electron microscopic image of Au-Ag-Cu nanoparticles at 200 nm scale bar shows spherical morphology of the nanoparticles.

Figure 2 (b). TEM image of Au-Ag-Cu nanoparticles at scale bar = 200 nm shows the nanoparticle size at the average diameter of 70.6nm. The surface potential of nanoparticles is the potential difference between the medium where nanoparticles are dispersed and the accessible surface of dispersed nanoparticles, which can be analyzed using a zeta sizer. The figure 3, demonstrates the zeta potential of the biosynthesized AgNPs which was found to be -30.8 mV. This shows that the CU-Ag-Fe Cu NPs synthesized from the leaf extract of *Moringa oleifera* are highly stable.

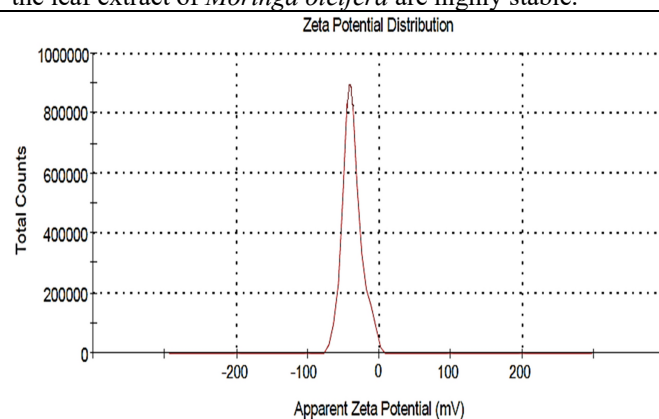


Figure 3. Zeta potential of Au-Ag-Cu nanoparticles

The bioactive compound of methanol extract of leaves of *Moringa oleifera* plant was traced out by FTIR spectrophotometer (Thermo electron Scientific). Totally 21 peaks were obtained for the FTIR analysis of *Moringa oleifera* methanol extract. The IR spectrum of *Cassia alata* sample showed an absorption band at 564.08 cm⁻¹ which corresponds to C-Cl stretching showed the presence of alkyl halides, band at 807.06 cm⁻¹ is C-H stretching exhibit the presence of, aromatic, bands at 1167.69 cm⁻¹, 1199.51 cm⁻¹, 1307.50 cm⁻¹, 1354.75 cm⁻¹, 1452.14 cm⁻¹, 1506.13 cm⁻¹, 1600.63 cm⁻¹, 1655.59

cm-1,1751.05cm-1,1999.82cm-1,2165.67 cm-1,2294.88 cm-1,2338.27 cm-1,2363.34 cm-1,2927.41 cm-1,2974.66 cm-1,3332.39 cm-1,3726.76 cm-1 and 3823.19 cm-1 indicate the presence of bioactive compounds such as sulfones, sulfonyl, chlorides, sulphates, sulfones, sulfates, sulfonamides, alkanes, aromatic, alkenes, ester, ketenes, isocyanates, isothiocyanates, acetylene, nitrile, phosphine, aldehyde, alkane, amide, and alcohol.

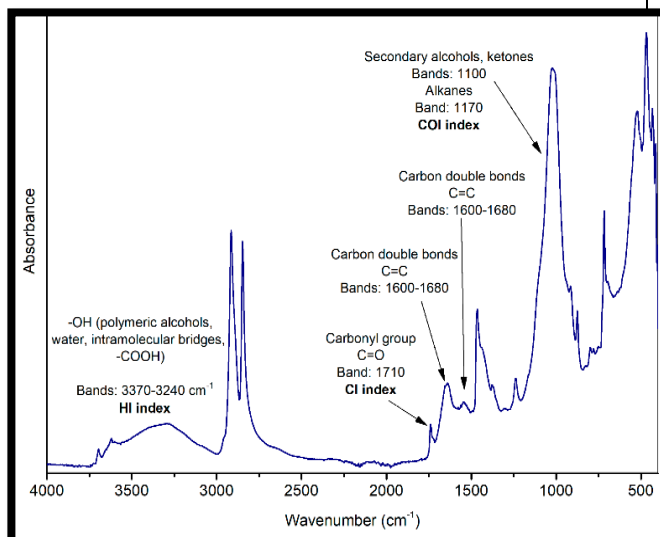


Figure 4. FTIR Spectrum of methanolic extract of *Moringa oleifera* shows the identity of functional groups

Table-1 FTIR Spectral shows functional groups

Wavelength [nm]	Wavenumber [cm ⁻¹]	Assignment
2857	3500	O-H stretch etch of hydroxyl groups
3125	3200	N-H stretch (amide A) of proteins
3379	2959	C-H stretch (asymmetric) of -CH ₃
3408	2934	C-H stretch (asymmetric) of >CH ₂
3423	2921	C-H stretch (asymmetric) of >CH ₂ in fatty acids
3450	2898	C-H stretch of C-H methine
3481	2872	C-H stretch (symmetric) of CH ₃
3506	2852	C-H stretch (symmetric) of >CH ₂ in fatty acids
5743	1741	>C=O stretch of esters
5830	1715	>C=O stretch of esters, RNA/DNA, OH-C=O
5899	1695	Amide I band components
5934	1685	resulting from antiparallel
5970	1675	pleated sheets and b-turns of proteins
6042	1655	Amide I of a-helical stretch ictures

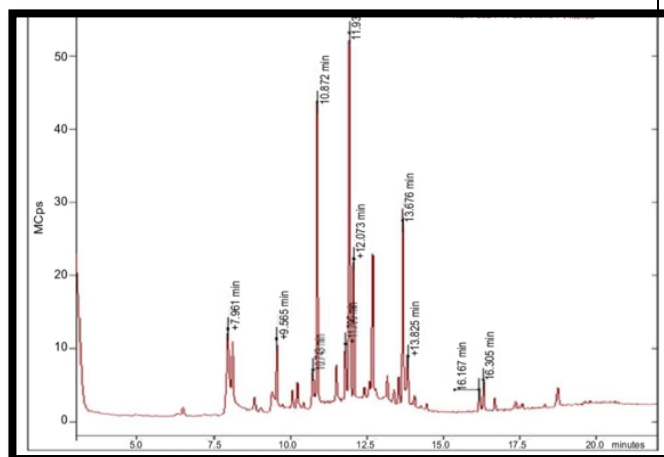


Figure 5. GC-MS analysis of *Moringa oleifera* leaf extract indicated the presence of compounds

The GCMS outline of *Moringa oleifera* indicated the presence of compounds in the plant extract shows, the retention time and compounds of 21.066-4,8,12,16- Tetramethylheptadecan-4- olide (C₂₁H₄₀O₂), 22.591 - Bis(2-ethylhexyl) phthalate (C₁₂H₁₄O₄), 24.232 - adamantane-1-carboxylic acid methyl ester (C₁₂H₁₈O₂),25.815 - Benzopyran-6-ol,3,4-dihydro-(C₂₂H₃₆O₂), 26.193 - gamma-Tocopherol (C₂₈H₄₈O₂),26.411 - dimethyl-5,5'-diphenyl-1H, (C₁₄H₇NO₃), 28.56 - 1Hbenzo[b]furo[2,3-f]indole (C₁₈H₁₆N₆S₂) and minimum presented retention time and compounds 16.303 Bicyclo [3.1.1]heptane, 2,6,6-trimethyl- (C₁₀H₁₈), 16.361 - 2- Pentadecanone, 6,10,14-trimethyl- (C₁₈H₃₆O),16.564 - Bicyclo [3.1.1]heptane, 2,6,6-trimethyl- (C₁₀H₁₈),16.637 phthalic acid (C₈H₆O₄), 16.739-3,7,11,15-Tetramethyl-2-hexadecen-1-ol (C₂₀H₄₀O), 17.145-5,9,13-Pentadecatrien-2-one, 6,10,14-trimethyl (C₁₈H₃₀O),17.595-2(3H)-Furanone,dihydro-5-methyl 5- pentyl (C₁₀H₁₈O₂), 17.828 - Hexamethyl-2,6,10,14,18,22-tetracosahexaen-1-yl]-6-methox (C₃₀H₅₀O), 18.525 - Oxalic acid, cyclohexyl isohexyl ester (C₁₄H₂₄O₄), 18.844 - 6-Octadecenoic acid (C₁₈H₃₄O₂), 19.077 - Phytol (C₂₀H₄₀O) were detected in the samples

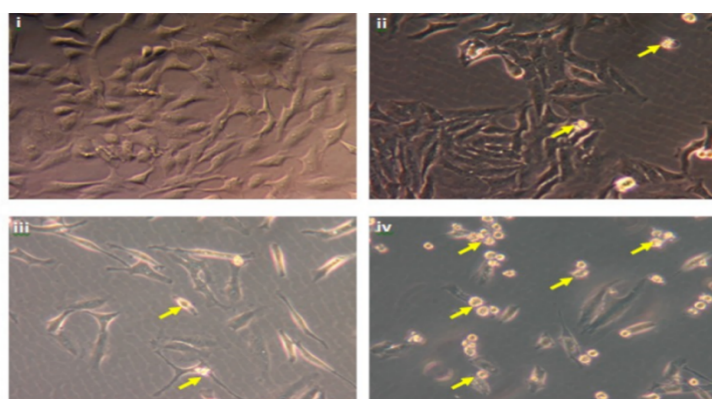
IUPAC name and 2D structure of bioactive compounds.

Dynamic light scattering (DLS) was used to evaluate the average size, size distribution, and possible aggregation of the synthesized Au-Ag-Cu nanoparticles in liquid medium. So the particle size in liquid medium is just few nano meters increased. As observed from the particle size distribution graph, an average particle size of 78.3 ± 0.9 nm and polydispersity index (PDI) of 0.574 were obtained. Regarding the zeta potential, the value found was -37.8 mV .

Poly dispersity describes the degree of “non-uniformity” of a distribution in liquid medium. This value was calculated based on the width of the size distribution. The range is from 0.0 (a perfect uniform sample) to 1.0 (highly polydisperse sample with multiple particle size). PDI values above 0.7 indicate a broad particle size distribution profile. The obtained PDI of 0.174 had a narrow particle size distribution and can be considered good polydispersity, with an acceptable value indicating moderate homogeneity. When particles are present in a

liquid, an electrical double layer (EDL) is usually formed, consisting of ions in the liquid, as shown in figure 6. This model explains the operation of the electrical repulsive force. The EDL surface consists of two layers. Usually, particles with ZP in the range of -30 mV to $+30$ mV are considered to be normally stable.

After treatment of HeLa cells with different concentration of Cu-Ag-Fe nanoparticles for 48 h, 46% of the cells were viable, 54% were in early apoptosis, 5% were in late apoptosis, and 1% exhibited necrosis (cell death). Treatment with 125 $\mu\text{g/mL}$ of the Cu-Ag-Fe nanoparticles fraction increased the percentage of cell death in late apoptosis, 70% of cells were died in late apoptosis when treated with 250 $\mu\text{g/mL}$ of the Cu-Ag-Fe nanoparticles. HeLa cells treated with 62.5, 125.0, and 250.0 $\mu\text{g/mL}$ of the Cu-Ag-Fe showed reduced cell viability with increasing dose when compared with untreated cells (figure 6), microscopic image showed that untreated HeLa cells were characterized by an organized monolayer with a regular polygonal appearance. After treatment, the cells became rounded and disconnected from each other and exhibited cellular shrinkage and nuclear condensation. Hoechst 33,342 staining then was used to determine if the Cu-Ag-Fe nanoparticles induced apoptosis in HeLa cells. Cells treated with 62.5 $\mu\text{g/mL}$ of the extract showed some chromatin condensation and DNA fragmentation, which are the hallmarks of apoptotic cells. A number of cells with similar changes were observed in the 125 $\mu\text{g/mL}$ treatment. At 250 $\mu\text{g/mL}$ of the extract, most cells showed apoptotic body formation with decreased cell viability, indicating that induction of apoptosis and cell death was Cu-Ag-Fe dose-dependent. Flow cytometry result based, Dot Plots of untreated HeLa cells (i) and treated HeLa cells (ii, iii, and iv) treated with 62.5, 125, and 250 $\mu\text{g/mL}$ of the Cu-Ag-Fe nanoparticles signify statistical significance at $p < 0.05$



compared to untreated HeLa cells.

Figure 6. Dose dependent anticancer activity of Cu-Ag-Fe nanoparticles against HeLa cells (i) Normal Hela cell (ii-iv) Various concentration of Cu-Ag-Fe nanoparticles shows apoptosis, and cell death in HeLa cells.

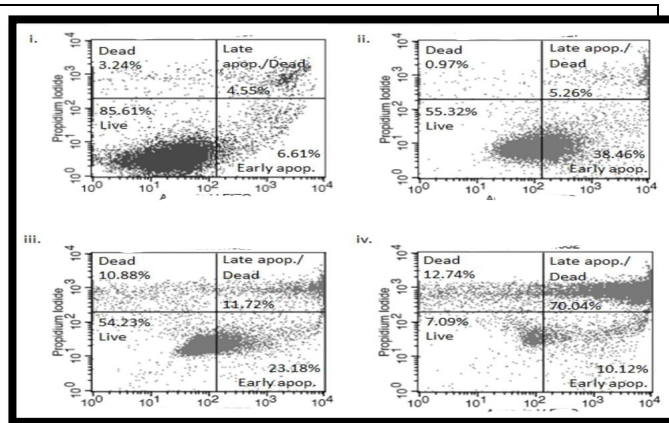


Figure 7. Flow cytometry analysis of Cu-Ag-Fe nanoparticles induced anticancer activity.

Cu-Ag-Fe nanoparticles made of inorganic molecules that act as linkers and one or more types of metal ions. Due to the versatility of its chemical composition, there are many zeolite-like structures (ZIF) using imidazoles as ligands, with nanoparticles in drug delivery systems due to their adjustable pore shape and size, ultrahigh surface area, and versatile functionalities. An endogenous copper metal-organic framework nanoenzyme has been demonstrated to mediate the synergistic interaction between H₂S-activated NIR PTT and chemodynamic therapy in the successful treatment of colon cancer, according to a recent study [12-15].

Among inorganic NPs, Cu-Ag-Fe nanoparticles have attracted significant attention in biomedical applications in recent years, particularly due to their antimicrobial, antioxidant, and anticancer properties. However, to safely exploit this potential, a detailed understanding of the cytotoxic effects of Cu-Ag-Fe nanoparticles is necessary. Such effects are especially pronounced in cancer cells, where they can trigger apoptosis and necrosis, thereby supporting the applicability of AgNPs, CuNPs, ZnNPs, promising anticancer agents [15-18]. Although the concepts of cytotoxicity and anticancer activity are related, the most fundamental difference between them is target specificity [19]. Previous studies have shown that Cu-Ag-Fe nanoparticles exhibit remarkable anticancer potential by inducing cytotoxic effects in various human cell lines. It is evidenced by us particularly, nano sized Cu-Ag-Fe nanoparticles demonstrate enhanced ability to trigger apoptosis, necrosis, or autophagy in cancer cells, leading to effective cancer or tumor cell elimination. Additionally, their size-dependent interactions can cause DNA strand breaks, ROS-mediated oxidative stress, and modulation of inflammatory cytokines, all contributing to their anticancer efficacy. Therefore, precise control over nanoparticle (NP) dose, size, and exposure conditions is crucial to maximize therapeutic benefits while minimizing toxicity.

4. Conclusion:

In conclusion, the development of eco-friendly Green synthesis of Cu-Ag-Fe nanoparticles is an important aspect in the field of nanotechnology and is also easily available starting materials, inexpensive method for nanoparticles biosynthesis and also easy to carry out in any laboratory, use of toxic reagent is avoided and pollution free. From our Study we report eco-friendly synthesis of

Cu-Ag-Fe nanoparticles using a leaf extract of *Moringa oleifera* and in our first research paper UV-Visible Spectrophotometric Analysis Cu-Ag-Fe nanoparticle was done in the wavelength range of 200-600 nm. In our study, various analysis methods, including MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), DHE (dihydroethidium) staining, were used on Cu-Ag-Fe nanoparticles. The results showed that Cu-Ag-Fe nanoparticles exhibited the strongest effects on HeLa cancer cells. Furthermore, they exhibited higher toxicity in HeLa cells than in normal cells. Additionally, as Cu-Ag-Fe nanoparticle decreased in size, their ability to inhibit cell migration and invasion also decreased. Our study observed, that spherical Cu-Ag-Fe with a size ranging from 70 to 76 nm had higher anticancer activity. This study also emphasized the importance of Cu-Ag-Fe morphology, beyond size, in biological activity. However, their cytotoxicity has led to debates about the use of this potent anticancer agent in clinical trials. While many studies have demonstrated selective cytotoxicity of Cu-Ag-Fe, especially compared to some chemotherapeutic drugs, some studies have observed that Cu-Ag-Fe and to overcome this, researchers have shown that selective cytotoxicity can be increased through methods such as using green synthesis methods. Our green synthesized, smart Cu-Ag-Fe nanoparticles are being used or have the potential to be exploited as drug delivery systems for advanced cancer therapies. Due to their special qualities, clinicians are now able to provide new treatments or add them as supplements to already-effective therapies. As this research paper has demonstrated, Cu-Ag-Fe nanoparticles have shown promise for developing safer and more effective cancer therapies, including targeted drug delivery, stimuli-responsive drug release, and co-delivery of combinational drugs. These capabilities may support long-term perspectives and the creation of novel cancer treatment approaches.

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