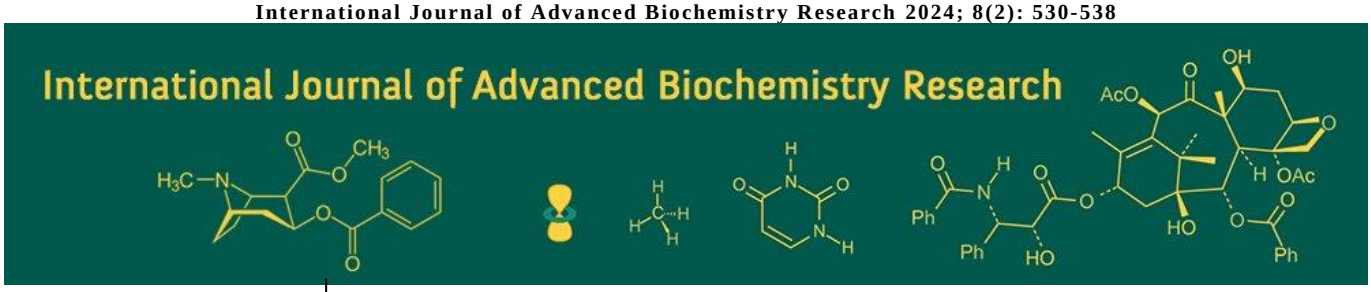

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Synthesis of Ag-Fe_3O_4 nanoparticles using *Aegle marmelos* leaf extract: Characterization and potential applications

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Abstract
The synthesis of bimetallic nanoparticles has emerged as a pioneering field with diverse applications. In this study, Silver-Iron (Ag-Fe_3O_4) bimetallic nanoparticles were successfully synthesized using $AgNO_3$ and FeO_2 as metal sources, along with an aqueous extract of *Aegle marmelos* (Bilva Patra) serving as a reducing and stabilizing agent. The synthesized nanoparticles, with a size of less than 8 nm, exhibited unique fluorescence properties. Various characterization techniques, including UV-visible spectroscopy, IR spectroscopy, dynamic light scattering (DLS) for size with zeta potential, X-ray diffraction (XRD), and scanning electron microscopy (SEM), were employed to confirm the formation of bimetallic quantum dots. The UV-visible spectroscopy results displayed an absorption peak in the range of 320 to 410 nm, indicating the coexistence of silver and iron in the bimetallic structure, forming an alloy. DLS analysis revealed the presence of smaller particles measuring between 6 and 8 nm, with a Z-average size and polydispersity index (PDI) of 10 nm and 0.32, respectively. The zeta potential value of -41 mV affirmed the stability of the synthesized nanoparticles. The unique properties of Ag-Fe_3O_4 nanoparticles synthesized via the green route make them promising candidates for antibacterial and anticancer applications. Ag-Fe_3O_4 exhibited remarkable anticancer efficacy against HeLa cells, with a mere 7% viable cells remaining at the highest concentration tested (100 µg/ml). This pronounced cytotoxicity underscores their potential as a potent anticancer agent. Additionally, these nanoparticles demonstrated noteworthy antimicrobial activity, highlighting their dual therapeutic potential in addressing both cancer and microbial infections. This research contributes to the understanding of bimetallic Nps, showcasing their potential in biomedical applications. The green synthesis approach, utilizing *Aegle marmelos* extract, emphasizes the sustainable and eco-friendly nature of the methodology, paving the way for the development of advanced nanomaterials with enhanced features.

Keywords: Bimetallic nanoparticles, green synthesis, *Aegle marmelos* extract, fluorescence, antibacterial, anticancer

1. Introduction
Bimetallic nanoparticles, characterized by the combination of two distinct metallic elements at the nanoscale, have emerged as focal points of scientific investigation due to their extraordinary properties and diverse applications [1]. These nanoparticles, often structured as alloys or core-shell arrangements, exhibit enhanced functionalities compared to their monometallic counterparts, finding utility across a spectrum of scientific domains including magnetic, optic, electronic, and catalytic applications [2-4]. The controlled synthesis of bimetallic nanoparticles through chemical methods has been instrumental in tailoring their properties, providing precise control over particle size, composition, and surface characteristics. This precision enables the optimization of their performance for specific applications, such as catalysis, where the synergistic effects of different metals contribute to enhanced reactivity and selectivity. Additionally, in electronic and optical applications, the ability to fine-tune the properties of bimetallic nanoparticles is crucial for achieving desired functionalities. Chemical synthesis methodologies, while effective, raise environmental concerns due to the use of toxic reagents and solvents. This has prompted the exploration of green synthesis strategies, which leverage natural sources like plant extracts, as reducing and stabilizing agents [5-7]. Green synthesis aligns with principles of sustainability, offering an environmentally benign alternative to traditional chemical methods.

The transition towards green methodologies is not only driven by environmental consciousness but also by the potential to discover new, biocompatible routes for nanoparticle synthesis.

In this context, our study focuses on the green synthesis of Silver-Iron (Ag-Fe) bimetallic nanoparticles, utilizing *Aegle marmelos* (Bilva Patra) extract as a reducing and stabilizing agent. The strategic choice of *Aegle marmelos* is underpinned by its rich phytochemical composition, known for its diverse bioactive compounds such as alkaloids, flavonoids, and polyphenols [8]. These compounds not only serve as effective reducing agents but also contribute to the stabilization of the synthesized nanoparticles. The dual functionality of *Aegle marmelos* extract, acting as both a reducing and stabilizing agent, enhances the efficiency of the green synthesis process. Also, *Aegle marmelos* is anti-inflammatory in nature. Its extracts when applied on the exposed area, help to cure inflammation. *Aegle marmelos* leaf juice with honey can prove useful for treating fever. *Aegle marmelos* can be used to treat tuberculosis. Our research endeavours to contribute to the evolving landscape of sustainable nanoparticle synthesis, recognizing the imperative for greener alternatives in the field of nanotechnology [9-10]. By showcasing the potential of green methodologies, our study aims to address and overcome the limitations associated with conventional chemical approaches. The use of plant extracts in green synthesis not only minimizes the environmental footprint but also taps into the inherent biocompatibility of natural compounds. This can potentially result in bimetallic nanoparticles with enhanced biocompatibility, making them suitable for applications in biomedicine and drug delivery where compatibility with biological systems is paramount.

This investigation will unfold by delving into the intricacies of chemical synthesis, emphasizing both its applicability and the environmental challenges it poses. Subsequently, the discussion will pivot towards green synthesis, exploring its principles and advantages. The detailed synthesis methodology of Ag-Fe_3O_4 nanoparticles utilizing *Aegle marmelos* extract will be elucidated, followed by a comprehensive characterization to understand the structural and morphological attributes of the synthesized nanoparticles. The study further extends to explore potential applications and the broader implications of adopting green synthesis in the fabrication of bimetallic nanoparticles [11-13]. Through this comprehensive exploration, our aim is to contribute to the evolving understanding of sustainable nanoparticle synthesis and pave the way for environmentally friendly nanotechnological advancements.

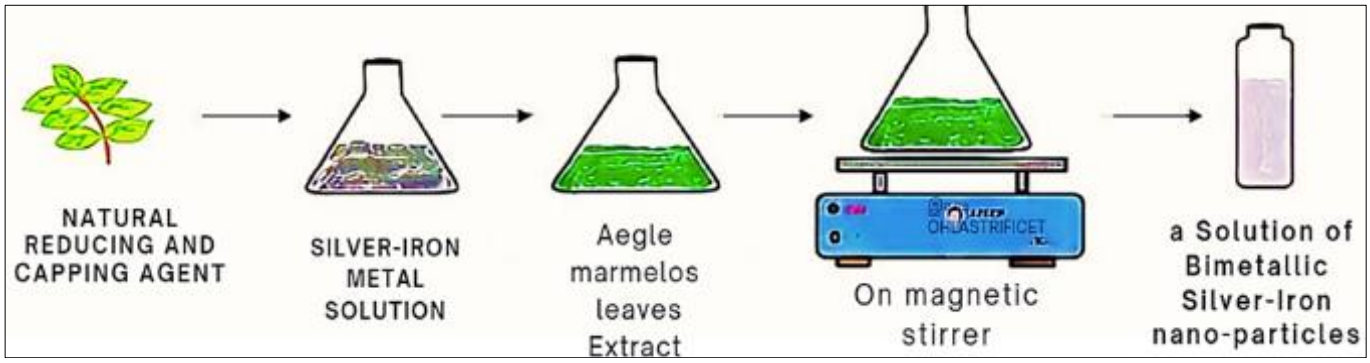

Fig 1: Schematic representation of one-pot Synthesis method of bimetallic nanoparticles

2. Materials and Methods

The materials used in this study include *Aegle marmelos* leaves (bilva patra), silver nitrate ($AgNO_3$) and Ferric chloride ($FeCl_2$), ethanol, and deionized water. The *Aegle marmelos* leaves were collected from a local source (Sindhudurg) and washed thoroughly with deionized water to remove any impurities. The leaves were then dried in an oven at 60 °C for 24 hours and ground to a fine powder using a mortar and pestle [14]. DI water was used to extract the phytochemicals from the powdered leaves. Silver nitrate and Ferric chloride were used as precursors for the synthesis of bimetallic Ag-Fe_3O_4 nanoparticles. All the chemicals used in the study were of analytical grade and purchased from Sigma Aldrich, INDIA.

2.1 Preparation of *Aegle marmelos* leaves Extract

The preparation of a plant extract from *Aegle marmelos* leaves involved a systematic procedure to extract bioactive compounds. Initially, the dried leaves of *Aegle marmelos* were meticulously ground into a fine powder using a mortar and pestle. Subsequently, 10 grams of the powdered leaves were combined with 100 mL of water in a round-bottom flask. This mixture underwent reflux at 70 °C for a duration of 2 hours, with continuous stirring to facilitate the extraction process. Following this, the resultant mixture was filtered using Whatman filter paper, and the filtrate was collected in a beaker. To concentrate the extract, a rotary evaporator was employed under reduced pressure at 40 °C. The resulting concentrated extract served as a reducing agent for the synthesis of Ag-Fe_3O_4 nanoparticles. Confirmation of the phytochemical content in the prepared extract was achieved through UV-Vis spectroscopy, which revealed a maximum absorbance at 280 nm, indicating the presence of flavonoids compounds and 267 nm for phenolic compounds. The characterized extract was then stored at 4 °C for future applications [15-18].

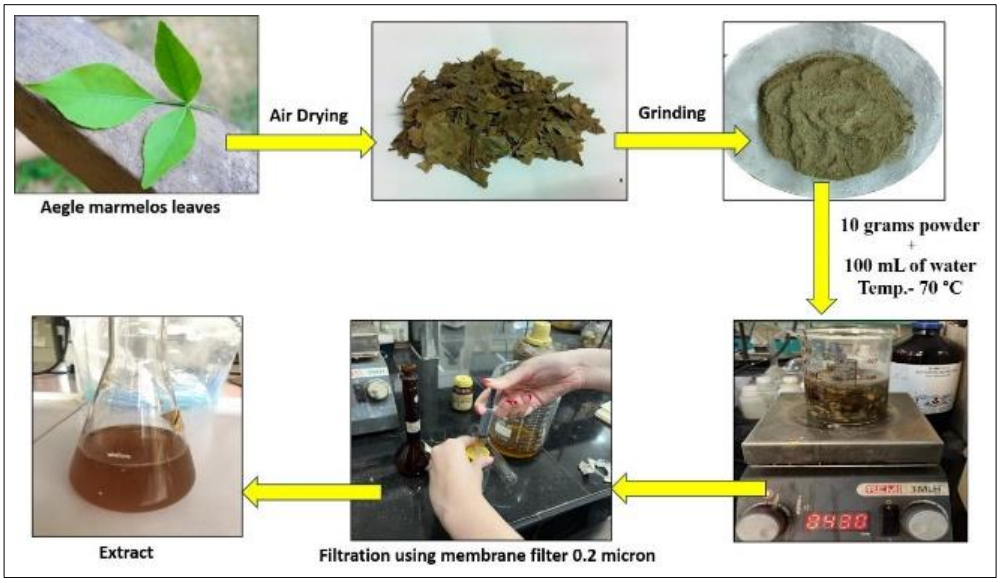

Fig 2: Plant extract preparation method

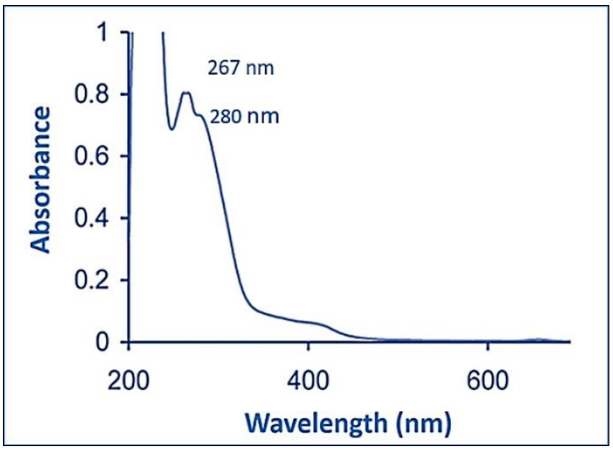

Fig 3: UV-Visible spectroscopy, for confirmation of presence of flavonoids compounds phenolic compounds

The synthesized nanoparticles were characterized using various techniques, including UV-Vis spectroscopy, X-ray diffraction (XRD), transmission electron microscopy (TEM), and Fourier-transform infrared spectroscopy (FTIR). These analyses provided insights into the size, morphology, and chemical composition of the nanoparticles.

2.2 Preparation of Bimetallic Ag-Fe_3O_4 NPs

The synthesis of bimetallic Ag-Fe_3O_4 nanoparticles utilizing *Aegle marmelos* leaves extract as a green reducing agent followed a meticulously controlled procedure. Initially, aqueous solutions of 10 mM silver nitrate (AgNO3) and 8 mM ferric chloride (FeCl3) were meticulously prepared. Subsequently, equal volumes (20 mL each) of these solutions were combined in a chemically inert round-bottom flask under continuous magnetic stirring at ambient temperature. Gradually, 14 mL of *Aegle marmelos* leaves extract, obtained through a rigorous extraction process, was introduced drop-wise into the reaction mixture. The ensuing reaction mixture was allowed to undergo uninterrupted stirring for a duration of 24 hours at room temperature. Notably, the visual observation of the reaction mixture transitioning from a colourless to a dark brown hue indicated the successful nucleation and growth of Ag-Fe_3O_4 nanoparticles. To ensure the removal of any residual precursors and organic matter, the synthesized nanoparticles were subjected to centrifugation at 10,000 rpm for 10 minutes. This step was critical in achieving the purification and isolation of the synthesized nanoparticles, readying them for subsequent characterization and application in various fields of research and technology [19-24].

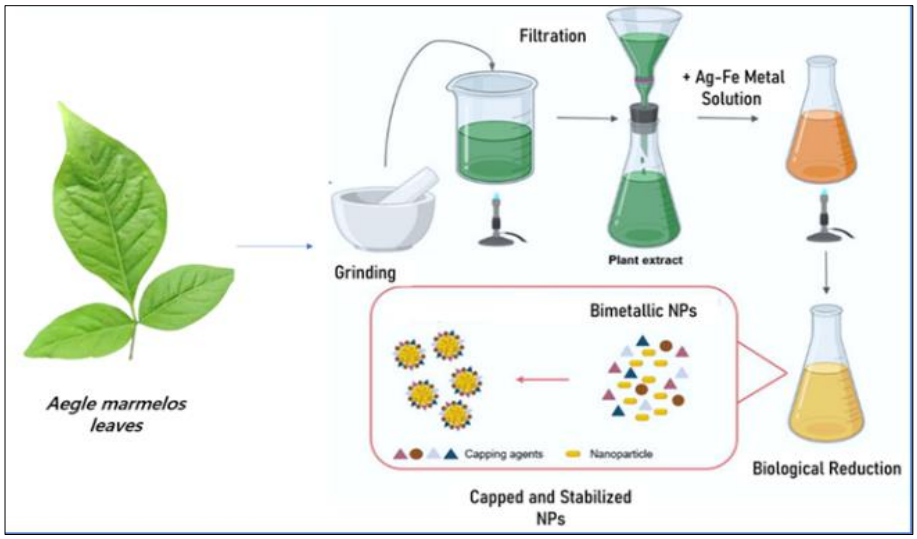

Fig 3: Bimetallic NPs synthesis method

2.3 Evaluation of Anticancer activity by MTT assay

2.3.1 Requirements

MTT Powder (the solution is filtered through a 0.2 µm filter and stored at 2-8 °C for frequent use or frozen for extended periods), DMSO, Trypan blue, Phosphate Buffered Saline (PBS), Trypsin, CO_2 incubator, Centrifuge, Hemocytometer.

2.3.2 Assay protocol

2.3.2.1 Cell Preparation: Cervical Cancer cells

HeLa cells (grown in DMEM media supplemented with 10% Fetal Bovine Serum), harvested using trypsin, and counted using Trypan blue and a hemo-cytometer.

- Collected the cells when they reach about 70-80% confluence.
- Checked for the viability and centrifuged the cells.

Procedure

1. Day 1, seed 2,500-5,000 cells/well (HeLa) in their exponential growth phase in 100 µL volume in a flat-bottomed 96-well polystyrene coated plate (Thermo Scientific Nunc 96-well plate, Nunclon Delta-white color with lid). Incubate overnight at 37 °C with 5% CO_2 to allow cell adhesion.
2. Day 2, after 24 hours of seeding, the concentration of nanodrugs (test compounds) were diluted in cell culture medium at different concentration (5, 25, 65 and 100 µg/ml) in DMEM without FBS containing a final volume of 100 µl/well. Incubated for 24 hour. Experiments done in triplicate.
3. Day 3, after 24 hour of incubation, added 10 µL of MTT solution (5 mg/10 ml of MTT in 1X PBS) into each well. Wrapped the plate in aluminium foil and incubated for 3-4 hours at 37 °C, 5% CO_2 incubator, protected from light.

Note: Depending on the individual cell type and concentration used, longer incubation times may be necessary.

1. Formazan crystals formed after 3-4 hours in each well were dissolved in 100 µl DMSO solution (solubilizing buffer).
2. Read absorbance at 590 nm. Read plate within 1 hour [32-34].
3. The positive control wells contain untreated cells, MTT solution and a solubilizing buffer.
4. The blank in the MTT assay is a cell-free medium plus an MTT solution and a solubilizing buffer.
5. Experiments were repeated three times for the average calculations.

Viability is defined as a percentage of live cells in a whole population or in other words, cell viability is defined as the number of healthy cells in a solution.

% Viability = Sample/Control x100

Sample

Mean value of the measured optical density (OD)/Absorbance of the Nanodrugs

3. Results and Discussion

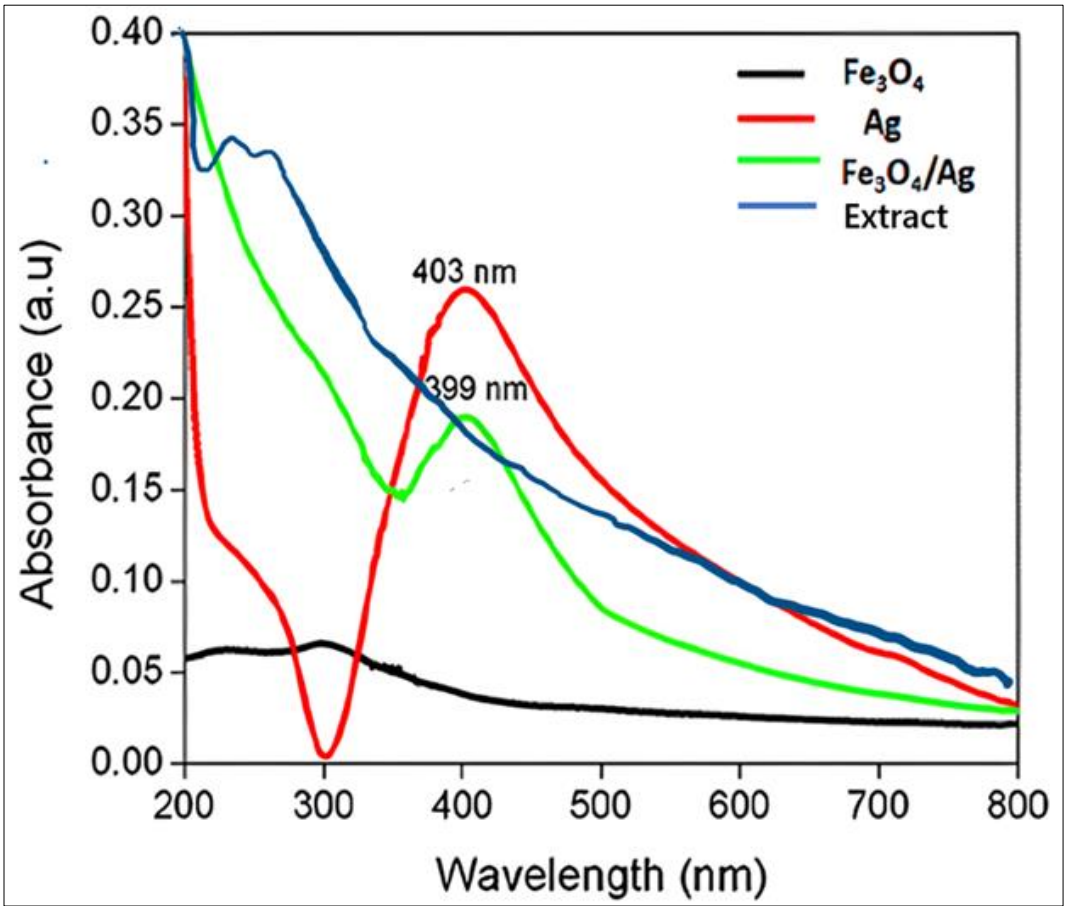

Fig 4: The UV-Vis spectroscopy analysis

The UV-Vis spectroscopy analysis conducted on the prepared extract and metal solutions, including Iron (Fe), Silver (Ag), and the bimetallic nanoparticle (NP) alloy, unveiled distinctive absorption peaks in the spectrum. Precise absorption maxima serve as spectroscopic fingerprints, providing intricate details about the specific phytochemical moieties and metal NPs present within the solution. Notably, the presence of flavonoid compounds was discerned through a prominent absorption peak at 280 nm, while a distinct peak at 267 nm signified the prevalence of phenolic compounds. The metal solutions exhibited characteristic absorption features, with Iron (Fe) displaying a well-defined peak at 310 nm, Silver (Ag) demonstrating a discernible peak at 403 nm, and the bimetallic NP alloy presenting a conspicuous peak at 390 nm [25-27].

The size of nanoparticles is measured using DLS, and their stability in suspension over time is also assessed.

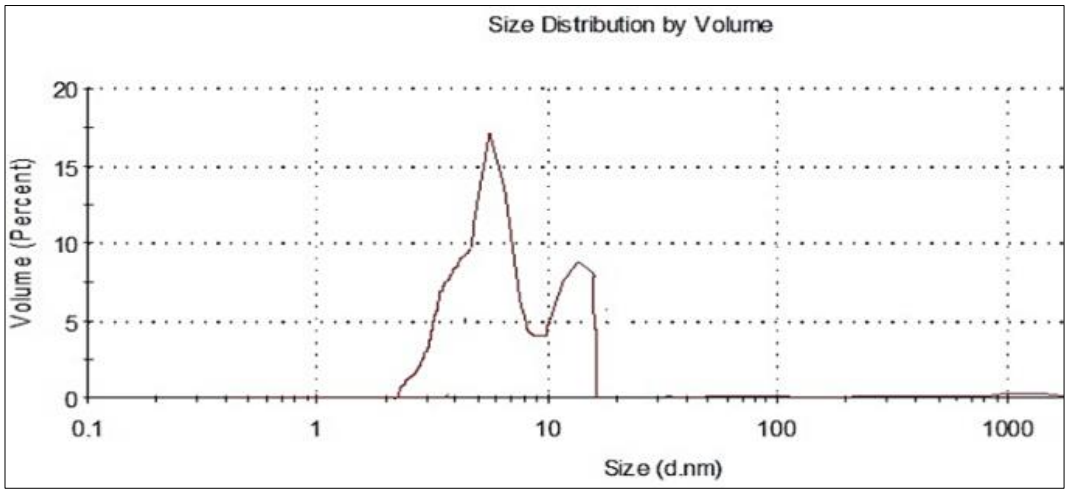

Fig 5: DLS Measurement for Fe-Ag core-shell

Figure 5 shows the size distribution of the prepared nanoparticles. The size distribution and polydispersity index (PDI) of Fe-Ag NPs were detected by DLS analysis. The results have indicated the presence of smaller particles of measures 9-11 nm.

Z-average size and PDI for the synthesized nanoparticles were observed to be 15.1 nm and 0.39 respectively [28-31].

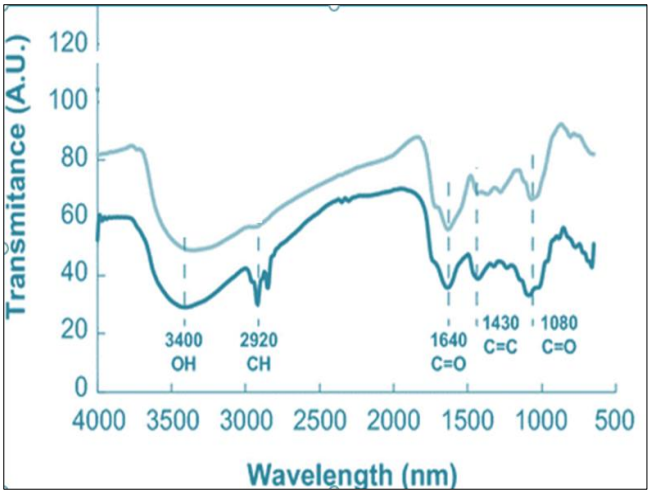

Fig 6: IR Spectrum of bimetallic Fe-Ag Nanoparticles

Fig.6 represents FT-IR spectrum of Synthesized Fe-Ag NPs. It shows the presence of functional groups in Fe-Ag particles. The IR spectrum of Ag-Zn observed broad and strong peaks at 3340 for OH,1640 for carbonyl, 2920 for -CH which confirmed the binding of polyphenols and amino acids [32]. The presence of polyphenols and amino acids shows the reduction of silver and iron ions into silver-iron bimetallic nanoparticles.

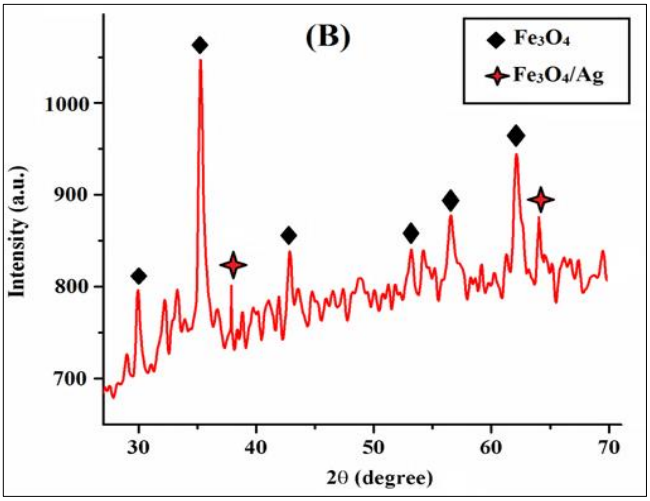

Fig 7: XRD Spectrum of bimetallic Fe-Ag Nanoparticles

The X-ray diffraction (XRD) patterns of the Fe_3O_4/Ag nanocomposite are depicted in Fig. 7. Analysis of Fig. 7 reveals distinctive diffraction peaks for Fe_3O_4/Ag nanocubes at 30.2°, 35.5°, 43.6°, 53.8°, 57.4°, and 62.8°, corresponding to crystal planes (220), (311), (400), (422), (511), and (440), respectively. The sharpness of these peaks is indicative of the cubic inverse spinel structure of iron oxide, in alignment with JCPDS card no. 19-629. The estimated particle size of Fe_3O_4 nanocubes using the Debye-Scherrer formula is 20.4 nm [28].

Upon examination of the Fe_3O_4/Ag nanocomposite, additional peaks for silver (Ag) are observed at 38.06° and 64.3°, corresponding to crystal planes (111) and (220). These peaks affirm the face-centered cubic structure of silver. Consequently, the XRD pattern provides clear evidence of the successful formation of the Fe_3O_4/Ag nanocomposite.

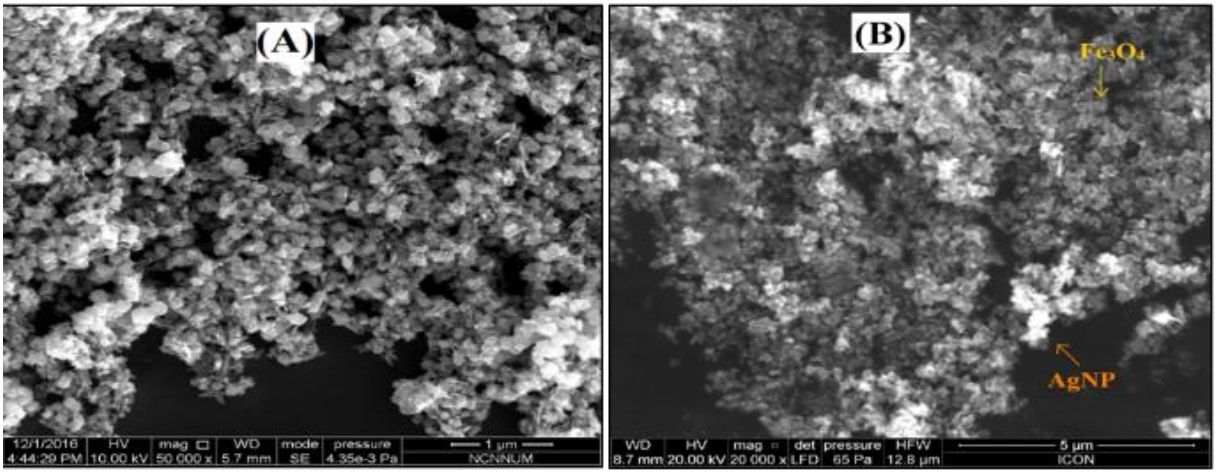

Fig 6: SEM s of bimetallic Fe-Ag Nanoparticles

The SEM techniques were employed to examine the surface morphology of the synthesized materials. In Fig.6 (A), the SEM image of Fe_3O_4 nanocubes reveals a homogeneous cubic structure. Fig.6 (B) shows the SEM image of the Fe_3O_4/Ag nanocomposite, confirming its formation with small, vivid spots of silver nanoparticles distributed regularly over the nanotubes [38].

3.2 Antimicrobial Activity

The antibacterial efficacy of various substances, including *Aegle marmelos* leaf extract, silver nitrate, ferric chloride, and bimetallic Ag-Fe_3O_4 nanoparticles, was assessed against both Gram-positive and Gram-negative bacteria. Results revealed that bimetallic Ag-Fe_3O_4 nanoparticles, at a concentration of 1000 µg/ml, demonstrated significant antibacterial activity against Pseudomonas aeruginosa, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, and Enterococcus faecalis, leading to inhibition zones measuring 31.2 mm, 24 mm, 30.1 mm, 29.7 mm, and 13.1 mm, respectively [39-42].

Notably, Bacillus subtilis exhibited the highest sensitivity to bimetallic Ag-Fe_3O_4 nanoparticles, with a minimum inhibitory concentration (MIC) of 15.32 µg/ml, while Enterococcus faecalis displayed the lowest sensitivity, with an MIC of 250 µg/ml. Additionally, the MIC values of bimetallic Ag-Fe_3O_4 nanoparticles against Escherichia coli, Pseudomonas aeruginosa, and Staphylococcus aureus were determined as 66.1 µg/ml, 101 µg/ml, and 43 µg/ml, respectively.

In contrast, *Aegle marmelos* leaf extract showed limited antibacterial activity, specifically against Escherichia coli and Bacillus subtilis. Neither silver nitrate nor Ferric chloride exhibited any antibacterial effects against the tested bacterial strains. These findings suggest that the effectiveness of bimetallic Ag-Fe_3O_4 nanoparticles cannot be solely attributed to their starting materials but rather to the unique properties and characteristics of the prepared nanoparticles themselves.

Table 1: Data for antibacterial activity of different samples

Bacterial Strain	*Aegle marmelos* leaf extract	Silver Nitrate	Ferric chloride	Bimetallic Ag-Fe_3O_4 NPs
Pseudomonas aeruginosa	0	0	0	31.2
Escherichia coli	X	0	0	24
Bacillus subtilis	X	0	0	30.1
Staphylococcus aureus	0	0	0	29.7
Enterococcus faecalis	0	0	0	11.9

(Please note that in the table above, "X" indicates limited antibacterial activity, and "0" indicates no antibacterial activity.

The mechanism underlying the antibacterial activity of bimetallic Ag-Fe_3O_4 nanoparticles involves various factors. Electrostatic interactions may damage bacterial cell membranes, disrupt proteins and enzymes, generate reactive oxygen species (ROS), and induce oxidative stress. The nanoparticles may also bind to proteins, disrupting cellular homeostasis, including the electron transport chain and signal transduction pathways, potentially leading to genotoxic effects. Moreover, the rough external surface of Ag-Fe_3O_4 nanoparticles may contribute to cell wall damage, enabling the nanoparticles to penetrate bacterial plasma membranes and exert toxic effects on the bacteria.

Table 2: MIC Values of Bacterial Strains

Bacterial Strain	MIC (µg/ml)
Escherichia coli	66.1
Pseudomonas aeruginosa	101
Staphylococcus aureus	43

3.3 Anticancer Activity

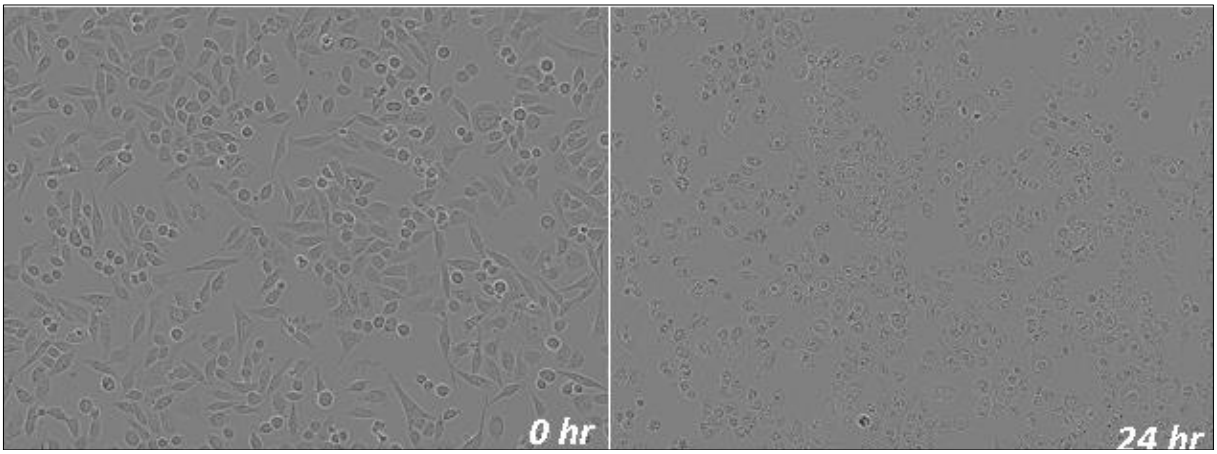

Fig 7: The microscopic images of bimetallic Ag-Fe_3O_4 Nanoparticles treatment with HeLa cells at 0 hr and 24 hr

The 20X magnification of the microscopic images provides a thorough look of the morphological changes that HeLa cells experienced after being treated with Ag-Fe_3O_4 Nps (Silver-Iron Nanoparticles). A detailed analysis and comparison of photos taken 24 hours and 0 hours after treatment reveal significant changes in the morphology of the cells. The observed changes in cell morphology clearly point to a major effect on the HeLa cells, with a clear transition toward an aberrant appearance. Crucially, the alterations that have been seen point to a possible outcome: cell death. The observed cell death after treatment with Ag-Fe_3O_4 Nps suggests that the nanoparticles may have a cytotoxic impact on HeLa cells over the studied period. The results of this experiment are very important because they provide insight into the therapeutic potential of Ag-Fe_3O_4 Nps in relation to HeLa cell survival. The noticeable alterations in cellular behaviour highlight the significant impact of the nanodrug on the cells that were treated. This data lays the groundwork for future research into Ag-Fe_3O_4 Np's potential as a therapeutic agent for the treatment of HeLa cells by offering insightful information about its mode of action [40].

Compound name	Conc. µg/ml	OD at 590 nm	% Viability
Control	0	1.590 ± 0.081	0.00
Ag-Fe_3O_4 NPs	100	0.133 ± 0.112	7

These results suggest that the NPs Ag-Fe_3O_4 showed good potency in Cervical cancer (HeLa cells) with only 7% viable cells present in maximum concentration i.e. 100 µg/ml.

Conclusion

In conclusion, this study successfully demonstrated the one-pot synthesis of bimetallic Ag-Fe_3O_4 nanoparticles using *Aegle marmelos* leaf extract as both a reducing and stabilizing agent. The utilization of natural plant extracts in NP synthesis, as an eco-friendly and sustainable alternative to conventional chemical methods, has garnered considerable attention due to their low toxicity and abundance of reducing agents. The characterized bimetallic Ag- Fe_3O_4 NPs underwent analysis through various techniques, including UV-Vis spectroscopy, dynamic light scattering (DLS), and Fourier Transform Infrared spectroscopy (FTIR). The results revealed spherical NPs with an average size of 8-11 nm, showcasing excellent stability and biocompatibility, making them well-suited for diverse biomedical applications. Furthermore, the synthesized nanoparticles exhibited potent antimicrobial activity against both Gram-positive and Gram-negative bacteria, suggesting their potential as effective antibacterial agents. Additionally, the study unveiled promising anticancer activity against the HeLa cell line, as evidenced by significant morphological changes observed at 20X magnification between images taken at 0 hours and 24 hours post- Fe_3O_4 Nps treatment. The distinct alterations observed in the HeLa cell morphology strongly indicate a consequential impact on cellular behaviour, including the occurrence of cell death with only 7% viable cells present in maximum concentration i.e. 100 µg/ml. In summary, the one-pot synthesis of bimetallic Fe_3O_4 NPs using *Aegle marmelos* leaf extract presents a promising and environmentally friendly approach for producing biologically active nanoparticles with applications in fields such as biomedicine, environmental remediation, and anticancer therapy. The multifaceted findings encompassing characterization, biocompatibility, antimicrobial and anticancer activities, and impact on HeLa cell morphology collectively highlight the versatility and potential of these nanoparticles in various scientific and practical domains.

Data availability

The corresponding authors can provide the supporting data for this study upon a reasonable request.

Conflicts of interest

The authors do not possess any conflicts of interest to disclose.

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