Green Synthesis: *In-Vitro* Anticancer Activity Of Silver Nanoparticles Against Mkn45- Human Gastric Cancer Cell Lines

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Abstract
Silver nanoparticles (Ag NPs) were synthesized by a green route using an aqueous *Phaseolus vulgaris* (green beans) extract. Water extracts of leguminous have the latent to be efficient anticancer ingredients, by means of copious mechanisms based on the raw material and the process. The characterization was conducted using a UV/Vis spectrophotometer (Jasco -model UV- 730), FT-IR, SEM (FEI Quanta FEG 200-High Resolution Scanning Electron Microscope), EDXA and TEM (JEOL 2100F FEGTEM). The cytotoxicity of the sample on AGS cells were determined by the method of Mosmann, (1983). The IC50 values were calculated and analysed based on the number of cells which exhibited suppression in cell growth on administration of *Phaseolus vulgaris* derived silver nanoparticles. Measurement of apoptotic induction using Acridine Orange/Ethidium Bromide (AO/EB) dual staining method is permeable and it stains all the Gastric Cancer (AGS) cells viable/nonviable cells. Using fluorescent rhodamine 123 (Rh 123) dye, mitochondrial membrane potential (MMP) was determined in this work. Our experimental results show that the Ag NPs can induce apoptosis and suppress the proliferation of AGS cells.

KeyWords: Silver nanoparticle., *Phaseolus vulgaris L..,* AGS Cells., *In-vitro* anticancer.

1. INTRODUCTION

Cancer is one of the foremost causes of death global and is characterized by proliferation of abnormal cells. Surgery, chemotherapy, and radiotherapy are typical methods used in cancer treatment. However, these are expensive and have side effects with limitations of their usage, so there is an imperative need for effective, inexpensive, and non-toxic treatments with negligible side effects that are acceptable by people (Chen, 2008).
Synthesis of silver nanoparticles by bioinspired synthesis using plant sources offers several advantages such as cost-effectiveness, eco-friendliness, and the elimination of high pressure, energy, temperature, and toxic chemicals necessary in the outdated synthesis methods (Aswathi *et al*., 2023). Dietary intake of silver is estimated at 70-90 µg\day. Silver in any form is not thought to be toxic to the immune, cardiovascular, nervous, or reproductive system and is not considered to be carcinogenic (Bayat, 2022).

Legume have been extensively studied to produce active peptides with a diversity of biological activities. (Ferlay *et al*.,2013). Generally, these Bioactive Peptides (BPs) are encrypted in proteins but can be released by means of enzymes during gastrointestinal transit or fermentation, germination, heating, and pressure (Yan, 2019). These peptides can generate a physiological effect against cancer cells and can induce cell death by different mechanisms like apoptosis, affecting the tubulin-microtubule equilibrium and inhibiting angiogenesis. Finally, anticancer therapy based on legume-derived peptides could play a significant role in the pharmaceutical and nutraceutical industry due to its benefits as functional ingredients which improves life quality by dropping the risk of cancer. (Tan *et al*., 2019).

2. MATERIALS AND METHODS

2.1. Materials

Phaseolus vulgaris pods were collected from the home garden. The pods were dried at room temperature for 3–4 days so that moisture gets removed completely. Dried pods were crushed to fine powder and stored in dry and airtight container for further use. We purchased silver nitrate (AgNO3) from Sigma, India. To avoid any photochemical reactions, all solutions for the experiment were produced fresh with double-distilled water and kept in the dark ((Rautela *et al.,* 2019).

2.2. Synthesis of Ag NPs

Two grams of plant extract powder were measured precisely and placed in a 250 ml flask containing 100 ml of double distilled water. The mixture was then heated for 15-20 minutes in an 80 °C water bath. After boiling, the extract was filtered and stored at 4°C and used within a week. A one millimolar AgNO3 solution in double distilled water was used as the reservoir for silver in this experimental setup. The silver nitrate was combined with *Phaseolus vulgaris* extract in a proportion of 1:9 and heated while being constantly agitated at 800 rpm using a magnetic stirrer. Over the following hour, the solution obtains a reddish-brown colour. Centrifugal force was applied to the solution at a speed of 15,000 rpm for 45 minutes, leading to the formation of pelletized Ag NP's. This product was rinsed repeatedly with deionized water (3-4 times) to remove any extract residue. The precipitant was lyophilized and stored in a cool and dark area.

2.3. Characterization of silver nanoparticles

In the experimental set up, silver nanoparticles are synthesized from the *Phaseolus vulgaris* fresh extract solution. Initially, the suspension's colour was green. After the addition of silver nitrate (AgNO3), the suspension's colour changed to reddish brown during 15 minutes of incubation. It is the quickest bio-reducing processes ever employed to produce silver nanoparticles. The characterization was conducted using a UV/Vis spectrophotometer (Jasco -model UV- 730), FT-IR, SEM (FEI Quanta FEG 200-High Resolution Scanning Electron Microscope), EDXA and TEM (JEOL 2100F FEGTEM).

2.4. Cell culture maintenance

Gastric Cancer – AGS cell lines were procured from the cell repository of National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco`s Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 µg/ml) were added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO2 at 37°C.

2.5.MTT assay- (3-(4,5 Dimethylthiozol-2-Yl)-2,5-Diphenyltetrazoliumbromide

MTT is reduced by mitochondrial dehydrogenase of viable cells yielding a measurable purple formation product. Viable cells contain NAD(P) H-dependent reductase, which reduce the MTT reagent to formazon, with a deep purple color. Formazon crystals are then dissolved using solubilizing solution and absorbance is measured at 500-600 nm by plate reader**.**

2.6.AO/EtBr staining:

The fluorescence microscopic analysis of apoptotic cell death was carried out according to the method of Baskic *et al.*, (2006). Gastric cancer cell lines AGS were cultured in 6 welled plates and at a cellular distribution rate of 1×10^6 cells per well, and dosages of 20µg/ ml and 40µg/ ml were administered, and an incubation period of 24 hours was observed. After the stipulate time was completed, the cells were collected from the wells and "AO-EtBr dye mix (1:1 v/v from 100 µg mL−1 in PBS)" was included and analysed using a fluorescence microscope (Biorad)(Jamali *et al*., 2018).

2.7. Evaluation of mitochondrial membrane potential (MMP)

Using fluorescent rhodamine 123 (Rh 123) dye, mitochondrial membrane potential (MMP) was determined in this work. AGS cells were seeded in 96-well plates, and the seeded cells were exposed to produced silver nanoparticles at IC50 concentrations for 24 hours. Cells were stained for 15 mins with the fluorescent dye Rhodamine 123 (5 mmol/mL) following incubation (Halliwell and Whiteman, 2004). The development of the cells was later observed using a fluorescent microscope after the labelled cells had been soaked in PBS.

2.8. DNA fragmentation:

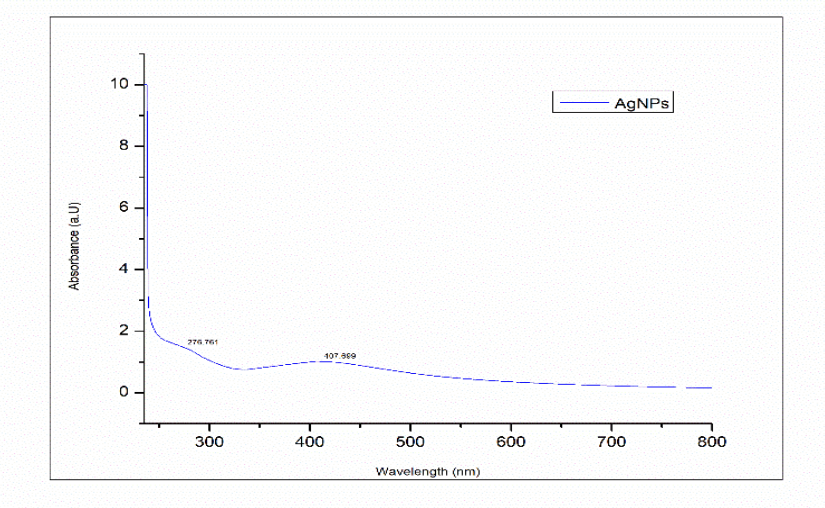
According to the manufacturer's instructions (Apoptotic DNA Ladder kit, Roche, Basel), DNA samples from untreated and treated AGS gastric cancer cell lines were collected for the study. AGS gastric cancer cell lines were initially seeded into six welled microplates, and then 35 ug/ ml of Ag NPs was added. The growth media is then removed after a 24-hour incubation period, and the cells are obtained by scraping with roughly 1 ml of phosphate buffered solution. To see the banding pattern in the treated cells, the final eluted DNA was electrophoresed in 1% agarose gel.

3.RESULTS

3.1. Characterization of silver nanoparticles

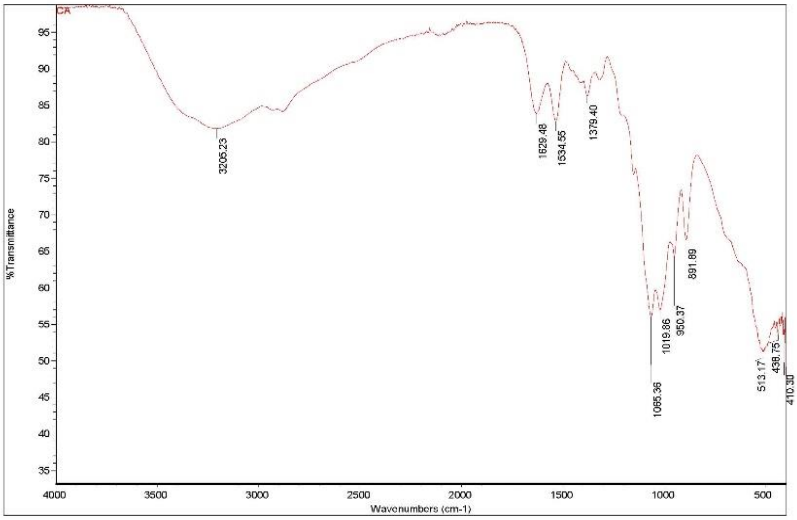
3.1.1. UV-visible spectrum of *Phaseolus vulgaris* using aqueous extract in silver nanoparticles

The UV absorption spectrum of silver nanoparticles with an increase in particle size, the SPR peak shifts to longer wavelengths. The resulting absorption spectra revealed a potent surface plasmon resonance band maximum at 407 nm with a vast surface of plasmon resonance, displayed a heightened SPR peak at 407.69 nm and 276.76 nm.



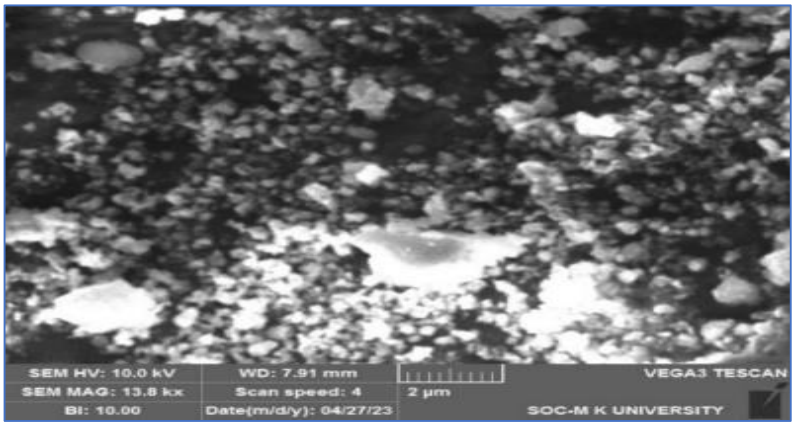
3.1.2. FTIR analysis for synthesized nanoparticles

Fourier Transform Infrared Spectroscopy (FTIR) was performed at the wavenumber range was 4000 cm-1 –500 cm-1 with a 0.05 mm slit. FTIR analysis expressed visible bands at 3205.23cm-1 , 1629.48cm-1 , 1534.55 cm-1 , 1379.40 cm-1 , 1637.68 cm-1 , 1065.36 cm-1 , 1019.86 cm-1 , 950.37 cm-1, 891.89 cm-1 and the functional groups of silver nano particles are=C-H & =CH2 Alkenes (usually sharp),NH2 scissoring (1°-amines)C=O (amide I band) med-str Amines Carboxylic Acids & Derivatives, C-N and N-H Stretching vibration Amide II bending,CH3 deformation Alkanes medium, C-O Stretching Alcohols & Phenols, Alkenes Stretching=C-H & =CH2 compound present



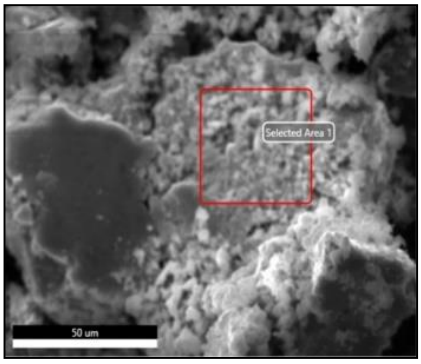
3.1.3. Scanning electron microscopy (SEM)

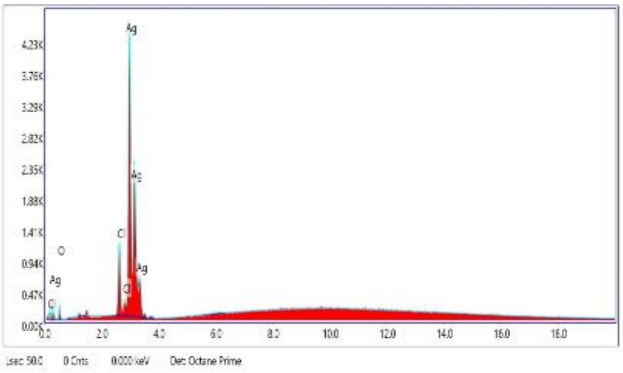
Scanning electron micrographs (SEM) was used to study Ag NP's surface morphology. The morphology of formed Ag NP's is broad, spherical and of 2µm size. The SEM images of synthesized Ag NP's indicating smooth surface.



3.1.4. Energy-dispersive X-ray spectroscopy (EDXA)

Silver nanoparticles were spherical, 10–40 nm in size. The dispersion of silver nanoparticles was 75.47% weight**,** whereas the absorbance in the chosen area was 50um. Strong peaks at 3 keV were seen in the EDX spectrum for silver nanoparticles. Strong signals were seen from the silver atoms in the silver nanoparticles during the EDX examination at 3 keV, which is a hallmark peak of metallic silver nanocrystal.

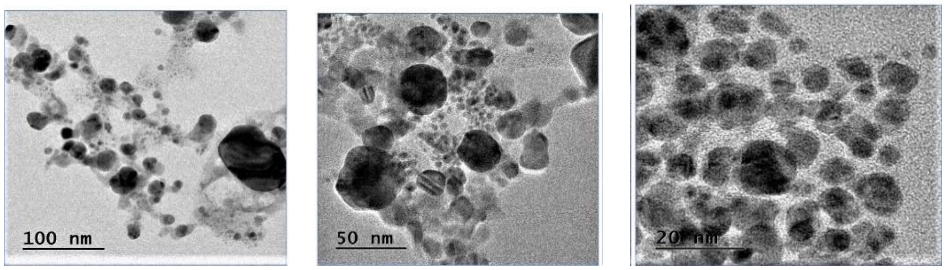




3.1.5. Transmission Electron Microscopy (TEM):

The grain particle and the boundaries of each grain were clearly demarcated in the TEM imaging. The diametrical measurement of each grain was found to vary from 50–70nm. On describing the TEM images elemental mapping of silver nanoparticles formulated using *Phaseolus vulgaris*, the elemental mapping pattern showed that the photomicrograph (Figure. A). The structure study is a polycrystalline structure, due to the presence of concentric circles (diffraction pattern) on imaging. The sample is highly porous (depicted in photomicrograph Figure. B) owing to the spaces found between the grains and the presence of dispersion of the grain particles (depicted in photomicrograph Figure. C). Presence of agglomeration like phenomenon is present in the images obtained from TEM, this might be due to the thin black coating of organic substances of the *Phaseolus vulgaris.*

A. 100 nm **B. 50nm** **C. 20 nm**

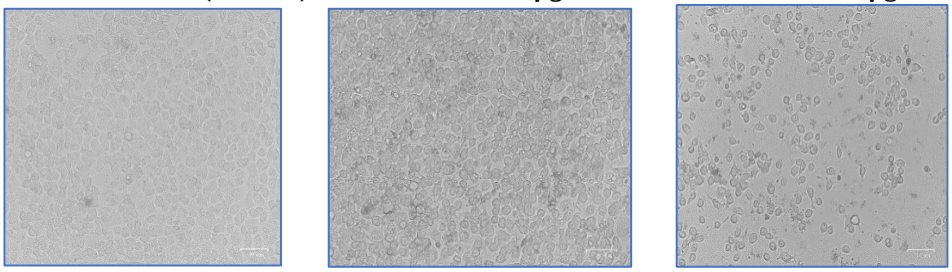


3.2. *In-vitro* Anticancer studies

3.2.1. MTT Assay

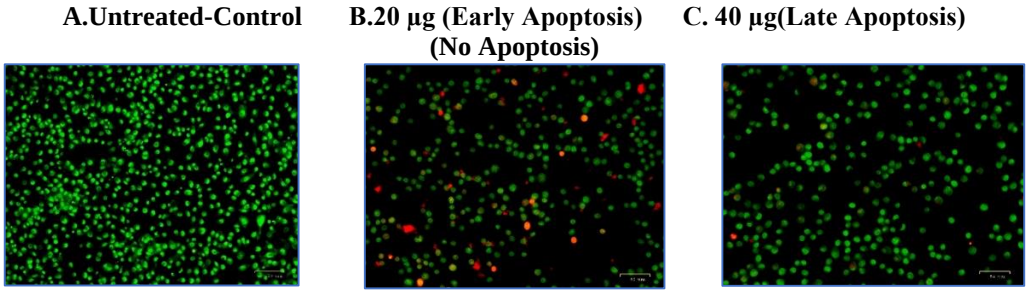
The 50% inhibitory concentration (IC50) value for biosynthesized Ag NP's against AGS (Gastric Cancer) cell lines recorded was 35.301±5.993 µg/ml respectively. The present results strongly indicate that the synthesized Ag NP's have a higher apoptotic activity of AGS (Gastric Cancer) cancer cell line compared to control.

A.Untreated (Control) **B. 20 µg** **C. 40 µg**



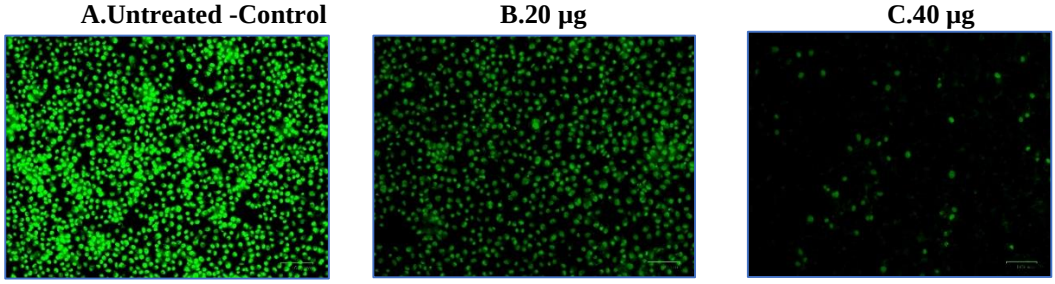
3.2.2. Acridine Orange and Ethidium Bromide (AO/EtBr) and Hoechst staining:

After AO/EtBr staining, the morphological view of the apoptotic, necrotic, and regular cells of AGS was diagnosed independently using fluorescence microscopy. The smear cells were identified by live cells in light green colour, initial apoptotic cells, belated apoptotic cells in orange colour and almost death cells in red coloured.



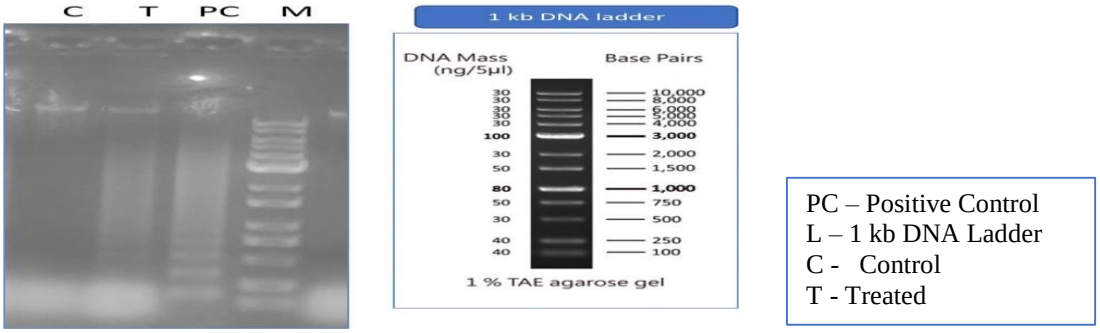
3.2.3. Mitochondrial membrane potential:

Higher mitochondrial depolarization was found to be silver nanoparticle (20 µg/mL) treated AGS cells over control cells (Figure 4.23 A, B, C). The greater MMP depletion was noticed at 40 µg value of the complexes. Such changes in mitochondrial membrane potential could be associated to the constant release of nanoparticles into AGS cancer cells.



3.2.4. DNA fragmentation:

DNA fragmentation showing the bands and the presence of laddering: Lane C - control or standard; lane T- Cells treated with 35 µg/ml of silver nanoparticles, Lane PC – Positive control, Lane L, which is the 1 kb DNA ladder which showed strong intense laddering.



3. DISCUSSION AND CONCLUSION

Ege et al. (2020) documented his research on AgNPs in *Phaseolus vulgaris*, whose concentration of the solution increases over time which can be qualitatively observed by taking a yellow-brown colour in their solutions due to vibration motions according to the theory known as surface plasmon resonance. In the present study, the resulting absorption spectra revealed a potent surface plasmon resonance band maximum at 407 nm with a vast surface of plasmon resonance, displayed a heightened SPR peak at 407.69 nm and 276.76 nm. In general, colour changes were observed with the application of silver nitrate solution to the colourless plant extracts, which initially approached yellow and then became brown in this analysis correlates the present work on the addition of AgNO3 caused the colour of the *Phaseolus vulgaris* filtrate to change from colourless to dark brown, which provides insight into the creation of silver nanoparticles.

After treatment, live cells contain higher polarity, whereas dead cells drop such polarity for the destruction of mitochondrial membrane reliability, thus, exhibit a suspension of the dye (Tan, 2023).

In-vitro anticancer results indicated that Ag NPs induced apoptosis (death of the cancerous cells) in a dose-dependent manner and significantly reduced gastric carcinoma colonies. This encouraging result provides useful information for designing a much better anticancer compound using a plant-mediated synthesis of Ag NPs with minimal side effects.

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