Synthesis and Characterization of Solanum Nigrum Derived Nanoparticles and Exploration if Its Antioxidant, Antibacterial And Anticancer Potentials in *in Vitro*

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Abstract

In recent years research in the synthesis of nanoparticles were investigated, for their exhibit in larger surface area, thus opening to many possibilities with respect to all technological applications. The Solanum nigrum leaf extracts were used to synthesize silver nanoparticles, which was confirmed by various spectral datas. The maximum absorption in UV spectrum for silver nanoparticle formation was at 458nm, the SEM image clearly indicates the shape of silver nanoparticles. The EDAX spectrum showed the presence of silver signal. All the characterization results revealed the synthesized silver nanoparticles size was between 100nm. After confirmation of silver nanoparticles, they were analyzed for its scavenging activity against free radicals. The synthesised silver nanoparticles were experimentally proved by its antibacterial activity using Well diffusion method. Further, to evaluate the cytotoxicity of silver nanoparticles in in vitro model, AGS (colon cancer cell line) and Hep-2 (Larynx cancer cell line) were used and analysed for its cell cycle arrest using Flow cytometer.

Keywords - *Solanum nigrum, silver nanoparticles, SEM, DPPH, Hep2cell line , AGS cell line, MTT and FACS*

I. INTRODUCTION

The development of green processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology [1,2]. In the past two decades the field of nanotechnology synthesized nanomaterials, had huge applications in the fields of physics, chemistry, biology and medicine [3]. The synthesized silver nanoparticles had considerable attention owing to their diverse properties such as catalysis [4], magnetic and optical polarizability [4], electrical conductivity[5], antimicrobial activity [6] and surface enhanced Raman scattering (SERS) [7].
The use of plants for the synthesis of AgNPs has gained importance in the last decade because it is rapid, doesn't affect the environment, no pathogens are used and the whole process involves a single-step technique. Plant extracts contain a combination of biomolecules (e.g.: enzymes, polysaccharides, alkaloids, tannins, phenols, terpinoids and vitamins) that are of great medicinal value and, although complex structures, and environmental benign [8]. It is presumed that the flavonone and terpenoid components from leaf broth are able to stabilize the formation of AgNPs while the polyol and water soluble heterocyclic components are responsible for the reduction of silver ions. Also, it is well known that AgNPs obtained from plant extract exhibit a brownish color in aqueous solution due to excitation of surface plasmon vibrations [9].

Among the biological alternatives, plants and plant extracts seem to be the best option. Plants are nature's "chemical factories". They are cost efficient and require little or no maintenance. A vast repertoire of secondary metabolites are found in all plants which possess redox capacity and can be explored for biosynthesis of nanoparticles. As a wide range of metabolites are presented in the plant products/extracts, nanoparticles produced by plants are more stable and the rate of synthesis is faster in comparison to microorganisms [10-11] . The main objectives of the study, to synthesize silver nanoparticles from *Solanum nigrum* leaf extracts and find out the potential applications in biological system.

II. MATERIALS AND METHODS

A. Collection and preparation of Solanum nigrum leaf extract

Fresh leaves of S*olanum nigrum* were collected from Madurai. The leaves were washed thrice with distilled water to avoid fine dust particles on its surface. Following thorough washing, the leaves are completely air dried for about 15 minutes to remove

residual moisture. About 20 grams of finely chopped leaves were boiled with 100 ml of distilled water for 30 minutes at 60°C. After cooling, the leaf extract is obtained as a clear filtrate using Whatmann No: 1 filter paper and it was used for further analysis.

B. Plant-mediated synthesis of silver nanoparticles [12]

The extract of S*olanum nigrum* leaves (5 ml) was mixed with 45 ml of 1mM silver nitrate ($AgNO_3$) solution in 1:9 ratio in a conical flask under aseptic conditions. A colour change -black suspended formation was observed within 2 minutes of adding the leaf extract, indicating the formation of silver nanoparticles. The mixture is incubated for 24 hours in a dark room to allow maximum precipitation. Following the incubation period, the mixture was centrifuged at 5000 rpm for about 15 minutes. The supernatant is discarded and the pellet is washed thrice with 70% ethanol to remove impurities and left for complete air drying. The silver nanoparticles were harvested as fine powder.

C. Optimization of Silver nanoparticle synthesis

Optimized silver nanoparticles synthesis was obtained by variation and fixation of different parameters such as temperature, time, ratio of plant extract to silver nitrate solution, concentration of silver nitrate solution and pH in order to achieve maximum yield.

D. Characterization of silver nanoparticles

1. UV-VISIBLE ABSORBANCE SPECTROSCOPY[13]

UV-Visible spectroscopy analysis was carried out on a Systronic UV-Visible absorption spectrophotometer 117 with a resolution of ±1nm between 200-1000nm processing a scanning speed of 200nm/min. The progress of the reaction between metal ions and the leaf extract were monitored by UV-Visible spectra of silver nanoparticles in aqueous solution with different wavelength. The reduction of silver ions and formation of silver nanoparticles occurred within an hour of reaction Control was maintained by using AgNO3.

2. Fourier Transform Infrared Spectroscopy

For FTIR measurements, Perkin Elimer-spectrum RXI model was used. Fourier transformed infrared spectra is generated by the absorption of electromagnetic radiation within the frequency range 400 to 4000 cm-1.

3. Scanning Electron Microscopy

The pellet was subjected for SEM analysis. Thin films of the sample were prepared on just dropping a very small amount of the sample on the plate, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry for analysis.

4. TEM analysis of synthesized silver nanoparticles

The pellet was subjected for TEM analysis. Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid, extra solution was removed using a blotting paper and then the film on the SEM grid were allowed to dry for analysis.

E. ANTI BACTERIAL ASSAY

The antibacterial assay were done by standard well diffusion method. Briefly Luria Bertani (LB) broth/agar medium was used to cultivate Bacteria. Fresh overnight culture of inoculum (100µl) of each culture was spread on LB plates. Sterile well were cut of 5mm diameter containing different concentration of silver nanoparticles along with standard antibiotic (30µg/ml) containing were placed in each well plate as control. The plates were incubated at 37oC for overnight. Next day the inhibition zones around the well were measured.

F. Antioxidant assay

1.Estimation of total antioxidant using DPPH photometric assay

The ability of the nanoparticles to bleach DPPH can be quantified using a spectrophotometric assay, the extent of scavenging causing a proportionate change in the absorption at 518nm. An exact amount (0.5ml) of the methanolic solution of DPPH was added with different concentration of the nanoparticles in 0.48ml of methanol, and allowed to stand at room temperature for 30 minutes. Methanol served as the blank. After 30 minutes, the absorbance was measured at 518nm and converted into percentage radical scavenging activity as follows

Scavenging activity (%) =
A_{518} [Control]–A_{518}[sample-treated]/A_{518} [Control] x 100

2. Nitric oxide-scavenging activity

The reaction mixture containing 0.3ml of sodium nitroprusside, 2.68ml PBS and (10-50µg) of silver nanoparticles was added and incubated at 25˚C for 15 minutes. Control tubes (100% generation) were prepared without the silver nanoparticles. After incubation, 0.5ml of the Griess reagent was added. The absorbance of the chromophore formed, indicative of the quantum of NO generated, was read at 546 nm.

G. Anticancer activity of synthesized silver nanoparticles

1. Cell viability assay

For the determination of cell viability, the cells were plated at a density of (1x10 6 cells/well) in a 96-well plate at 37˚C in 5% CO2 incubator. After 24 h of culture, the medium in the wells was replaced with the fresh medium containing nanoparticles in varying concentrations. After 24 h, 20 µl of MTT dye solution (5 mg/ml in phosphate buffer pH 7.4) was added to each well. After 4 h of incubation at 37˚C and 5%

CO2, the medium was removed and formazan crystals were solubilized with 200 µl of DMSO and the solution was vigorously mixed to dissolve the reacted dye. The absorbance of each well was read on a micro plate reader at 545 nm. The spectrophotometer was calibrated to zero absorbance, using culture medium without cells. The relative cell viability (%) related to control wells containing cell culture medium without nanoparticles was calculated by the following formula:

% of cell viability = 100 x (Sample absorbance / Control absorbance)

2. Cell cycle analysis by flow cytometry

The distribution of Hep-2 and AGS cells in various phases of the cell cycle was analyzed by flow cytometry using PI stain.

Hep-2 and AGS (1x106 cells) treated with the test compound of silver nanoparticles (400 and 100µg/ml) were harvested after 24 hours and washed with PBS. The cells were resuspended in 1ml of the PI reagent and allowed to stainin the dark for 30 minutes. At the end of the incubation period, the cells wereanalyzed using the FACSverse flow cytometer (BD Biosciences, USA). TheFACSuite software was used to determine the percentage of cells in different phases of the cell cycle.

III. RESULTS AND DISCUSSION

Eco friendly methods of green mediated synthesis of nanoparticles are the present research in the limb of nanotechnology. Plant extracts are good source of biologically active substances but knowing the side effects before therapeutic application is essential to know the safety of the extract. Many Ayurvedic formulations are in use without valid scientific data on safety and efficacy[14-19].

In this study we are reported the biosynthesis of silver nanoparticles by using natural plants extract of Solanum nigrum. The aqueous silver ions were reduced to silver nanoparticles when added to plant extract of *Solanum nigrum*. Biologically synthesized silver nanoparticles have a wide range of applications because of their remarkable physical and chemical properties. They have potential applications as optical receptors, catalysts in chemical reactions.The role of plant biochemical's for the synthesis of nano silver particles is directly related to the mechanisms of nanotechnology and green chemistry. Quite a few reports are available in the literature on the extracellular biosynthesis of Ag nanoparticles using several plants, plant pure compounds, microorganisms and enzymes [21-24].

Here we used fresh aqueous leaf extracts of Solanumnigrum mixed with the AgNO3 solution for the formation of nano-Ag and it was confirmed by the change in the color of the extract to dark brown color immediatelyafter 24 hours incubation in a dark room as shown in Figure 1.

The use of plant-derived compounds, leaf extract has been extensively studied over the past years in the synthesis of AgNPs. The phytochemicals present in plant extracts such as proteins, polyphenols and sugars have been reported to play a significant role in reducing and stabilizing the Nanoparticle synthesis. However, it is obvious that determining the specific role of these phytochemicals is ruled out since most of them have a dual function during synthesis. For example, proteins and sugars can actively participate in both reductions of metal ions and in the stabilization of nanoparticles formed .

A. *Solanum nigrum* plant extract **B.** silver nitrate and **C.** after addition of silver nitrate **D.** Silver nanoparticles post 3 hours **E.** Silver nanoparticles post 24 hours **F.** Dried form of silver nanoparticles

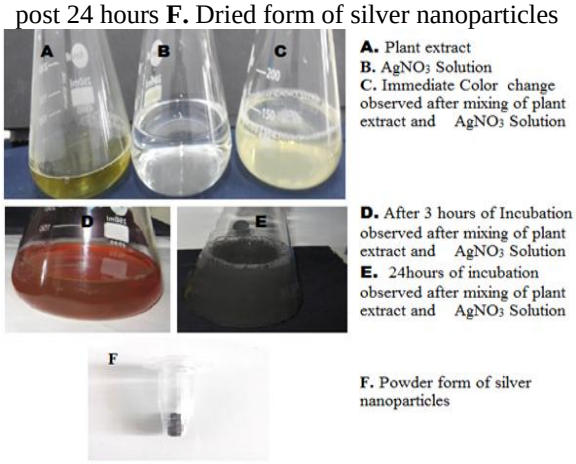

Fig.1 Synthesis of Silver nanoparticles from Solanum nigrum leaf extract

A. Optimisation of silver nano particles synthesis

Silver nanoparticles was synthesized by using Solanumnigrumleaf extracts and optimized by various parameters such as difference in concentration of silver nitrate (0.5, 1.0, 1.5, 2.0mM), temperature (37^0C, 60^0C, 90^0C,120^0C), pH (6,7,8,9,), time of incubation (3, 6, 12, 24hrs) and ratio (1:1,1:9, 3:7). It is clear which is indicated in Figure 2, the overall results showed that the optimized reaction condition for the synthesis of plant-mediated nanoparticle for various parameters such as temperature-600C, time- 24 hours, concentration of silver nitrate-0.5mM, pH-neutral and the ratio of silver nitrate solution and Solanumnigrumleaf extract was 1:9. Figure 2 A shows 0.5 mM concentration. maximally favoring the formation of silver nanoparticles. The rate of silver nanoparticles increased at 600C (Figure 2B). Figure C shows that a neutral pH condition enhances and accelerates the formation of silver nanoparticles but the acidic condition deteriorates the formation of silver nanoparticles. Figure D showsthe optimum time required for the completion of reaction from our study

is 24hrs. Figure E shows the ratio of silver nitrate solution (0.5mM) and the leaves extract was altered to investigate the optimum composition to maximize the yield of silver nanoparticles. It was found that the optimum ratio for the reaction is 1:9.

A. Optimization of various concentration B. Temperatures C. Various pH D. Time of incubation E. Plant extract and silver nitrate ratio

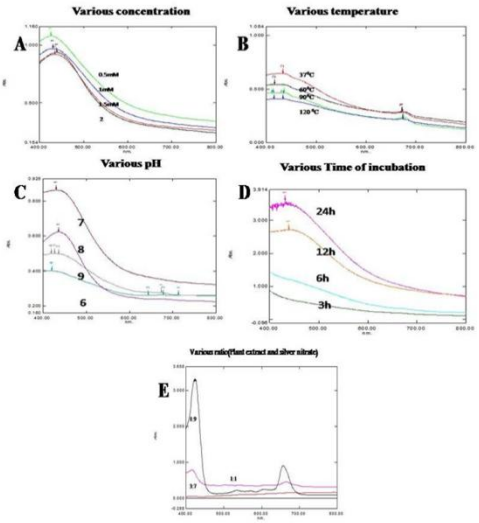

Fig.2 Optimisation of silver nanoparticles formation using various parameters

Various factors affecting biological synthesis of Nanoparticles and a number of controlling factors are involved in the nucleation and subsequent formation of stabilized nanoparticles. These factors include pH, concentrations, reaction time and temperature. The pH value of the reaction medium plays a significant role during the formation of nanoparticles Many studies have shown that varying the pH of the reaction medium tends to produce variability in shape and size of nanoparticles synthesized. In particular, larger particles tend to be produced at a lower acidic pH which compared to high pH values[25].

B. Characterization of synthesized silver nanoparticles

1. UV-Vis Spectrophotometer

UV–Vis spectroscopy is an important technique to establish the formation and stability of nanoparticles in aqueous solution. The shape and size of nanoparticles in aqueous suspension can be assessed by UV-Visible absorbance studies.

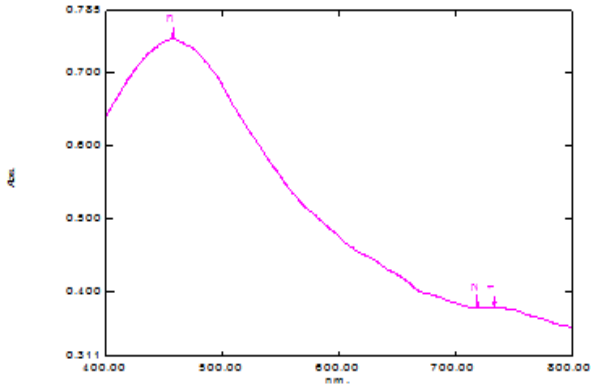

Fig.3 UV-Vis Spectrophotometer analysis of synthesized silver nanoparticles

Figure 3 shows the maximum absorbance of synthesized silver nanoparticles at 458 nm which confirmed the formation of silver nanoparticles.Same results observed by a large number of plants are reported to facilitate silver nanoparticles synthesis[26]. Plant-mediated synthesis of biomedical silver nanoparticles by using leaf extract of Citrullus colocynthis and were characterized by using UV-Vis spectroscopy, FTIR and AFM and the synthesized nanoparticles were generally found to be spherical in shape and 31 nm in size and FT-IR peaks were in the extract ranging from 1000-4000cm-1 which confirmed the presence of polyphenols with aromatic ring and bound amine region for the synthesis and stabilization of silver nanoparticles[27].

2. FTIR Spectrum of Silver Nano Particles

FTIR measurement was carried out to identify the potential biomolecules and functional groups responsible for reducing the silver ions. Different functional groups and structural features in the molecule absorb at characteristics frequencies. The frequency and intensity of absorption are the indications of the band structures and structural geometry of the molecule. The FTIR spectra of silver nanoparticles are shown in **Figure 4.** The interaction of nanoparticles with phytochemicals of *Solanum nigrum* showed intense peaks at 3350.90cm-1 corresponds to N–H stretch (1°, 2° amines, amides), 2928.04cm-1 corresponds to =C–H stretch (alkenes), 2360.95cm-1 correspond to H–C=O: C–H stretch (aldehydes), 1700-500cm-1 corresponds to many fingerprint regions and relative shift in position and intensity distribution were confirmed with FT-IR. These include single bond stretches and a wide variety of bending vibrations. Same result observed in rapid biosynthesis of silver nanoparticles using *Eichorniacrassipes* and which was confirmed by Fourier Transform Infrared spectrum (FTIR) revealed that the phenolic groups present in the plant extract were responsible for the reduction of silver nitrate into silver nanoparticles [28].

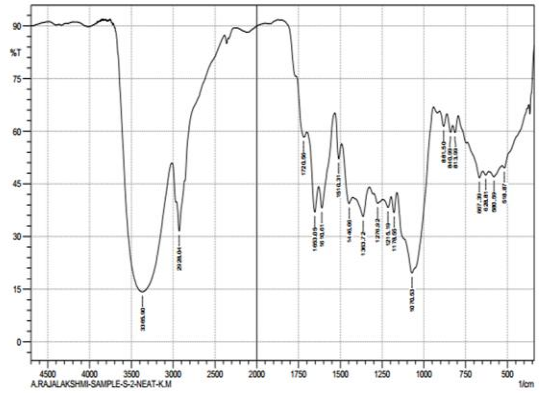

Fig.4 FTIR Spectrum of Synthesized silver nanoparticles

3.SEM, EDAX and TEM analysis for synthesized silver nanoparticles

The inspection of SEM images clearly indicates that synthesized silver nanoparticles size is in the range of 71.80nm and the representative SEM images are shown in **Figure 5**. The analysis through energy dispersive X-ray spectrophotometers confirmed the presence of an elemental silver signal of silver nanoparticles.

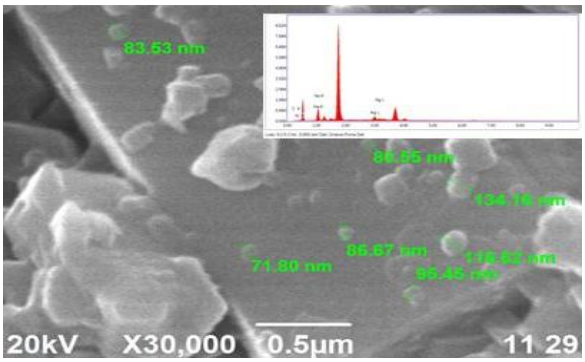

Fig.5 SEM and EDAX Profiling of Synthesized silver nanoparticles

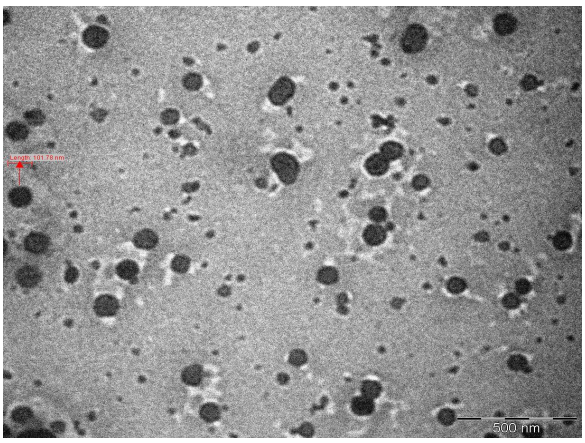

Fig.6 TEM Profiling of Synthesized silver nanoparticles

The TEM analysis implies the particle minimum average size around 34.29nm which results are shown in **Figure 6.**

Synthesized silver nanoparticles were well-characterized by UV-Visible Spectroscopy (UV-Vis), Fourier-Transform Infrared Spectroscopy (FT-IR), Transmission Electron Microscopy analysis (TEM). It was found that spherical shape nanoparticle, size 20 nm were found in TEM analysis [29].

4. Antioxidant activity of synthesized silver nanoparticles

The radical-scavenging activity of synthesized silver nanoparticles of *Solanum nigrum* was estimated by comparing the percentage inhibition of formation of DPPH radicals with that standard ascorbic acid. The DPPH scavenging activity of silver nanoparticles increased with increase in concentration and the nanoparticles have a good radical scavenging ability this result was shown in **Figure 7.** Oxidative diseases are due to free radicals resulting in oxidative stress. Free radicals such as superoxide anion, hydroxyl radicals and non-radical species such as hydrogen peroxide and singlet oxygen are different forms of activated oxygen constituting reactive oxygen species (ROS) **[30].** The active anti-oxidative defense system is required to balance the production of free radicals. The oxidative damage created by free radical generation is a critical etiological factor implicated in several chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis and neurodegenerative diseases and also in the aging process. In the treatment of these diseases, antioxidant therapy has gained an enormous importance.

A. Dot blot assay B. DPPH assay C. Nitric oxide scavenging assay D. Hydrogen radical scavenging assay

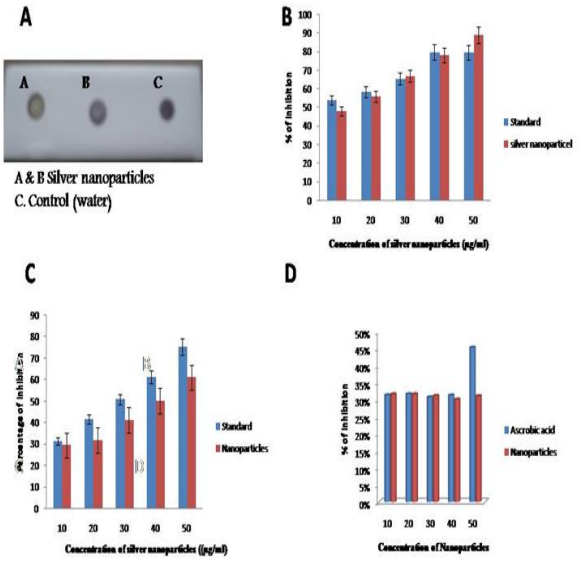

Fig.7 Antioxidant activitiy of synthesized silver nanoparticles

Figure 7 A shows the dot blot assay of synthesized silver nanoparticles in the TLC plate wherein the violet background change into a white color which

indicates the radical property of the silver nanoparticles. Figure 7B shows the DPPH scavenging activity of silver nanoparticles increased the inhibition rate with increase in concentration. Figure 7C shows the Nitric Oxide scavenging activity of the synthesized silver nanoparticles. Figure 7D shows the hydrogen peroxide scavenging activity of the synthesized silver nanoparticles and caused a strong dose-dependent inhibition of hydrogen peroxide.

C. Antibacterial activity of synthesized silver nanoparticles

These nanoparticles have been shown to accumulate in the membrane and can subsequently penetrate into the cells causing damage to the cell wall or cell membranes. Our results of antibacterial activity of synthesized Ag nanoparticles against four different bacteria such as *Pseudomonas aeruginosa, Staphylococcus aureus, E.coli* and *Bacillus subtilis*. It showed a clear inhibition zone, standard antibiotic (100µg/ml) tetracycline was used as a control and these results are shown in **Figure 8**.

- Figure 8A shows the well diffusion method using synthesized silver nanoparticles while Figure 8B shows the zone of inhibition around the well.

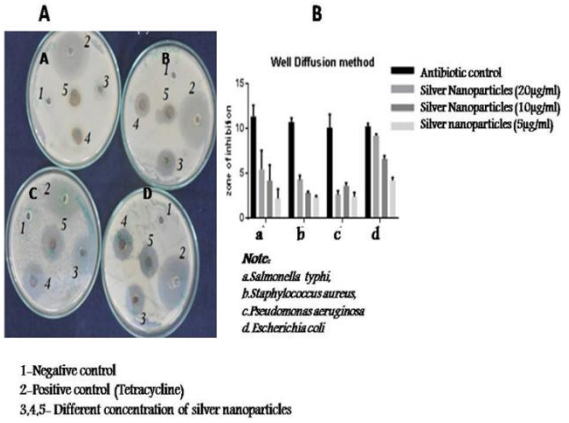

Fig.8 Antibacterial activity of synthesized silver nanoparticles

Silver was generally used in the nitrate form to induce antimicrobial effect but when silver nanoparticles are used, there is a huge increase in the surface area available for the microbes to be exposed to silver nanoparticles synthesized using plant extracts (from different sources) [31-32].

D. in vitro cytotoxicity assay

The effects of synthesized silver nanoparticles on the viability of Hep-2 cell line were checked using MTT assay. The synthesized silver nanoparticles were able to reduce the viability of the Hep-2 cells in a dose-dependent manner, as shown in Figure 9. After 24 hours of treatment, the synthesized silver nanoparticles were found to be cytotoxic to Hep-2 cells at concentrations of 400µg/ml and higher. AgNPs, at the concentration of 400µg/ml, decreased the viability of Hep-2 cells to 50% of the initial level, and hence this was chosen as the IC50. For further experiments, the 400 µg/ml was carried out for analyzing the effect of AgNPs. Cytotoxicity was found to be dependent on the nanoparticles used. The synthesised silver nanoparticle effect on a cell-culture model was quantified using MTT assay and to determine the viability for 24 hour incubation period.

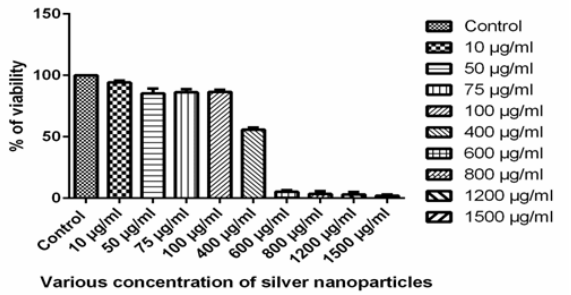

Fig.9 MTT assay of synthesized silvernanoparticle using Hep-2 cells

The induction of synthesized silver nanoparticles mediated cell apoptosis was observed by using dyes such as Acridine Orange/Ethidium Bromide double staining for treated the nuclei in the with an optimized dose of silver nanoparticles. Microscopic images of the dual stained cells, which was showed in **Figure 10**. Here the live cell nuclei stained green due to Acridine Orange uptake and their numbers gradually decreased with time owing to more cell death treated with the IC-50 concentration of nanoparticle, the dead cells are stained with red color. **Figure 10A** shows the phase contrast microscopy observation of Hep-2 cells in the control and IC-50 value treated groups. **Figure10B** shows the Hep-2 control and IC_{50} value treated cells observation using Fluorescence microscopy. Live and untreated cells had well-organized chromatin structures, whereas the treated cells had fragmented or condensed chromatin indicating apoptotic nuclei. Therefore, the nuclear staining experiment confirms the occurrence of apoptosis after the addition of synthesized silver nanoparticles to Hep-2 cancer cells.

A. Phase contrast microscopy B. Fluroescence microcopy observation of Hep-2 cells

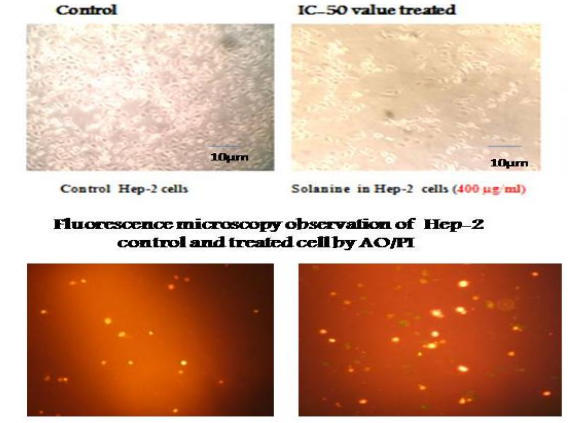

Fig.10 Morphological observation of Hep-2 cells

E. Cell cycle analysis

Cell cycle arrests are associated with the anticancer activity of synthesized silver nanoparticles. FACS cell cycle analyzes showed the anticancer regulatory action of silver nanoparticles. Anticancer activity of silver nanoparticles can be accompanied with the cell cycle arrest in any of the four phases. The total DNA content of the controlled, untreated and treated cancer cells (Hep-2) with 400µg /ml nanoparticles was estimated through FACS using PI staining. **Figure 11** A shows Control Hep-2 cells and **Figure B** shows Hep-2 cells treated with IC50 silver nanoparticles. **Figure C** indicates the DNA content quantified in each stage of cell cycles and the inhibition activity against cancer cells compared with the control. Recent literature demonstrated many drugs showing cell cycle arrest in both G0/G1and G2/M phase.

A. Control B. IC-50 value treated Hep-2 cells C. Bar diagram of cells in each stage

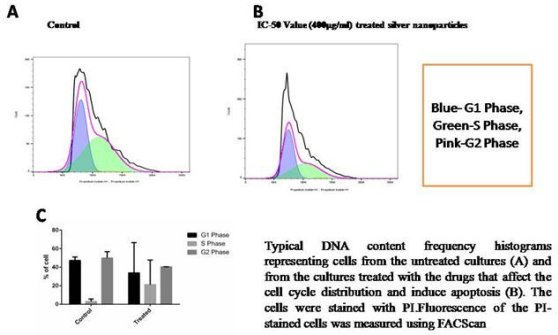

Fig.11 FACS analysis for Hep-2 cell lines

F. Apoptotic activity of synthesized silver nanoparticles in AGS cell lines

The effect of synthesized silver nanoparticles on the viability of AGS cells was checked using MTT assay. The synthesized silver nanoparticles were able to reduce the viability of the AGS cells in a dose-dependent manner, which was shown **in Figure 12.** After 24 hours of treatment, the AgNPs were found to be cytotoxic to AGS cells at concentrations of 100µg/ml.

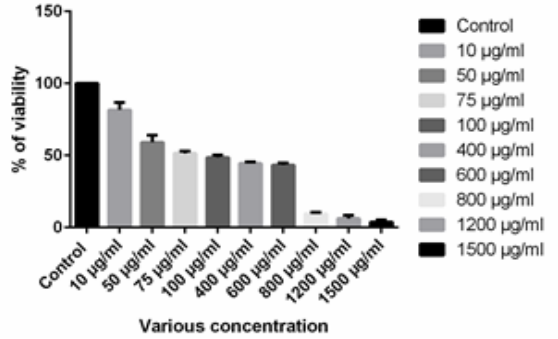

Fig.12 MTT assay of synthesized silver nanoparticles using AGS cell line

The induction of synthesized silver nanoparticles mediated cell apoptosis was observed by Phase contrast and fluorescence microscopy. Acridine Orange/Ethidium Bromide double staining of treated cell nuclei with an optimized dose of silver nanoparticles. Microscopic images of the dual stained cells, presented **in Figure 13.**

A. Phase contrast microscopy B. Fluroscence microscopy observation in control and IC-50 value treated with silver nanoparticles

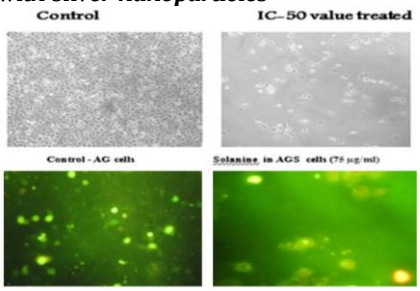

Fig.13 Morphological observation of AGS cell lines

The treated Hep-cells contained more apoptotic cells when compared to the untreated monolayer (Figure 13 A and B control cells). There was characteristic nuclear fragmentation of nuclei in treated HEp2 and the untreated control cells did not show any nuclear fragmentation. The apoptotic cells displayed the characteristic features of condensed nuclear chromatin and formation of membrane blebs. The nuclear changes were observed in the Hep-2 cells exposed to presence and absence of synthesized silver nanoparticles.

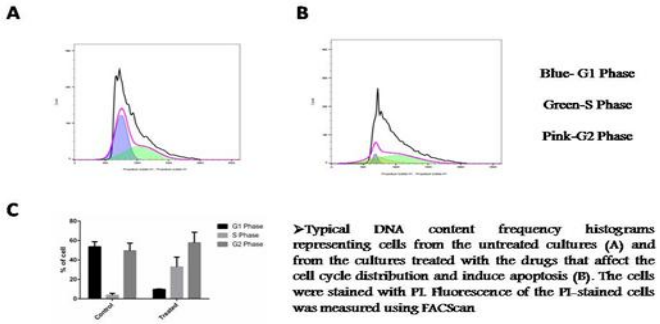

Fig.14 FACS analysis of AGS cell lines for Synthesized silver nanoparticles

Further Cell cycle arrest is associated with the anticancer activity of silver nanoparticles. FACS analyzed cell cycle regulatory action of silver nanoparticles towards cancer cells. Anticancer activity of silver nanoparticles can be accompanied with the cell cycle arrest in any of the four phases. The total DNA content of controlled untreated and treated cancer cells (AGS cells) with 100µg /ml estimated through FACS using PI staining. The inhibition activity against cancer cells compared with control is shown in **Figure 14**.

IV. CONCLUSION

In the present study, eco-friendly and environment benign silver nanoparticles (AgNPs)

were successfully synthesized by reduction of silver metal atoms in to silver nano particles by utilizing fresh plant leave extract of Solanum nigrum. Nanoparticles have great promises of antioxidant, antibacterial and apoptotic activity to the cancer cells (AGS and Hep-2 cell lines).

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