

MURRAYA KOENIGII: BIOGENIC SYNTHESIS OF SILVER NANOPARTICLES AND THEIR CYTOTOXIC EFFECTS AGAINST MDA-MB-231, HUMAN BREAST CANCER CELL LINES

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ABSTRACT

Silver nanoparticles were successfully synthesized using silver nitrate and *Murraya koenigii* leaves extract by varying the concentration of hydroalcoholic extract. The *Murraya koenigii* leaves extract was containing carbohydrates, flavonoids, phenolic and alkaloids as phyto-constituents. Formation of Silver nanoparticle with phytochemicals was confirmed by UV-Vis spectra by observing peak absorption at 411.0 nm & 423.5 nm. FT-IR spectroscopy showed the capping of silver was due to phenols and alkaloids. Dynamic light scattering of the prepared formulations revealed all the formulations were within nano range, SN1 showed average particle diameter 170.7 nm with zeta potential -22.51 mV, SN2 showed particle diameter was 67.5 nm with zeta potential -18.14 mV, and SN3 showed average particle diameter of 64.1 nm with zeta potential -12.56 mV and SN4 formulation showed the average particle diameter of 58.4 nm with zeta potential -11.89 mV, SN5 formulation showed the average particle diameter of 46.9 nm with zeta potential -8.92 mV, out of the above formulation SN5 was having least average particle diameter. Atomic force microscope showed that the particles were having smooth surface in the range from 29.4 nm to 52.0 nm for SN5 formulation. X-ray diffraction of the formulation showed the structural information of silver nanoparticle which were FCC in shape. Haemolytic studies revealed that there was negligible haemolysis of erythrocytes when nanoparticles were used in the concentration of 100 µg/ml thus prepared formulation were bio-compatible. MTT assay of optimized formulation SN5 compared with pure hydroalcoholic extract showed a dose dependent anticancer activity against MDA-MB-231 cell lines. The IC₅₀ for hydroalcoholic extract was 2.40 mg/ml whereas for synthesized AgNps the IC₅₀ was 6.522 µg/ml only.

INTRODUCTION

In pharmaceuticals, about 90% of the active ingredients are in the form of solid particles. The development in nanotechnology, has opened a new avenue for nanoparticles by a variety of innovative ways. Nanotechnology is frequently applied in fiber textiles, agriculture, electronics, forensic science, space and medical therapeutics, namely in disease detection, controlled drug delivery, as biosensors in tissue engineering and so on. Nanoparticle drug formulation reduces the patient expenses and risks of toxicity.^[1]

Nanoparticles are solid colloidal particles ranging from 1 to 100 nm. These Nano materials can be used therapeutically as drug carriers, in which the active principle (drug or biologically active material) is dissolved, entrapped, or encapsulated or to which the active principle is adsorbed or attached.^[2] As nanoparticles size and surface characteristics can be easily manipulated, this could be used for both passive and active drug targeting. Nanoparticles can be used in control and sustained release of the drug during the transportation as well as at the location of the release. An

increase in drug therapeutic efficacy and reduction in dose can be achieved. Choosing an appropriate matrix helps in increasing the efficacy of drug and reducing side effects associated with dose. Nanoparticles can be developed for various routes of administration including oral, nasal, injection, intraocular (within the eyes) etc.^[3]

Metal nanoparticles like gold, silver and platinum are widely prepared nanoparticles in industries. Silver nanoparticles have been given more attention due to their numerous applications in catalysis, bio molecular, detection and diagnostics, and therapeutic, micro-electronics fields etc. The silver nanoparticles formulation was first marketed in 1907. From then methods of synthesizing and its applications increased year by year. The silver nanoparticle itself has got many activities like anti-cancer,^[4] anti-microbial^[5] etc. So development of silver nanoparticles with different natural extracts will enhance the biological activity.

There are several research articles on silver nanoparticles synthesized by green synthesis using plant extracts, fungus, etc. Silver nanoparticles of *Abelmoschus*

esculentus (L.) pulp extract,^[6] *Ficus krishnae*,^[7] has anti-cancer activity. Silver nanoparticles by *Micromonospora* species,^[8] *Pedaliium murex* leaf,^[9] bitter guard fruit,^[10] *Loquat* leaf,^[11] Edible mushrooms^[12] has anti-bacterial activity. Silver nanoparticles using *Azadirachta indica*.^[13] Silver nanoparticles synthesized using *Urtica dioica* Linn. Leaves and their synergistic effects with antibiotics.^[14] Silver nanoparticles synthesized using Papaya fruit extract, *Zea mays*,^[15] *Murraya paniculata* leaf^[16] has anti-microbial activity.^[17] All these synthesis of silver nanoparticles have novelty with regard to variation in size, shape, synthesis conditions, stability and their biological activity. The potential of plants for the rapid synthesis of nanoparticles is to be explored.

MATERIALS AND METHODS

Materials

Fresh leaves (*Murraya koenigii*) were procured from the local market of Bagalkot. Silver nitrate of analytical grade was purchased from SDFCL, Mumbai. Double distilled water was used throughout the study.

Preparation of extract

The leaves were washed with 2% KMnO₄ (potassium permanganate) solution followed by double distilled water for 2-3 times and dried at room temperature in shade. 10gm of dried leaves of *Murraya koenigii* was weighed and warmed with 70ml double distilled water and 30ml absolute ethanol at 60°C for 15 min in RBF later it was sonicated for 15 min at 60°C. After sonication the solution was filtered through muslin cloth, and again filtered by Whatmann filter paper No.1 and was centrifuged at 5000 rpm to remove leaf debris for 5 min and kept in refrigerator for further use.

Synthesis of silver nanoparticles

For the reduction of silver ions, extract was mixed to 2mM aqueous silver nitrate. A change from yellow to reddish color was observed and kept for 2h in dark amber glass bottle. To obtain the dry powders of green synthesized silver nanoparticles, the mixture was centrifuged at 10,000 RPM for 15 min. The pellet was resuspended in double distilled water and centrifugation process was repeated thrice to get rid of any un-reacted biological materials. The purified pellets were then dried in hot air oven at 60°C to get silver nanoparticles, these nanoparticles were further used for characterization studies.^[18]

Characterization of AgNPs

The formulation of silver nanoparticles in aqueous suspension was analyzed by UV-Vis spectrophotometer (Shimadzu, Japan). The hydrodynamic size distribution and polydispersity index of nanoparticles are analyzed by using dynamic light scattering (Brookhaven instrument corp.) instrument. FTIR spectra was recorded by placing pellet in holder of Fourier transform infrared (FTIR) spectrophotometer (Shimadzu IR Affinity- 15). X-ray diffraction crystallography (Advanced brucker instrument) technique was performed for determination

of the dimension of biologically synthesized silver nanoparticles using X- ray diffractometer. The particles size and shape in 2D and 3D was analyzed by using AFM dimension ICON system. Haemolytic activity was done using rats blood.

MTT assay

Cells cultured in T-25 flasks were trypsinized and aspirated into a 5mL centrifuge tube. Cell pellet was obtained by centrifugation at 300 x g. The cell count was adjusted, using DMEM HG (Dulbecco's Modified Eagle Medium with High Glucose) medium, such that 200µl of suspension contained approximately 10,000 cells. To each well of the 96 well microtitre plate, 200µl of the cell suspension was added and the plate was incubated at 37°C and 5% CO₂ atmosphere for 24 hr. After 24 hr, the spent medium was aspirated. 200µl of different test concentrations of silver nanoparticles formulation (25, 50, 100, 200 and 400 µg/mL serially diluted from stock) and standard drug Cisplatin (50 µg/mL diluted from stock) were added to the respective wells. The plate was then incubated at 37°C and 5% CO₂ atmosphere for 24 hr. The plate was removed from the incubator and the silver nanoparticles containing media was aspirated. 200µl of medium containing 10% MTT reagent was then added to each well to get a final concentration of 0.5mg/mL and the plate was incubated at 37°C and 5% CO₂ atmosphere for 3 hr. The culture medium was removed completely without disturbing the crystals formed. Then 100µl of solubilisation solution (DMSO) was added and the plate was gently shaken in a gyratory shaker to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 570 nm and also at 630 nm. The percentage growth inhibition was calculated, after subtracting the background and the blank, and concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) was generated from the dose response curve for the cell line.^[19-22]

RESULTS AND DISCUSSION

UV-Vis spectrometer analysis

The absorbance spectroscopy is a rapid way to obtain data of colloidal silver nanoparticle solution. The UV-Vis spectroscopy is most widely used techniques for structural characterization of silver nanoparticles.^[23] Synthesized silver nanoparticles exhibit strong absorption of electromagnetic waves in the visible range due to their optical resonant to its collective oscillation of conduction electrons, combined with the incident light Fig.1.a shows the UV-Vis absorption spectra of the hydroalcoholic extract & fig 1.b and shows after 2 hours AgNPs from *Murraya koenigii*. The peak of the above spectra was due to Surface Plasmon resonance property of silver nanoparticles. Surface Plasmon resonance (SPR) is defined by Link and Elligh^[24] wave which induces a polarization of the electrons with respect to the much heavier ionic core of a nanoparticle. SPR is easily and rapidly measured by UV absorbance and peak widths can indicate particle size and distribution from

band widths and the peak maxima absorbance. This phenomenon complies with Mie's theory for spherical particles. This method, however, has a narrow working size range of 2-20 nm, although the upper limit could be as large as 50-80 nm. The spectra recorded before addition of silver nitrate & after 2hrs of addition observed increased intensity in absorption spectra of

silver solution with time, indicating the formation of increased number of silver nanoparticles in the solution; this is similar with the findings of Daizy *et al.* The prepared AgNPs showed strong absorption band at 423.5 and 411 nm which is typical absorption band of nanoparticles due to their surface Plasmon thus synthesized AgNPs may be polydispersed.

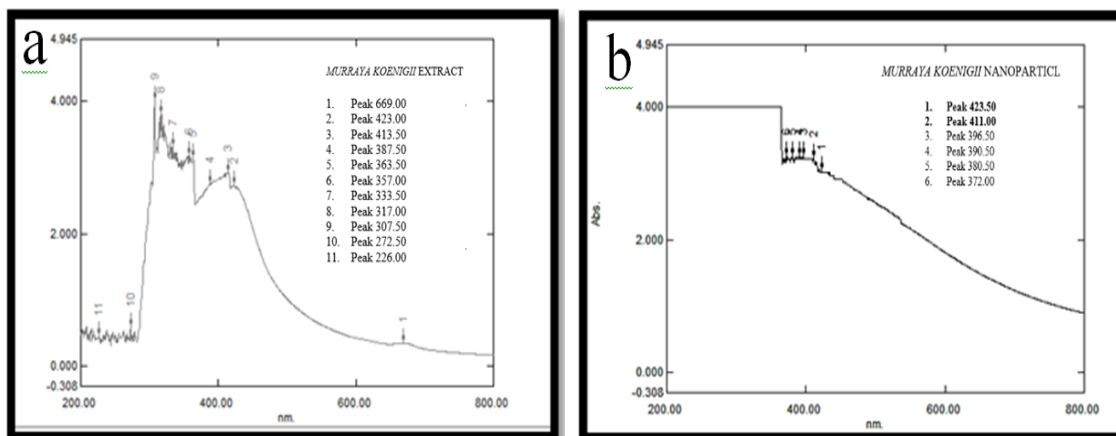


Fig-1: UV-Vis peaks of (a) pure hydroalcoholic extract of *Murraya koenigii*, (b) Silver nanoparticles (SN5 formulation).

FT-IR analysis of silver nanoparticle

FTIR analysis was used for the characterization of the extract and the resulting nanoparticles. FTIR absorption spectra of hydroalcoholic extract before and after reduction of Ag ions are shown in fig 2 a & b. Absorbance bands were observed in the region of 1633.78 cm^{-1} may be due to -C=C- stretching vibration. Weak intensity of 1062.82 cm^{-1} can be assessed as absorption band of C-O-C. 1388.81 cm^{-1} absorption may be due to -C-O stretching, -C=C- stretching vibration

can be allotted to 1525.76 cm^{-1} . The above bands can be corresponding to -C=C- (ring), -C-O-C- and C=C (chain) which are derived for hydroalcoholic phenols. Weak stretching band of 1250.89 cm^{-1} may be due to C-N. the strong band of 3432.48 cm^{-1} is due to CH-NH-CH these two bands clearly indicate the presence of alkaloids. The relative shift in position and intensity distribution of IR bands in the two spectra show that the biomolecules are responsible for capping and stabilization of silver nanoparticles.^[25]

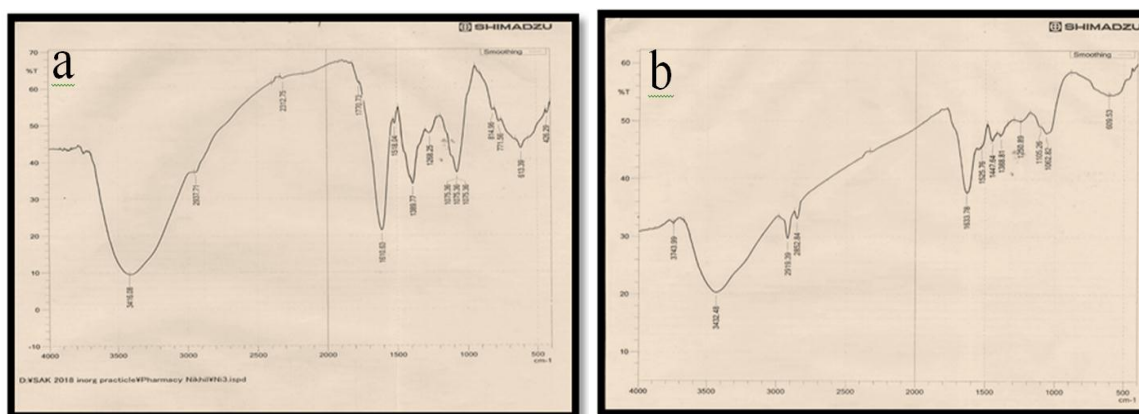


Fig. 2: FTIR peaks of (a) Hydroalcoholic extract of *Murraya koenigii* leaves, (b) Silver nanoparticles of *Murraya koenigii*.

Dynamic light Scattering and Zeta potential

The DLS size distribution of silver nanoparticles from SN1, SN2, SN3, SN4 and SN5 are as follows, SN1 showed mean particle diameter 170.7 nm with zeta potential -22.51 mV , SN2 showed mean particle diameter was 64.1 nm with zeta potential -18.14 mV , SN3 showed mean particle diameter of 58.4 nm with zeta

potential -12.56 mV , SN4 formulation showed the mean particle diameter of 67.5 nm with zeta potential -11.89 mV and SN5 formulation showed the mean particle diameter 46.9 nm with zeta potential -8.92 mV . Above all the formulation. SN5 was having least average particle diameter. From observed particle size distribution graph it was confirmed that nanoparticle

were having a broad size distribution of particles. This indicated synthesized nanoparticles were poly dispersed with negative charge in the concentration of hydroalcoholic extract from SN1 to SN5. It was observed that as the concentration of hydroalcoholic extract was increased the particle size of silver nanoparticles were decreased these values were in good agreement with the reported literature.^[26] The high negative zeta potential of the silver nanoparticles confirms the repulsion among the particles and proves that they are stable. As SN5 formulation was having least average particle diameter so it was further chosen for evaluation.

X-Ray Diffraction

The crystalline nature of silver nanoparticles was confirmed from X-ray diffraction (XRD) analysis. The XRD pattern of silver nanoparticles in colloid SN5 is shown in fig 3. The diffraction peaks are indexed as (111), (200), (220), (311) and (222) planes of FCC silver. The feature indicates the nanocrystals are (111) oriented. The Bragg peaks representative of FCC silver nanocrystals, additional unassigned peaks (400), (420) and (422) were also observed suggesting that the crystallization of bio-organic phase has occurred on the surface of the nanoparticles. Similar results were reported in silver nanoparticles synthesized using geranium leaf extract^[27] and mushroom extract.^[28]

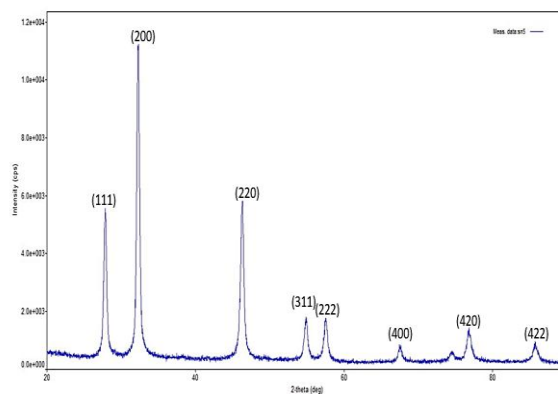


Fig. 3: X-ray diffraction of SN5 formulation.

Atomic force microscope

AFM studies showed that the AgNPs were similar in shape and size, ranging between 29.4 nm and 52.0 nm. The two-dimensional and three-dimensional views of the AgNPs are shown in fig 4. AFM analysis was performed to analyse the size and shape of the AgNPs. From the AFM images, it was found that the biosynthesized AgNPs were mostly FCC in shape with narrow size distribution as shown in fig 4(a). Most of the AgNPs are sized between 29.4 nm and 52.0 nm and were in aggregated form. A similar result was reported by Sahana *et al.*^[29] On the synthesis of AgNPs from *Cassia auriculata* flower extract. But, in their studies synthesized NPs were hexagonal and irregular narrow size distribution was observed. The average particles size was found to be 42.01 nm by AFM. DLS (Dynamic light Scattering) results show that the mean particle size of the biosynthesized AgNPs was around 46.9 nm which is shown in fig 4(b). The DLS measures the hydrodynamic radii of the NPs and hence the particle size value was found to be little high.^[30]

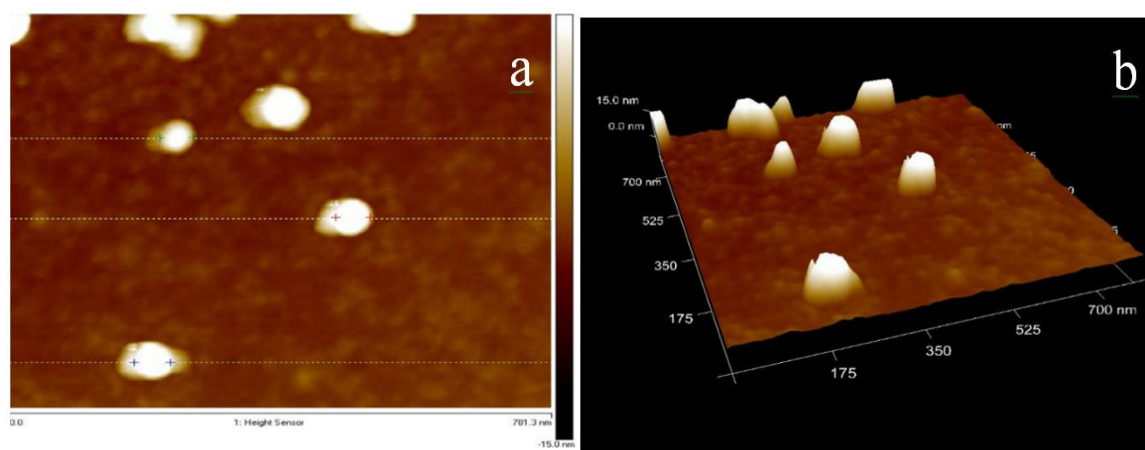


Fig. 4: AFM 2D and 3D images.

Biocompatibility

Haemolytic activity of silver nanoparticles was performed to evaluate the biocompatibility of the silver nanoparticles on normal cells, especially erythrocytes. The hemolytic assay was performed using different concentrations of SNPs (25, 50, 100, 200 and 400

$\mu\text{g/mL}$) on erythrocytes. The absorbance values of erythrocytes in PBS and in 1% triton X-100 was used along with the values of erythrocytes treated with SNPs on the formula. It was found that at below 100 $\mu\text{g/ml}$ there was negligible haemolysis and moderate increase in the haemolysis was observed at 200 $\mu\text{g/ml}$.

MTT Assay

The *in vitro* anti-cancer activity of hydroalcoholic extract of *Murraya koenigii* and SN5 formulation was tested on human cancer breast cell line MDA-MB-231. A dose dependent decrease in the viability of MDA-MB-231 cells was observed on treatment with hydroalcoholic extract and AgNps as shown in the fig.5 the half maximum inhibitory concentration (IC_{50}) for hydroalcoholic extract was greater than the dilution used for the test (25-400 $\mu\text{g/ml}$). Swee et al.,^[31] performed the MTT assay of *Murraya koenigii* leaves extract on MDA-MB-231 (breast cancer cell lines) and reported IC_{50} as 2.40 mg/ml , whereas for synthesized AgNps the IC_{50} was 6.5 $\mu\text{g/ml}$ only. There was change in percentage of cell

viability in MDA-MB-231 cells treated with hydroalcoholic extract, 2 % mortality rate was noted at 100 $\mu\text{g/ml}$ concentration for hydroalcoholic extract of *Murraya koenigii* whereas there was 97.3 % mortality rate at 100 $\mu\text{g/ml}$ concentration for synthesized silver nanoparticles (SN5). Above 100 $\mu\text{g/ml}$ silver nanoparticles did not exhibit profound mortality on MDA-MB-231 cells, this may be due to the saturation effect of silver nanoparticles. An increased mortality of MDA-MB-231 cells was observed for synthesized AgNps as compared to hydroalcoholic extract. This may be due to the carbazole alkaloids and phenolic compounds present in *Murraya koenigii*, which capped silver ions.

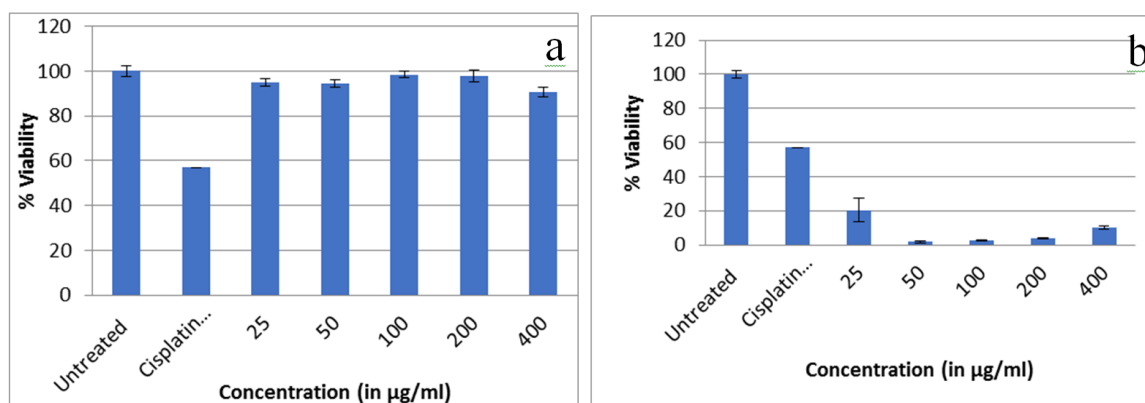


Fig.5. (a) % viability of MDA-MB-231 cell line Vs plant extract, (b) % viability of MDA-MB-231 cell line Vs SN5 formulation.

CONCLUSION

In the present study we have successfully synthesized the silver nanoparticles using *Murraya koenigii* from green synthesis. By DLS, X-ray, AFM, UV and FTIR, we have characterized the shape, size, crystallinity and time required to synthesize nanoparticles. The DLS showed size of nanoparticles was 46nm & polydispersed, the X-ray & AFM studies showed silver nanoparticles was FCC, UV revealed the formation of AgNPs and FTIR confirmed the reduction of silver was due to polyphenols & carbazole alkaloids. The study revealed that the synthesized AgNPs showed the potent anticancer activity on breast cancer cell lines (MDA-MB-231), further investigation is needed to reveal potency of synthesized silver nanoparticles on different cancer cell lines.

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