



Original Research Article

Cyto-toxicity and oligodynamic effect of bio-synthesized silver nanoparticles from plant residue of *Artocarpus altilis* and its spectroscopic analysis

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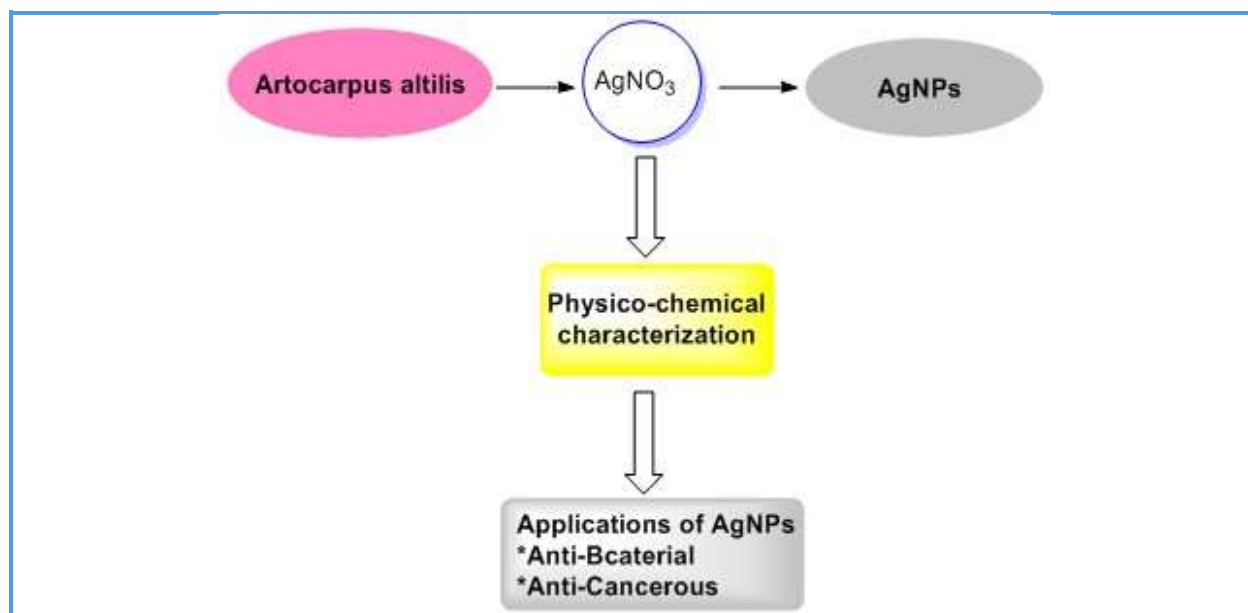
Crystalline

Artocarpus altilis

ABSTRACT

The medicinal plant residue obtained to synthesis AgNPs is the thrust area of research today. The present research work emphasis on the AgNPs synthesized from a medicinal plant residue *Artocarpus altilis* whose secondary metabolites bear responsible for the confined size of the AgNPs. Further, the AgNPs were analyzed for Physico-chemical analysis, where FT-IR Peak value gives the functional groups of *A. altilis*. FESEM analyses show surface morphology with 44 nm. EDAX analyses of show metal precursor involved in the process. XRD patterns show the crystalline structure. The AgNPs was analysed for the antibacterial assay against five human pathogens. Finally, cyto-toxic activity of AgNPs was analyzed with two human cancer cell lines namely MCF 7 lung cancer cell line and A549 breast cancer cell line. Hence, the novel and eco-friendly AgNPs are safe with its biocompatibility which becomes a promising agent in the biomedical precisely.

Graphical Abstract



Introduction

Synthesis of nanomaterials has been widely the area of research interest in the biomedical industry nanotechnology can provide promising applications *via* bio-sensing, biomedical, food, feed, and drug delivery, cosmetics and cancer therapy [1]. The antimicrobials applications have tremendous potentials to come out of microbial multidrug resistance [2]. The AgNPs have been a useful bio-compound that acts against some stains of human pathogens [3, 4]. Due to the physiochemical properties, the biosynthesized AgNPs do provide the fundamental substratum for various applications [5]. In recent past, the medicinal plants have been a choice material for synthesis of the NPs because of its ecofriendly nature, low time consuming, and large production. To name few extract such as *ocimum sanctum*, *petroselinum crispum*, *murraya koenigii*, *coriandrum sativum*, for the synthesis of metal NPs [6–9]. Here the important aspect is the obtaining a confined size and shape of AgNPs. The temperature,

concentration of ions, and time duration are the prime important factors, determining the fate of the NPs synthesis [10]. As the industry demands more AgNPs, there is another element comes in to dispose those synthesized NPs. The NPs are toxic and reactive at all levels. The cellular and sub cellular level toxic more prominent [11]. Since environment gets accumulated with NPs, the AgNPs are toxic and *in vitro* level experiments proved that the NPs can increase the oxidative stress, apoptosis, and genotoxicity [12]. The biosynthesized NPs procedure has to be standardized as the demand NPs production is increased. The so-called physical and chemical methods do have its negative side [13]. Due to the toxic nature, the AgNPs have a potential effect on the cancer cells. Because of nano size, the particles using the surface area provide promising effects as anti-cancer and anti-microbial compounds [14, 15]. The supreme aspects in the AgNPs have a minimal toxic effect compared to its counterparts. Since this modern science is at infancy stage, there should be effective protocols towards the

monitoring of NPs disposal in open environment [16–18]. Hence, it is befitting to have green method to synthesis and characterize AgNPs which are less toxic and large production is possible. As present study promptly emphasis on its eco method in the production of AgNPs; *A. altilis* do provide a confined size and shape of AgNPs when plant material incorporate with Ag. Having a AgNPs synthesis by green method, there is wide range of applications in the row which are eco friendly by nature. Moreover AgNPs from *A. altilis* serve as an anti-cancer and anti-microbial agent. The investigations are pure evident that AgNPS are promising bio compounds that acts upon various applications for human.

Experimental

Leaf extract

The plant *Artocarpus altilis*, was collected from the Tiruchirappalli, Tamil Nadu, South India and its identification is confirmed by the experts of Botanical Survey of India, Southern Regional Centre, Coimbatore, India (Ref. Number:BSI/SRC/5/23/2015/Tech.2500). The leaves were washed in running water and blotted dry and shade dried 4 days before pulverized into fine powder. One g of the fine filtered powder was boiled in 100 mL distilled at 60°C water for 5 minutes and cooled to room temperature. The filtrate was filtered first through Whatman No.1 filter paper (0.45 µm) and then through 0.22 µm sized filters. The filtrate was stored in sterile vials and kept in ambient temperature.

Chemicals

Pure and analytical grade chemicals used in the experiments were purchased from Hi-Media laboratories Pvt. Ltd., Mumbai. The water used was obtained through glass double distillation.

Glassware

Borosil (India) glass ware (Conical flasks, Measuring cylinders, Beakers, Petri plates and Test tubes etc....) were washed and sterilized as per standard microbiological procedures.

Synthesis of the AgNPs from *A.altilis*

The aqueous solution of 1mM AgNO₃ stock was prepared. From the stock, appropriate aliquots were allowed to react with plant extract (leaves) for the synthesis of AgNPs. The optimal mixing volumes were determined after a series of preliminary studies. The reaction was allowed at room temperature for an incubation period ranging between 1 h and 24 h to form the AgNPs. Here the filtrate of the plant extract acts as reducing/stabilizing and/or capping agent for the developing AgNPs.

Conformation and characterization of AgNPs

The conversion Ag ions to AgNPs indicates a change of colour in solution that turns from yellowish to brownish red, thus said to be the visible observation that confirms the formation AgNPs. Characterization of the AgNPs was conducted using Fourier transform infrared spectroscopy (FT-IR), field emission scanning electron microscopy (FE-SEM), energy dispersive X-ray spectroscopy (EDX), and X-ray diffraction (XRD) analysis. These experiments indicate the nanomaterials' synthesis is based on the reduction of metal-salts is the easiest and most widely used method in obtaining the uniform sized metal NPs.

Fourier transform infrared spectroscopy (FT-IR)

Infrared spectroscopy was used to find out possible physico-chemical interactions. The measurements were taken for the AgNPs synthesized after 24 h of reaction and were done using a FT-IR SHIMADZU 8400S with a

wavelength range of 4000 ~ 400 nm, where the samples were incorporated with KBr pellets to acquire the spectra. The results were compared for shift in functional peaks of critical value.

Field emission scanning electron microscopy (FE-SEM)

The FESEM was performed to characterize the morphology, shape, and topography of the AgNPs. The powder sample and the freeze-dried sample of the AgNPs was sonicated, and a small drop of this sample was placed on a glass slide and allowed to dry. Then, a thin layer of platinum was coated on the specimen to make it conductive. FE-SEM studies were performed using Hitachi model S3000H, Hitachi Ltd, Tokyo, Japan.

Energy dispersive x-ray spectroscopy (EDX)

The system for EDX, namely Bruker offers a powerful range for energy-dispersion of AgNPs. The new generation of QUANTAX EDAX features the XFlash® 6 detector series with active areas from 10 mm² to 100 mm². 10, 30, 60, and 100 mm² active area detectors offer ideal solutions for micro- and nano-analysis. Energy resolutions for detectors are usually specified in accordance with ISO 15632:2002.

X-ray powder diffraction (XRD)

XRD studies were carried out using XPERTPRO multipurpose x-ray diffractometer procured from the Netherlands, using Cu K α radiation with a wavelength of 1.540 Å. With the model of D8-A25, the XRD at is high (up to 1200 °C) in air or vacuum with double laser alignment system. The dried powder of AgNPs was further analyzed under X'Pert Pro X-ray diffractometer operated at a voltage of 40 kV and a current of 30 mA with Cu K α radiation in θ -2 θ configurations and its crystalline domain size was calculated from the width of the XRD

peaks using the Scherrer's formula, $D = 0.94 \lambda / \beta \cos \theta$.

Anti-bacterial assay

Anti-bacterial assay was experimented for the zone of inhibition studies. The bacterial strains were collected with MTCC number from division of microbiology, school of life sciences, and Bharathidasan university, India. The bacterial species tested were (five) *Rhodococcus rhodochrous* (MTCC-265); *Vibrio cholera* (MTCC-3904); *Staphylococcus aureus* (MTCC-2940); *Pseudomonas aeruginosa* (MTCC-1934); *Escherichia coli* (MTCC-739).

The tests bacteria were maintained in Nutrient Agar slants. The cultures were sub cultured and the same were allowed to grow two days and they were stored at 5 °C for future studies. Nutrient Agar medium was used for antibacterial studies. After having added all the ingredients in the distilled water, the substances were boiled for dissolving the broth completely and sterilized by autoclaving at 121 °C for 20 min at 15 lb. Then cooled it for few mints and then bacterial cultures were sub cultured in the liquid medium (Nutrient Broth) at 32 °C for 24-48 h. Then used for the experiments. The laminar air flow chamber was used for before inoculation with 70% alcohol. The sterile Petri-dish was used. The cabinet was irradiated with UV lamp is on for 25 min.

Disc diffusion assay

Disc diffusion method was used for the rapid determination of the drug and substances with a specific microbe. This method consists of impregnating small circular disc of standard filter paper with given amount of a chosen concentration of substance. The discs are placed on plates of culture medium that has been seeded with a test microbial inoculum. After incubation the diameter of the clear zone of

inhibition surrounding the deposit of substance is taken as a measure of the inhibitory power of the AgNPs against the particular test pathogen.

Cytotoxicity assay

The cytotoxic activities of all the complexes have been investigated against the cancer cell lines by using MTT assay. The MCF-7 human breast cancer cell line and A549 human lung cancer cell line were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were cultured in DMEM high glucose medium (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum (Gibco), and 20 mL of penicillin/streptomycin as antibiotics (Gibco), in 96 well culture plates, at 37 °C in a humidified atmosphere of 5% CO₂ in a CO₂ incubator (Thermo scientific, USA). All experiments were performed using cells from passage 15 or less.

Cell viability assay

$$\frac{\text{Mean of absorbance of untreated cells (control)} - \text{Mean of absorbance of treated cells}}{\text{Mean of absorbance of untreated cells}} \times 100$$

Acridine orange (ao) and ethidium bromide (eb) staining

Apoptotic morphology was investigated by AO/EB double staining method following Spector *et al* with some modifications. Briefly, the cells treated with IC₅₀ Concentration of compounds for 24 h. After incubation, the cells were harvested and washed with cold PBS. Cell pellets were resuspended and diluted with PBS to a concentration of 5X10⁵ cells/mL and mixed with 25 µL of AO/EB solution (3.8 µM of AO and 2.5 µM of EB in PBS) on clean microscope slide and immediately examined under fluorescent microscope (Carl Zeiss, Axioscope2plus) with UV filter (450–490 nm). Three hundred cells for each sample were scored for viable, apoptotic or necrotic by staining the nucleus structure

The complexes, AgNPs, AgNO₃ and plant extract were first dissolved in dimethyl sulfoxide (DMSO) to make a stock. These stock solutions were diluted separately with media to get various concentrations of the complex. Two hundred micro liters of these samples were added to wells containing 5 X 10³ MCF-7 cells and A549 cells per well. DMSO solution was used as solvent control. After 24 h, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and the plates were wrapped with aluminum foil and incubated for 4 h at 37 °C. The purple formazan product was dissolved by addition of 100 µL of DMSO to each well. The absorbance was monitored at 570 nm (measurement) and 630 nm (reference) using a 96-well plate reader (Bio-Rad, iMark, USA). Data were collected for three replicates each and used to calculate the respective mean. The percentage inhibition was calculated, from this data, using the formula:

and membrane integrity and the percentage of apoptotic and necrotic cells were calculated accordingly.

Results and Discussion

Characterization of AgNPs

The biogenic AgNPs were successfully synthesized from *A. altilis* plant residue at ambient temperature and right concentration of Ag. The obtained AgNPs were subjected to various physico-chemical analysis. The first one was the FT-IR spectrum of the AgNPs and the observed peaks indicated the occurrence of flavonoids and terpenoids. The functional group present in the plant extract converts the Ag ions to AgNPs. The peaks were observed with (Table

1) AgNPs at 1596.5cm⁻¹ (C=C aromatic rings), 1383.4 cm⁻¹ (methyls), and 1043 cm⁻¹ (ether linkages) imply the presence of flavonoids or terpenoids on the surface of synthesized AgNPs (Figure 1).

Table 1. FT-IR Peak values of AgNPs

Peak area	Mode of vibration	Functional groups
2917.97	CH ₃ ,CH ₂ and CH Stretch	Alkanes
2395.91	P-H	Phosphine
1980.53	Si-OR	Silicon
1596.52	C=O Stretch	Carboxylic acids
1383.62	C-H def	Alkanes
1043.53	C-O Stretch	Ethers

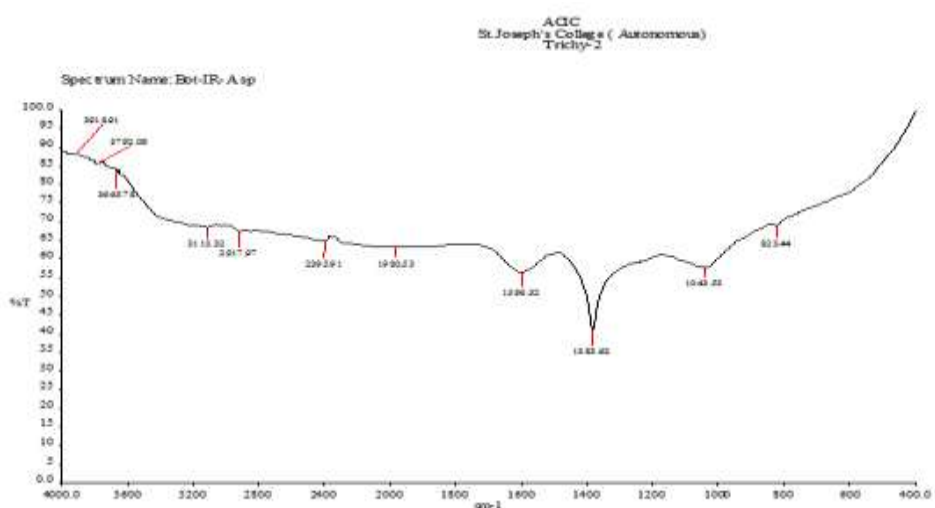


Figure 1. FT-IR Peak value and its functional groups of AgNPs

The particle size was obtained to be 44 nm (Figure 2). The EDX analysis of AgNPs show the percentage of pure Ag present in the solution (Figure 3).

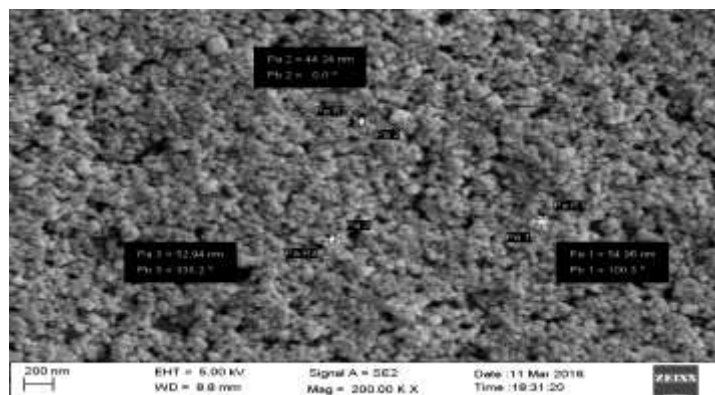


Figure 2. FESEM analyses of AgNPs

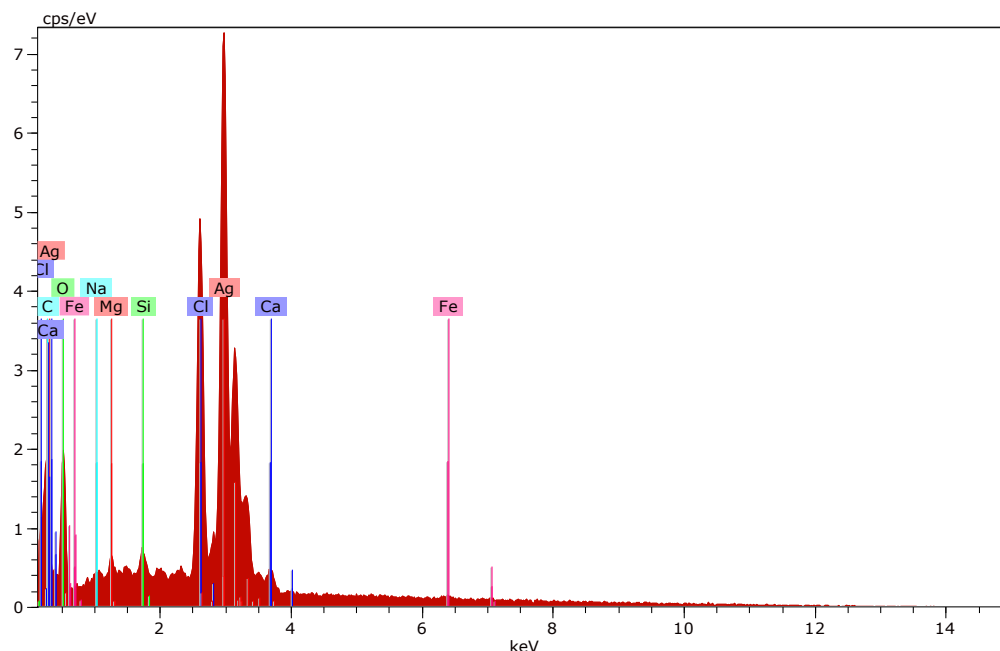


Figure 3. EDAX analyses of AgNPs

The obtained results show along the graphical representation that the Ag occupies maximum percentage 58% that constitute the AgNPs along other precursors and nearly 12.7% of silver was detected from ED-X [19]. Therefore, the amount of Ag in the converting solution is important to have a confined size of AgNPs. The absorption peak at 3 KeV showed the peak signals recorded for other metallic elements in the EDX spectra, it is apparent that

the biogenic AgNPs are at purity grade point. XRD Peak values of AgNPs indicate the crystalline nature of the AgNPs. The major four peaks (Table 2) 27.78, 32.17, 38.09, and 46.24 in the X-ray diffraction pattern of the synthesized AgNPs corresponds to cubic silver with 2θ values as per the JCPDS card N0 89-3722. The XRD pattern showed that the AgNPs obtained by the reduction of Ag had a FCC crystal structure (Figure 4).

Table 2. XRD Peak values of AgNPs

Pos. [$^{\circ}2\theta$.]	Height [cts]	FWHM [$^{\circ}2\theta$.]	d-spacing [\AA]	Rel. Int. [%]
10.6734	2.98	0.8029	8.28889	1.36
24.2404	36.93	0.0010	3.67178	16.89
27.7867	106.55	0.2007	3.21070	48.74
32.1701	218.60	0.2676	2.78252	100.00
38.0901	49.18	0.5353	2.36258	22.50
46.2465	101.21	0.4015	1.96312	46.30
54.8293	38.58	0.3346	1.67440	17.65
57.3837	26.99	0.9792	1.60446	12.34
64.5814	3.87	0.1486	1.44312	1.77
76.8356	22.68	0.3079	1.24066	10.38
85.6351	20.74	0.1496	1.13429	9.49

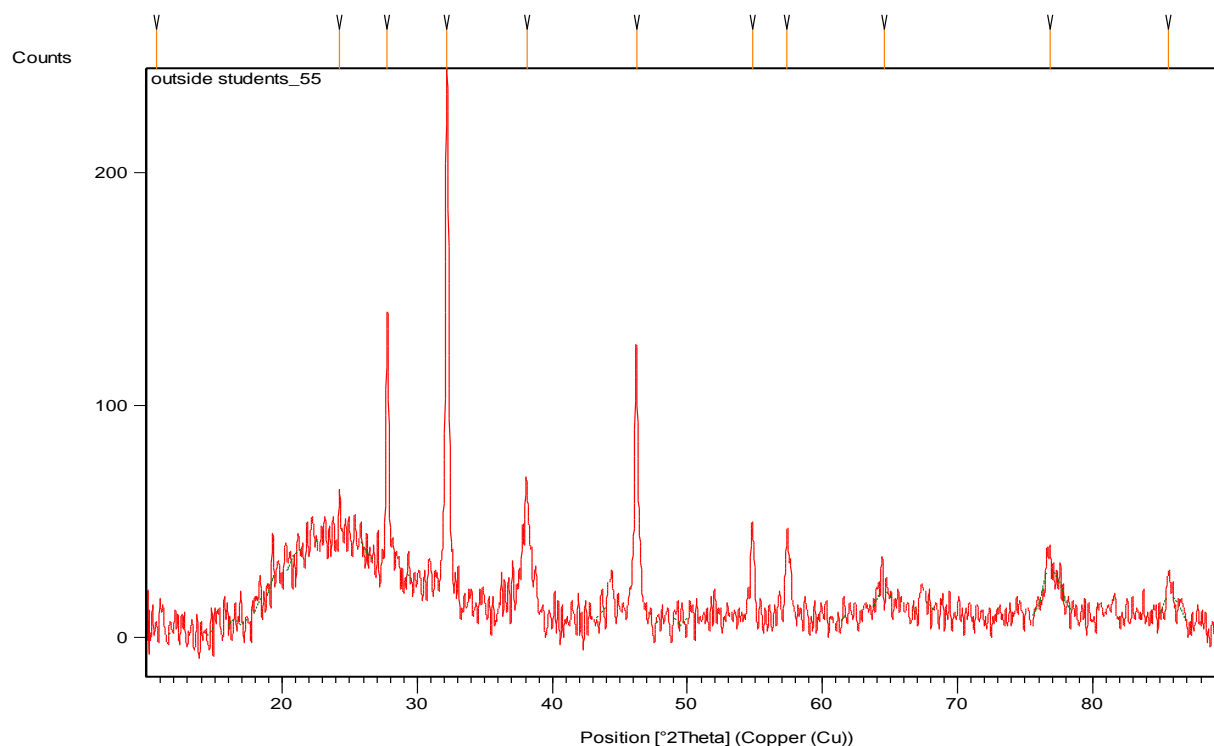


Figure 4. XRD patterns of AgNPs

Anti-bacterial assay

The anti-bacterial assay was evaluated with five human pathogens using the disc diffusion

method. The bacterial strain *P. aeruginosa* showed a maximum zone (13 ± 1.17) of inhibition (Table 3).

Table 3. Evaluation of antibacterial effects of silver nanoparticles (*Artocarpus altilis*) against pathogens by agar disc diffusion method

S. no	Test Bacteria	Zone of inhibition(mm)		
		AgNPs	Streptomycin	AgNO ₃
1	<i>Rhodococcus rhodochrous</i> MTCC - 265	11.33±0.577	30.66±4.725	6.66±4.77
2	<i>Vibrio cholera</i> MTCC - 3904	10.62±0.577	31.23±3.214	6.69±5.76
3	<i>Staphylococcus aureus</i> MTCC - 2940	10.66±1.154	28.33±1.527	3.33±6.77
4	<i>Pseudomonas aeruginosa</i> MTCC - 1934	13±1.17	25.35±4.725	6.60±5.79
5	<i>Escherichia coli</i> MTCC - 739	12±1.25	28.30±0.577	6.61±5.70

The antibiotic streptomycin and a negative control AgNO₃ also significantly relative zone of

inhibition. The cumulative answers for measurements were done based on triplicate

method. The second measurement for zone of inhibition was with *E. coli* bacterial strain (12 ± 1.25) (Figure 5).

Many metal salts and metal NPs have been found to be effective in inhibiting the growth of

many infectious bacteria. Ag and AgNPs occupy a prominent place in the series of such metals which are used as antimicrobial agents from time immemorial [20].

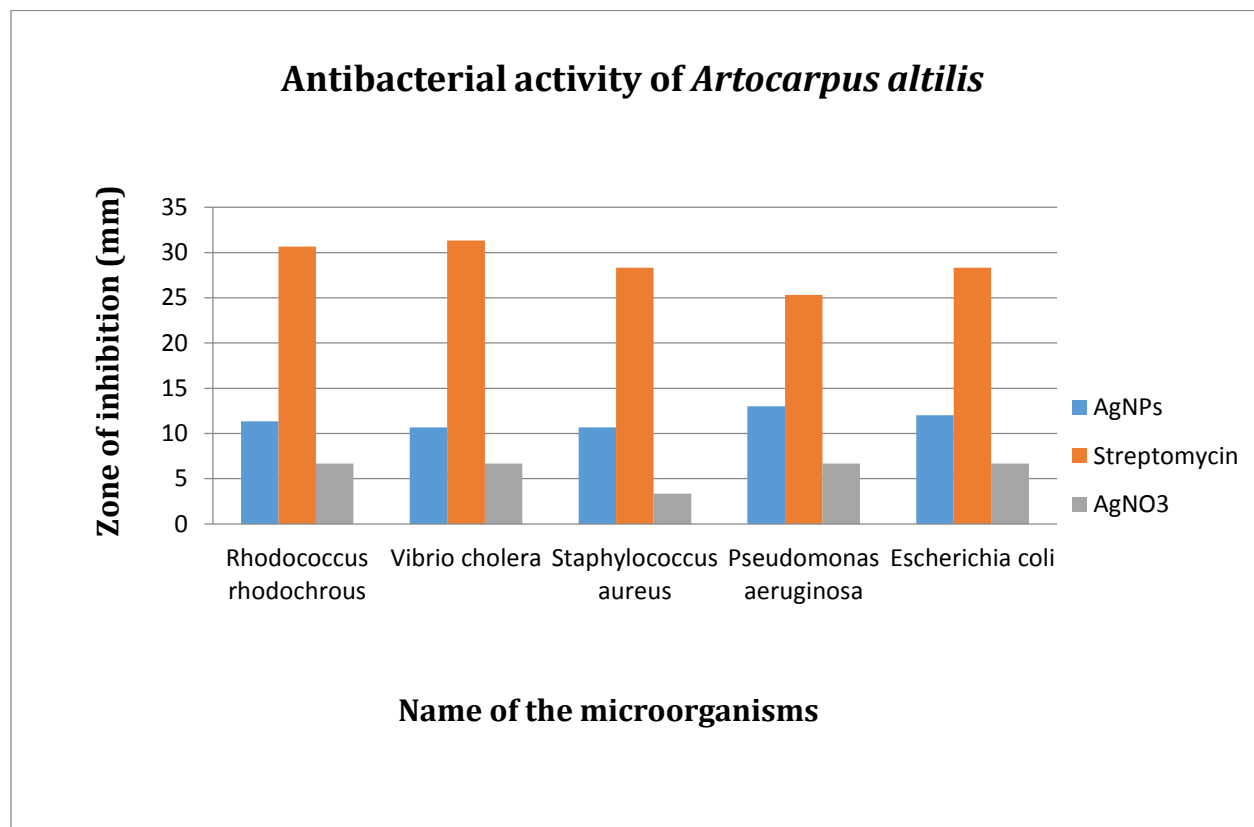
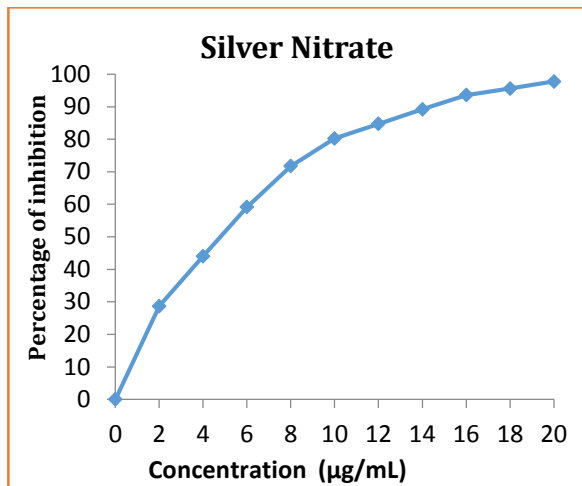


Figure 5. Graphical representation of comparison of zone of inhibition recorded for silver nanoparticles (*Artocarpus altilis*) against pathogens by agar disc diffusion method

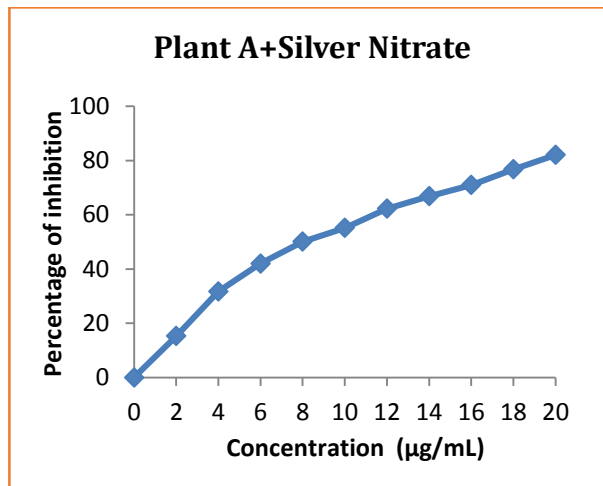
The mechanism of growth inhibition is due to electrostatic interaction, adsorption and penetration of nanoparticles into the bacterial cell wall [21, 22]. Toxicity of nanoparticle also depends on composition, surface modification, intrinsic properties and type of microorganisms. Hence present study on antibacterial gives prominent evidence that AgNPs from plant extract is suitable bio-compound for curing of various disease related to microbes.

Cyto-toxic assay

The cytotoxicity of AgNPs from *A. altilis* was analysed with two human cancer cell lines. Namely human lung cancer cell line (A549) and human breast cancer cell line (MCF-7). The cytotoxic activities of all the complexes have been investigated against the A549 human lung cancer cell line and MCF-7 human breast cancer cell line by using MTT assay. The cyto-toxic activity was determined according to the dose values of the exposure of the complex required to reduce survival to 50% (IC_{50}), compared to untreated cells (Figure 6 and 7)

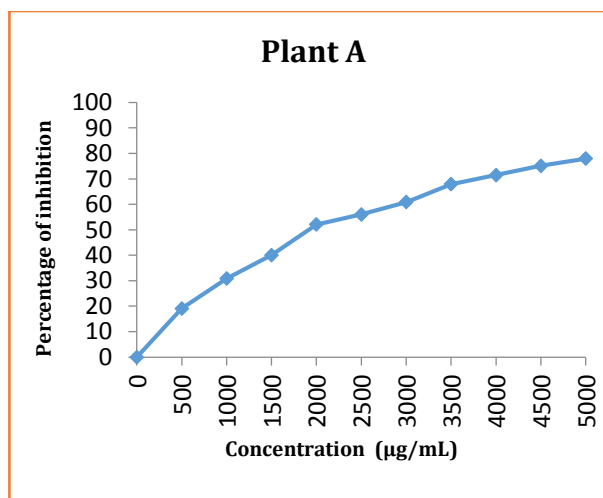


(a)

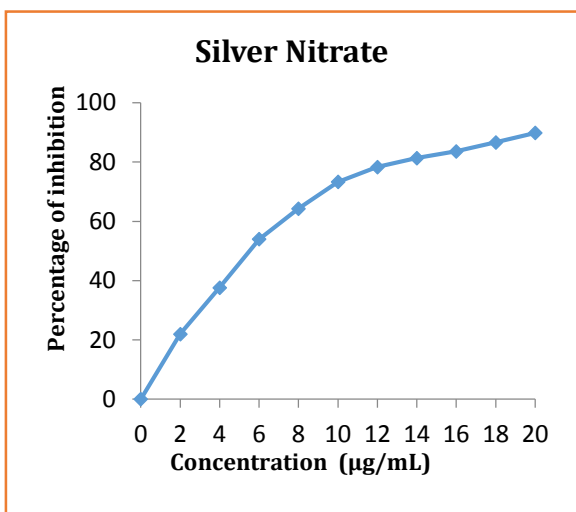


(b)

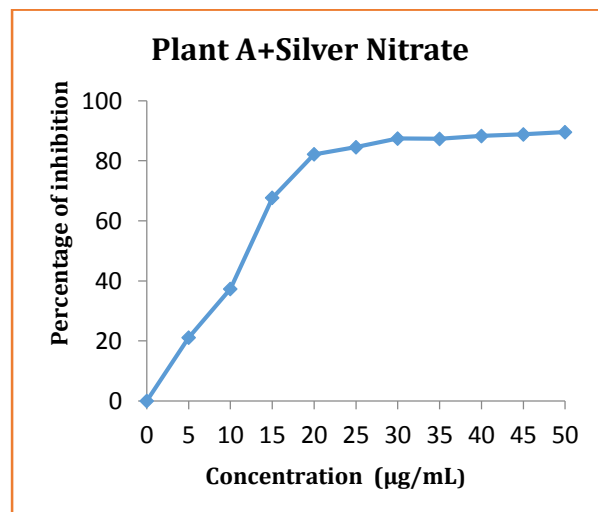
Figure 6. Graphical representation A549 human lung cancer cell line (a)-AgNO₃- IC₅₀ = 5.5 ± 0.05 µg/mL; (b)-AgNPs- IC₅₀ = 8 µg/mL (A549); (c)- *A.altilis*- IC₅₀ = 1990 ± 0.05 µg/mL



(c)

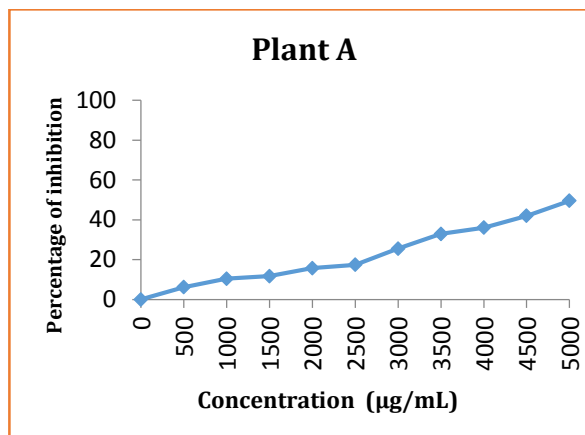


(a)



(b)

Figure 7. Graphical representation MCF-7 human breast cancer cell line. (a)-AgNO₃- IC₅₀ = 5.8± 0.05 µG; (b)-AgNPs- IC₅₀ =11.95± 0.05 µG; (c) *A.altilis* - IC₅₀ = 5000 µg/mL



(c)

The observed IC₅₀ values for 24 h reveal that all the complexes exhibit lower range of cytotoxicity. The ability of the complexes to kill the cancer cells at 24 h incubation vary as Silver Nitrate > AgNPs (Silver Nitrate+ Plant A); Plant A (*A. altilis*). The results of the MTT assay indicated that these silver nitrate conjugated plant samples are very toxic to A549 cells; MCF-7 cells and plant A is less toxic (Table 4 and 5).

Table 4. *In vitro* cytotoxicity assays for the complex against human lung cancer cell line (A549)

Compound	IC ₅₀ Values (24 h)
Silver Nitrate	5.5± 0.05 µg/mL
AgNPs(Silver Nitrate+ Plant A)	8 µg/mL
Plant A(<i>Artocarpus altilis</i>)	1990± 0.05 µg/mL

Table 5. *In vitro* cytotoxicity assays for the complex against human breast cancer cell line (MCF-7)

Compound	IC ₅₀ Values (24 h)
Silver Nitrate	5.8± 0.05 µg/mL
AgNPs(Silver Nitrate+ Plant A)	11.95± 0.05 µg/mL
Plant A(<i>Artocarpus altilis</i>)	5000 µg/mL

Acridine orange (AO) and ethidium bromide (EB) staining

The most important characteristics of apoptosis are morphological changes during cell death. This aspect represents that AO/EB double-stained A549 human lung cancer cell line and MCF-7 human breast cancer cells treated with test substances 24 h underwent both early apoptosis (cells with red arrows) and late apoptosis. The control or viable cells shows green fluorescence and normal cell features of uniform chromatin with an intact cell membrane, whereas, the early apoptosis cells showed bright green region with yellowish green nuclear fragmentation and membrane bubbles and apoptotic bodies outside. The late apoptosis cells exhibited orange-yellow or red nuclei with condensed or fragmented chromatin [26]. The results demonstrate that all substances induce majority of cell death through apoptosis mode and very fewer in necrosis for 24 h treatment (Table 6 and 7).

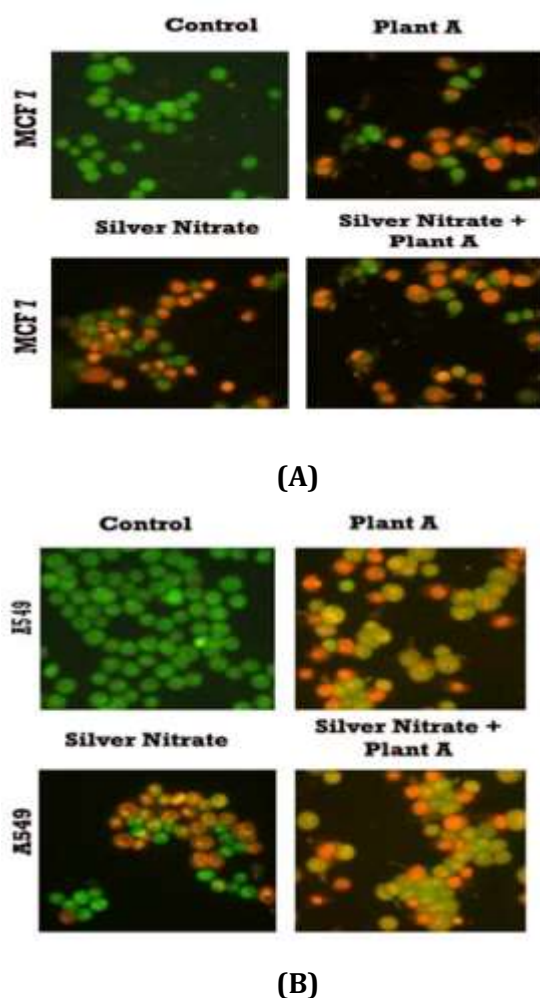
Table 6. Acridine orange (AO) and ethidium bromide (EB) staining -A549 Human lung cancer cell

	Normal	Apoptosis	Necrosis
Control	88	7	5
Silver Nitrate	49	42	9
AgNPs(Plant A+Silver Nitrate)	48	37	15
Plant A	47	41	12

Table 7. Acridine orange (AO) and ethidium bromide (EB) staining MCF -7 Human Breast cancer cell

	Normal	Apoptosis	Necrosis
Control	89	6	5
Silver Nitrate	49	37	14
AgNPs(Plant A+Silver Nitrate)	48	47	5
Plant A	51	38	11

Chromatin condensation and fragmentation were majorly observed in Silver Nitrate, AgNPs (Silver Nitrate+ Plant A), and Plant A (*Artocarpus altilis*) treated cells (Figure 8).

**Figure 8.** Ao/Eb: control, silver nitrate, AgNPs (Silver nitrate+ Plant A), plant A (*Artocarpus*

altilis) treated cells. Green colour cells are live cells and red colour cells showing apoptotic morphology-A-B (A) MCF7 cells and (B) A549 cell reports that AgNPs fabricated fruit extract also exhibited cytotoxic effect against MCF-7 breast cancer cell lines with an IC50 of 67 $\mu\text{g}/\text{mL}/24$ h [23]. The details on A549 adenocarcinoma lung cancer is elaborated [24]. Ag NPs obtained from *C. collinus* aqueous extract exhibit dose dependent effects against human lung cancer cell (A549) and normal cell (HBL-100) [25]. Hence AgNPs seems to be potential drug that works at cancer cells treatment. Since the precursor from green method, it promise safe and ecofriendly approach for treatment of various human disease. There are many experiments on different cancer cells that have been experimented with plant based NPs. The optional choice for the cancer cell treatment is AgNPs. Since it contains bio-compound embedded with Ag metal with large surface area to work with, it is suitable method to find standard methods to improve this mordent science.

Conclusion

The plant extract mediated AgNPs from *Artocarpus altilis* is purely a green method that employ the metallic compound mixed to plant bio-compound in order to use it for various useful applications. Less time consuming, large scale production, Eco-friendly are some of the positive side towards the AgNPs of plant origin. Though is no standard protocol towards the proper disposal AgNPs, this present study makes an emphasis on the monitoring system in disposal of AgNPs in open environment. Clinically, AgNPs do promise many useful aspects for human welfare, but after the unitization the NPs deposited in environment are hazardous since many living organism would encounter the same. Therefore it is befitting to have AgNPs from plant residue and the applications for clinical purpose are much

demand since bio medical industry largely depends on biogenic AgNPs.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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